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The effect of cationic albumin-conjugated PEGylated tanshinone IIA nanoparticles on neuronal signal pathways and neuroprotection in cerebral ischemia

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ABSTRACT

Targeted treatment of ischemic stroke remains problem due to the complex pathogenesis of this disease and the difficulty in drug delivery across the blood–brain barrier (BBB). In the present study, the delivery efficiency of cationic bovine serum albumin-conjugated tanshinone IIA PEGylated nanoparticles (CBSA-PEG-TIIA-NPs) in rat brain was investigated. We further explored whether the protective mechanism of CBSA-PEG-TIIA-NPs in cerebral ischemia was associated with modulating neuronal signaling pathways. The experimental cerebral ischemia model was established to evaluate the treatment efficacy of CBSA-PEG-TIIA-NPs. The pharmacokinetics demonstrated that CBSA-PEG-TIIA-NPs could obviously prolong circulation time and increase plasma concentration compared with intravenously administrated TIIA solution. The biodistribution and brain uptake study confirmed that CBSA-PEG-TIIA-NPs possessed better brain delivery efficacy with a high drug accumulation and fluorescence quantitative level in brain. CBSA-PEG-TIIA-NPs effectively reduced infarction volume, neurological dysfunctions, neutrophils infiltration and neuronal apoptosis. Moreover, CBSA-PEG-TIIA-NPs significantly suppressed the expression of pro-inflammatory cytokines TNF-α and IL-8; upregulated the expression of anti-inflammatory cytokines IL-10 and increase TGF-β1 level in the ischemic brain. In addition, treatment with CBSA-PEG-TIIA-NPs markedly inhibited the mRNA expressions of GFAP, MMP-9, COX-2, p38MAPK, ERK1/2 and JNK, down-regulated the protein levels of GFAP, MMP-9 and COX-2, as well as decreased the phosphorylation of ERK1/2, p38MAPK and JNK. These results demonstrated that CBSA-PEG-TIIA-NPs displayed remarkable neuroprotective effects on ischemic stroke through modulation of MAPK signal pathways involved in the cascades of neuroinflammation.

1. Introduction

Ischemic stroke has a high morbidity and mortality, which is recognized as a major public health problem worldwide [1,2]. Although different mechanisms are involved in the pathogenesis of cerebral ischemia, converging evidence suggests that the inflammatory injury plays critical role. Inflammatory cascades are triggered by cerebral ischemia/reperfusion (I/R) and further amplify secondary brain injury due to cytotoxic neuronal cell death and neurological dysfunction [3–5].

The major mediators in the inflammatory injury after the onset of ischemia are cytokines, cyclooxygenase-2 (COX-2), adhesion molecules, matrix metalloproteinases (MMPs), and eicosanoids, which contribute to irreversible damage [3,6,7]. Pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), IL-6 and IL-8 have been implicated in the disruption of the blood–brain barrier (BBB) and the invasion of inflammatory cells into the central nervous system (CNS) [8–10]. Anti-inflammatory cytokines, IL-10 and transforming growth factor (TGF)-β1, have been identified to suppress the production of pro-

Abbreviations: TIIA, Tanshinone IIA; PEG, poly ethylene glycol; PLA, poly lactic acid; CBSA, cationic bovine serum albumin; MCAO, middle cerebral artery occlusion; MPO, myeloperoxidase; TNF-α, tumor necrosis factor alpha; IL-8, interleukin-8; IL-10, interleukin-10; TGF-β1, transforming growth factor-β1; GFAP, glial fibrillary acidic protein; MMP-9, matrix metalloproteinase-9; COX-2, cyclooxygenase-2; p38MAPK, p38 mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase-1 and 2; JNK, c-Jun N-terminal kinases.

