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RESEARCH ARTICLE

Novel Lipid Mediators as a Promising Therapeutic Strategy for Ischemic Stroke

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ABSTRACT

Despite displaying efficacy in experimental stroke studies, neuroprotection has failed in clinical trials. The translational difficulties include a limited methodological agreement between preclinical and clinical studies and the heterogeneity of stroke in humans compared to standardized strokes in animal models. Promising neuroprotective approaches based on a deeper understanding of the complex pathophysiology of ischemic stroke, such as blocking pro-inflammatory pathways plus pro-survival mediators, are now evaluated in preclinical studies. Combinatorial therapy has become increasingly attractive in recent years as recognizing the complexity of stroke progression becomes evident. The paper aimed to test the hypothesis that blocking pro-inflammatory platelet-activating factor receptor (PAF-R) with LAU-0901 plus administering a selected docosanoid, aspirin-triggered neuroprotectin D1 (AT-NPD1), which activates cell-survival pathways after middle cerebral artery occlusion (MCAo), would lead to neurological recovery. We have demonstrated that LAU-0901 plus AT-NPD1 treatment affords high-grade neuroprotection in MCAo, equaling or exceeding that afforded by LAU-0901 or AT-NPD1 alone at considerably moderate doses, and it has a broad therapeutic window extending to 6 hours after stroke onset.

Introduction

Stroke continues to be one of the most detrimental diseases. Despite ongoing advances in stroke research, the consequences of death and disability remain substantial, and identifying effective therapeutics continues to present a challenge. Therefore, the development of new therapeutics for stroke is an unmet need.

Following cerebral ischemia, a cascade of biochemical events occurs, ultimately leading to cell damage and the death of neurons[1]. N-methyl-D-aspartate (NMDA)-mediated glutamate excitotoxicity follows the onset deprivation of ischemic stroke, leading to the activation of intracellular signaling cascades that trigger calcium-mediated excitotoxicity. When the blood flow is restored, the surge of oxygen sets in motion excessive oxidative stress that triggers inflammatory responses involving astrocytes, microglia, and blood-brain barrier (BBB) permeability perturbations. Molecular targets can be pharmacologically modulated within this cascade to produce neuroprotection. Some of the events targeted include glutamate release, excitotoxicity, mitochondrial dysfunction, activation of intracellular enzymes, nitric oxide production, free radical production, apoptosis, and inflammation[1]. Increasing evidence suggests that inflammation following stroke plays a primary role in secondary brain damage following reperfusion[2].

Stroke pathophysiology displays multiphasic changes. For example, the vascular response to ischemia activates endothelial cells and upregulates circulating leukocytes and adhesion molecules, including E-(endothelial surface) and P-(platelet surface), and L-(leukocyte surface) selectins, ICAM-1, and integrins. Leukocytes can travel across endothelial cells to the brain by interacting via these adhesion molecules and secrete pro-inflammatory cytokines into the brain parenchyma[3]. The acute inflammatory response after stroke, therefore, primes interactions between platelets, leukocytes, lymphocytes, and endothelial cells responsible for BBB injury and infiltration of immune cells into the brain parenchyma[4]. Whereas excitotoxicity and exaggerated oxidative stress are present from the first hours to days after stroke, inflammation at different magnitudes and forms of expression, on the other hand, can last for months. The neurovascular unit (endothelia, neurons, astrocytes, and pericytes) and microglia, astrocytes, oligodendrocytes, neutrophils, monocytes, and lymphocytes are involved in the post-stroke injury[5].

Current status of stroke therapies

Tissue plasminogen activator (tPA) administered within 3 to 4.5 hours of symptom onset is still the only thrombolytic agent for patients with ischemic stroke [6]. However, the narrow therapeutic time window and the risk of intracerebral hemorrhage after tPA pose hurdles to its clinical use. The last 25 years of stroke research have brought considerable progress concerning animal experimental stroke models, therapeutic drugs, clinical trials, and post-stroke rehabilitation studies, but significant

knowledge gaps about stroke treatment remain. In preclinical studies, over 1026 potential therapies have been trialed, pointing to some of the events mentioned above, with many providing protection[7]. Unfortunately, after nearly 200 clinical trials, all attempts at neuroprotection for ischemic stroke clinically have failed. Short-term experimental trials often result in failed therapeutic development due to false-negative outcomes in clinical settings[3]. One factor that could be listed for the failure of clinical trials is the enrollment of patients outside the optimal time window of 4 hours after the onset of stroke, which points to the need for therapies with efficacy beyond 4 hours after the onset of stroke[2]. Understanding the functional and behavioral output which might mislead true recovery is problematic in clinical trials wherein animal models have a more remarkable ability to mask the functional benefits[8].