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inflammatory cytokine in protection of damaged brain tissues after ischemic stroke. Also, anti-inflammatory cytokines are associated with repairing damaged brain tissues [11–13]. Therefore, the imbalance between pro-inflammatory cytokines and anti-inflammatory cytokines, play an important role in the modulation of immune system and contributes to the inflammatory cascade in cerebral ischemia [14]. MMPs have also been upregulated, in particular, MMP-9 degrades the matrix components of the basement membrane, which results in neuron-inflammation, brain edema, and hemorrhagic transformation in cerebral ischemia [15,16]. Genetic and pharmacological inhibitions of MMP-9 significantly reduced neuronal damage in brain ischemia [17,18]. Besides MMP-9, cyclooxygenase-2 (COX-2) has an important role in ischemic brain injury. Numerous studies have found a dramatic increase of COX-2 following ischemia [19]. Increased COX-2 may product of prostanoid synthesis, PGE_2 and free radicals [20].

Cerebral I/R injury is associated with alterations of signal transduction pathways involved in inflammation and cell apoptosis. Many studies indicate that mitogen-activated protein kinases (MAPKs), including p38 kinases (p38MAPK), extracellular signal-related kinases-1 and 2 (ERK1/2), and c-Jun N-terminal kinase (JNK) play pivotal function in regulating inflammatory gene production, cell death and survival [21–23]. These signal pathways are activated by hypoxia, oxidative stress, inflammatory cytokines, increases of intracellular Ca^{2+} levels and glutamate receptor stimulation in cerebral I/R injury [2,24]. Activated p38MAPK is involved in inflammatory responses by producing pro-inflammatory cytokines from activated microglia or astrocytes, and ultimately resulted in neuronal cell death in hippocampal CA1 region [25]. Recent studies have indicated that ERK1/2-mediated signals play a major role in ischemia-induced apoptosis through regulation of Bax/Bcl-2/Bcl-xL expression [26–28]. Activation of JNK pathway is involved in neuronal death in neurodegenerative diseases and cerebral ischemia [29]. Generally, MAPKs signaling pathways contribute to activating downstream pro-inflammatory mediators, resulting in inflammation cascade in ischemic stroke [2,5,23]. Pharmacological intervention of these pathways may reduce apoptosis and downstream inflammatory mediators, resulting in neuroprotection [30–32]. Therefore, therapeutic agents that can target multiple pathophysiological mechanisms can be extremely useful in preventing inflammation and neuronal death in ischemic cerebral injury.

Tanshinone IIA (TIIA) is the major active ingredient of a traditional herbal medicine *Salvia miltiorrhiza*, which has been widely used for treatment of cerebrovascular diseases [33]. However, the *in vivo* brain distribution of TIIA is severely limited by its poor solubility and short half-life, which reduces the plasma-free drug in the blood circulation [34]. This significantly decreases the permeability of drug across the BBB [35]. Moreover, the BBB is the most serious obstacle limiting the development of new drugs for the central nervous system (CNS). During the past decade, many strategies have focused on this pivotal problem by designing different methods to improve drug passage across the BBB. Among these, nanotechnology and biotechnological approaches have gained significant momentum, since they can effectively enhance the brain-specific delivery of drugs [36]. One potential strategy is the use of polymeric nanoparticles modified with specific targetors for controlled drug delivery and release. Biodegradable polymeric nanoparticles (NPs) have been adopted as potential carriers for drug delivery to the CNS [37]. Biodegradable polymeric nanoparticles offer a higher stability when they are in contact with biological fluids, such as poly (lactic acid; PLA), poly (glycolic acid) and poly (D, L-lactide-*co*-glycolide acid). Among them, PLA had been approved by FDA in USA to be widely used in nanoparticles [38]. Pegylation of these biodegradable polymer increases the blood circulation time of NPs, presumably due to decreased uptake and elimination by the reticuloendothelial cells [39]. PEG also confers flexibility on the conjugated antibody in the interaction with its ligand, and minimizes steric hindrance. Moreover, PEG-NPs show no toxicity toward neuron cells [40]. The surface embellishment of cationic bovine serum albumin (CBSA) attached to NPs was demonstrated to exhibit greater capacity for attachment to the negative charged luminal side of the brain microvessels and increase flux into the cerebrospinal fluid. Furthermore, pegylated NPs modified with CBSA has proved to be a better drug carrier for brain delivery through absorptive mediated transcytosis [38]. In our previous study, we have developed an effective brain targeting drug delivery system, which was CBSA conjugated PEGylated nanoparticles (CBSA-PEG-NPs) [41].