Combination therapy for stroke

Combinatorial therapy has become increasingly attractive in recent years as recognizing the complexity of stroke progression becomes evident[9,10]. Some drug combinations display pharmacological potentiation (i.e., synergism), which may result in lower doses, few adverse side effects, and an extended treatment window[11]. Treatment outcomes involve the re-establishment of blood flow to ischemic tissue, with the reintroduction of oxygen transiently adding to the injury due to the generation of inflammatory mediators and toxic levels of oxidative free radicals, protein synthesis arrest, and eventually cell death[1]. Therefore, potentially successful treatment options are required to address several critical mediators of neuronal death.

Lipid mediators in the resolution of inflammation

We first put in perspective the physiological and pathological significance of the bioactive phospholipid platelet-activating factor (PAF). PAF is a hippocampal excitatory synapse messenger that regulates LTP and participates in memory formation. Several studies were initiated after we used ischemia-reperfusion (I/R) in gerbils that uncovered that naturally occurring PAF antagonists exerted remarkable neuroprotection[12]. This resulted in a search for signaling and receptors. We identified specific PAF binding sites in the synaptic nerve-ending membranes and intracellular membranes[13]. Then, we found that intracellular PAF binding sites activate a Fos/Jun/Ap-1 transcriptional signaling system[14].

An outcome was to experimentally formulate the question: Is there a physiological role of PAF and its synaptic receptor? Sure enough, we found enhancement of hippocampal excitatory synaptic transmission by PAF[15] and its role as a potential retrograde messenger in CA1 hippocampal long-term potentiation (LTP)[16] by a novel presynaptic receptor that modulates the release of excitotoxic neurotransmitters[15]. Therefore, PAF is a synapse messenger and an intracellular modulator of gene expression[16]. The activation of phospholipase A2 (PLA2) and

release of arachidonic acid (AA) and other lipid mediators at the synapse are partly driven by PAF[17]. With D. Jerusalinsky and I. Izquierdo, we uncovered that PAF has a role in memory formation[18,19].

Moreover, we began assessing the significance of over-produced brain PAF based on our previously published studies[16,17]. So we designed synthetic platelet-activating factor receptor (PAF-R) antagonists[20]. A degraded PAF enzyme was also identified as a PAF signal terminator[21].

Platelet-activating factor receptor antagonists

In recent years, PAF-R antagonists have gradually attracted international attention in stroke prevention and treatment. During brain ischemia and in other pathologic conditions involving oxidative stress, PAF concentration increases, and, in turn, it becomes a pro-inflammatory messenger and a mediator of neurotoxicity[22]. Excessive PAF production promotes neuronal damage; inhibition of this process plays a critical role in neuronal survival and prevention of ischemic brain injury[23–25]. Within the past 30 years, many structures have been identified as PAF antagonists. These include not only PAF analogs but also antagonists derived form natural products and non-lipid synthetic compounds[26]. PAF-R antagonists have different mechanisms of action compared to previous antiplatelet drugs and a variety of anti-inflammatory and neuroprotective effects[27]; they are currently widely used for blood circulation disorders and accumulating evidence has been shown them to be an effective treatment for ischemic cerebrovascular diseases[28] . We have recently synthesized and characterized a highly selective PAF-R antagonist, LAU-0901 (2,4,6-trimethyl-1, 4-dihydro-pyridine-3, 5-dicarboxylic acid), that provides neuroprotection when administered in moderate dosages in mice and rat models of focal cerebral ischemia[29,30]. LAU-0901 improved neurological scores, indicating improved functional and histological outcomes and restoring local cerebral blood flow[29,30]

Aspirin-triggered neuroprotectin D1: A novel lipid mediator

Mechanisms to resolve the acute inflammatory response following I/R injury after stroke have been targets of interest due to the complex signaling events that have the potential to be mediated, improving functional outcomes and reducing neuronal death[31]. We have discovered that the brain produces a novel lipid mediator, aspirin-triggered neuroprotectin D1 (AT-NPD1; 10R, 17R–dihydroxy-docosa-4Z, 7Z,11E,13E,15Z, 19Z-hexaenoic acid), which is a novel aspirin-triggered derivative of docosahexaenoic acid (DHA), which we have shown to have anti-inflammatory and pro-resolving bioactivity[31,32]. AT-NPD1, when administered in a rat model of middle cerebral artery occlusion (MCAo), allowed for neurobehavioral recovery at 3 hours after the onset, reducing brain edema and infarction and improving neurobehavioral recovery[32]. A reduction in polymorphonuclear neutrophil (PMN) recruitment and decreased PMN transendothelial migration were observed in a murine model of peritonitis treated with AT-NPD1[31].

Suggested therapeutic options to protect the brain after experimental ischemic stroke.

The pathophysiologic role of inflammation in stroke encompasses the initial injury and the sustained signaling in the acute phases following stroke, which progresses the damage and affects recovery [33]. Homeostatic disruptions resulting from ischemic stroke-induced injury cause excitotoxicity increase in intracellular calcium sustained inflammatory cell signaling and cytokine release and disruptions to the BBB [34]. Current therapeutic options do not fully address these disruptions, further indicating that combination therapy may be necessary to protect against I/R injury adequately.

We considered a combinatory approach to address multiple components of stroke pathophysiology, primarily focusing on blocking pro-inflammatory signaling while concurrently aiding in neuroprotection and the resolution of the inflammation [23,26,35,36] (**Figure 1**). Mitochondria usually remove excessive Ca^{2+}, but in ischemic tissue, excessive accumulation of calcium in these mitochondria leads to membrane depolarization and dysfunction. Mitochondrial apoptosis is mainly regulated by B-cell lymphoma-2 (Bcl-2) family proteins which are either pro-apoptotic (e.g., Bax, Bok) or anti-apoptotic (e.g., Bid, Bcl-2) [37]. Neuroprotectin D1 (NPD1) has targeted the Bcl-2 family of proteins [38]

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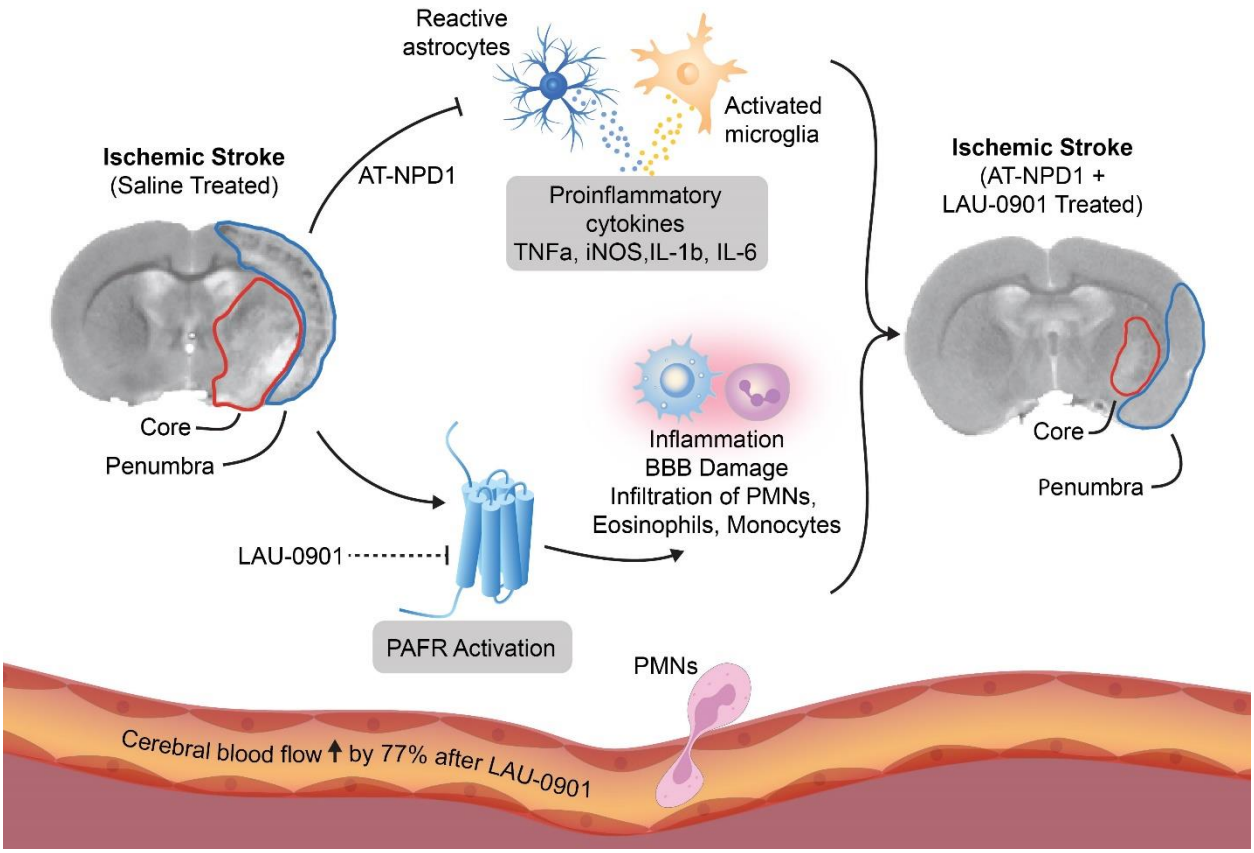