In the present study, we synthesized methoxy PEG–PLA and maleimide PEG–PLA by ring opening polymerization. PEGylated TIIA NPs were prepared by emulsification and solvent evaporation method, and subsequently conjugated CBSA through the maleimide function. The protective effects of CBSA-PEG-TIIA-NPs on the cerebral I/R model were assessed by infarct size, neurological score and by determination of pro-inflammatory cytokines TNF-a and IL-8 and anti-inflammatory cytokines IL-10 and TGF-β1 in ischemic brain tissue. The neutrophils infiltration, microglia activation and neuronal apoptosis were also evaluated. In order to elucidate the probable mechanisms of CBSA-PEG-TIIA-NPs in ameliorating cerebral ischemia injury, the chemokines involved in inflammation such as MMP-9, COX-2 and three major subgroups of MAPK signal pathway, including p38MAPK, ERK1/2 and JNK were evaluated by Western blotting and real-time quantitative PCR, respectively.

2. Materials and methods

2.1. Materials

Tanshinone IIA (purity > 98%) was obtained from Xi'an Guanyu Biotechnology Co., Ltd. (Shanxi, China). MPEG (MW 2000) and maleimide PEG (MW 4300) were purchased from RAPP POLYMERE (Tubingen, Germany) and Creative PEGWorks (USA), respectively. Bovine serum albumin fraction V (BSA) was purchased from Biosharp Co. Ltd. (USA). 5, 5′-Dithio-bis (2-nitrobenzoic acid) (DTNB, Ellmann's reagent) was purchased from Acros Organics (Belgium). Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Deionized water was purified using a Millipore-Q water-purification System (Bedford, USA). All other reagents and solvents were analytical grade. TdT-mediated dUTP nick end labeling (TUNEL) cell apoptosis detection kit was obtained from Roche Systems, Inc. (Basel, Switzerland). The double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kits of TNF-α, IL-8 and IL-10 were supplied by R&D systems (Minneapolis, MN, USA). The primers for real-time RT-PCR were synthesized by Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

2.2. Animals

Male Sprague-Dawley rats (220 ± 20 g) were supplied by Slaccas Laboratory Animal Co. Ltd. (Shanghai, China). The animals were maintained in standard cages in a controlled room (temperature 25 ± 1 °C, relative humidity 75 ± 5%, 12 h light/dark cycle) and fed with standard rodent diet. All animal experiments were approved under animal protocol number SCXK (Zhe) 2008-0033 by the Institutional Animal Care and Use Committee of Zhejiang University.

2.3. Preparation of CBSA-PEG-TIIA-NPs

The copolymers of methoxy PEG–PLA (MPEG–PLA) and maleimide PEG–PLA were synthesized by ring opening polymerization based on previously described procedures [41]. Thiolated-CBSA was prepared according to the published method with a minor modification [38]. The thiolated CBSA fractions were identified using UV spectrophotometer at 280 nm, and then collected. The extent of thiolation determined using Ellman's reagent [42]. In brief, thiolated-CBSA was conjugated through the maleimide function to the NPs as follows: PEGylated NPs loading TIIA were dispersed in 0.01 M PBS buffer (pH 7.4), followed by the addition of thiolated CBSA in the same solvent at the molar ratio of 1:1 (maleimide: thiolated-CBSA). The conjugation of thiolated CBSA to the NPs loaded with TIIA was performed overnight under gentle magnetic stirring. In order to eliminate free thiolated-CBSA, the reaction mixture was then applied to Sephadex PD10 column and was eluted with 0.01 M PBS buffer (pH 7.4). The CBSA-NPs fractions were visually identified and collected [43], and then concentrated by centrifugation at 4 °C, 15,000 rpm for