Figure 1. Inhibition of inflammatory signaling and PAF-R activation induced by I/R injury by LAU-0901 and AT-NPD1. I/R injury triggers the activation of microglia and astrocytes, causing the release of pro-inflammatory cytokines (TNFα, iNOS, IL-1β, IL-6). PAF-R activation is associated with increased inflammation, BBB damage, and infiltration of PMNs, eosinophils, and monocytes. Treatment with LAU-0901 plus AT-NPD1 reduced penumbra and core lesion volumes after MCAo.

AT-NPD1 = aspirin-triggered neuroprotectin D1, BBB = blood-brain barrier, IL-1β = interleukin 1 beta, IL-6 = interleukin 6, iNOS = inducible nitric oxide synthase, PAF-R = platelet-activating factor receptor, PMNs = polymorphonuclear neutrophils, TNFα = Tumor Necrosis Factor alpha

The activation of inflammatory factors during the reperfusion phase after stroke presents a significant barrier when considering treatment options due to the homeostatic disruptions. Inhibiting inflammatory signaling and targeting the resolution of the processes together would yield superior neuroprotection than either alone.

Synergistic neuroprotection by platelet-activating factor receptor antagonist plus aspirin-triggered neuroprotectin D1 in experimental ischemic stroke

The present study was prompted by our earlier finding demonstrating the neuroprotective efficacy of LAU-0901 and AT-NPD1 in focal cerebral ischemia [29–32]. We discovered that these lipid mediators

promote neuronal cell survival with important anti-inflammatory activity.

The use of LAU-0901 to block inflammatory signaling and AT-NPD1, which is known to contribute to the activation of cell survival pathways and potentially have anti-inflammatory effects, were assessed to determine if synergistic neuroprotection was observed [35,36]. Sprague-Dawley rats were subjected to 2 hours of MCAo, behavior testing (days 1-7), and *ex vivo* MRI on day 7 was conducted. Different doses of LAU-0901 (45 and 60 mg/kg), AT-NPD1 (111, 222, 333 µg/kg), and combinatory treatment with LAU+AT-NPD1 or vehicle were administered at 3h after the onset of stroke. In the therapeutic window, vehicle, LAU-0901, AT-NPD1, and LAU+AT-NPD1 were administered 3, 4, 5, and 6 hours after the onset of MCAo. The combination of LAU-0901+AT-NPD1 following 2 hours of MCAo improved neurobehavioral deficits and the cortical and subcortical lesions were significantly decreased when the combination was administered compared to either treatment alone [36]. In addition, LAU+AT-NPD1, when administered at 3, 4, 5, and 6 hours, improved behavior and reduced lesion volumes on day 7 compared to the vehicle. This 6-hour time frame is clinically relevant in that it is logistically challenging to institute therapy in many patients with acute ischemic stroke at early times. Taken together, this finding suggests that this combinatory treatment offers great promise in the treatment of patients with acute ischemic stroke.

Conclusion

I/R damage after stroke may trigger various molecular cascades associated with the dysregulation of numerous neuroinflammatory pathways and disruption of neuronal circuits, which aggravate brain damage.

Therefore, developing neuroprotection strategies to safeguard the brain from cerebral ischemia and I/R is an important goal. Although stroke is a complex disorder with activation of multiple detrimental signaling cascades, almost all neuroprotective strategies to date have attempted a monotherapy against a single target. The use of a single agent is limited in its applications to stroke due to the multifaceted pathophysiology following brain I/R. When therapeutics targeted both the sustained inflammatory response and treatments that aid in restoring homeostatic metabolic signaling in the brain, greater degrees of neuroprotection were observed. We have demonstrated that LAU-0901 plus AT-NPD1 treatment affords high-grade neuroprotection in MCAo, equaling or exceeding that afforded by LAU-0901 or AT-NPD1 alone at considerably moderate doses, and it has a broad therapeutic window extending to 6 hours after stroke onset. The exact mechanisms of LAU-0901 plus AT-NPD1 remain obscure. We can speculate that joint inhibition and inflammation resolution by LAU-0901 and AT-NPD1 provides additive neuroprotection in experimental stroke.

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Conflict of Interest

The authors report no conflicts of interest.

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