Deep Succinylproteomics of Brain Tissues of Intracerebral Hemorrhage with Inhibition of Toll-like Receptor 4 Signaling

Yan-Jing Liang

Chengdu University of Traditional Chinese Medicine <https://orcid.org/0000-0002-3164-6694>

Yuan-Rui Yang

The general hospital of western theater command <https://orcid.org/0000-0002-5114-4915>

Chuan-Yuan Tao

Sichuan University West China Hospital <https://orcid.org/0000-0002-4233-4178>

Su-Hao Yang

Chengdu University of Traditional Chinese Medicine <https://orcid.org/0000-0002-6097-4830>

Xin-Xiao Zhang

Chengdu University of Traditional Chinese Medicine <https://orcid.org/0000-0002-7657-3409>

Jing Yuan

Chengdu University of Traditional Chinese Medicine <https://orcid.org/0000-0002-6695-163X>

Zhan-Qiong Zhong

Chengdu University of Traditional Chinese Medicine <https://orcid.org/0000-0002-3847-9104>

Shu-Guang Yu

Chengdu University of Traditional Chinese Medicine <https://orcid.org/0000-0002-1413-8989>

Xiao-Yi Xiong (✉ xiongxy_89@163.com)

Chengdu University of Traditional Chinese Medicine <https://orcid.org/0000-0003-0720-907X>

Research Article

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Abstract

The details of Toll-like receptor (TLR) 4 signaling affects protein succinylation in intracerebral hemorrhage (ICH) brains remains completely unclear. In this study, we constructed mice ICH models to investigate the changes in ICH-associated brain protein succinylation with the treatment of TLR4 antagonist, TAK242, using a high-resolution mass spectrometry-based, quantitative succinyllysine proteomics approach. We characterized a concentration of approximately 6700 succinylation events and quantified approximately 3500 sites, highlighting 139 succinyllysine site changes in 40 pathways. Further analysis showed that TAK242 treatment induced an increase in 29 succinyllysine sites of 28 succinylated proteins and reduction of 24 succinyllysine sites on 23 succinylated proteins in ICH brains. Both the TAK242 treatment induced hypersuccinylated and hyposuccinylated proteins in ICH brains were mainly located in mitochondria and cytoplasm. GO analysis showed that TAK242 treatment induced changes in ICH-associated succinylated proteins were mostly located in synapse, membrane, vesicle, etc., and enriched in many processes, such as metabolism, synapse, myeline, etc.. KEGG analysis showed that TAK242 induced downregulation of succinylation was significantly linked to fatty acid metabolism and lysosome. Moreover, a combination analysis of our succinylproteomic data with previously published transcriptome data identified that most of the differentially succinylated proteins induced by TAK242 treatment were mainly distributed into neurons, astrocytes and endothelial cells; and 7 and 3 of these succinylated proteins significantly high express in neurons and astrocytes, respectively. In conclusion, our analyses uncover a number of TLR4 signaling affected succinylation processes and pathways in mouse ICH brains and provide new insights for understanding ICH pathophysiological processes. Data are available via ProteomeXchange with identifier PXD025622.

1 Introduction

Protein posttranslational modifications (PTMs) are one of the most efficient biological mechanisms due to the expansion of protein functions (Walsh et al. 2005; Witze et al. 2007; Karve and Cheema 2011) and have been shown to be involved in regulating biological processes of human diseases, such as Alzheimer's Disease (Marcelli et al. 2018), brain injury (Klimova et al. 2018), cancer (Hsu et al. 2018), etc.. Protein succinylation is a novel kind of PTMs identified in 2011 and tricarboxylic acid (TCA) cycle intermediate succinate derived succinyl-CoA has been shown as a cofactor for lysine succinylation (Zhang et al. 2011). In addition, succinate as a metabolite plays an important role in contributing innate immune signaling might via increasing protein succinylation (Tannahill et al. 2013), suggesting that protein succinylation is an important mechanism for the activation of inflammatory immune cells (e.g., macrophages, dendritic cells, and T cells), because which would display a metabolic switching from oxidative phosphorylation (OXPHO) to aerobic glycolysis enabling rapid cell proliferation when they activated (Everts et al. 2014; Jha et al. 2015; Shi et al. 2011). For example, studies showed that TLR4 activation by LPS in macrophages induce the accumulation of the succinate (Everts et al. 2014; Mills and O'Neill 2014; Tannahill et al. 2013; Wang et al. 2017) and protein succinylation (Tannahill et al. 2013), while inhibiting the protein succinylation could suppress the pro-inflammatory response in macrophages

(Wang et al. 2017). Furthermore, protein succinylation also has been shown might contribute to cellular energy regulation via increasing the activity of enzymes involved in glucose and lipid metabolism (Park et al. 2013; Mills and O'Neill 2014). Overall, protein succinylation through the addition of succinyl groups to lysine residues is critical to regulate metabolic processes, and immunity and inflammation (Mills and O'Neill 2014; Liu et al. 2016).

Intracerebral hemorrhage (ICH) is a life-threatening disease with a poor prognosis and few proven treatments (Cordonnier et al. 2018). Increasing evidence shows that the ICH-induced secondary brain injury could be aggravated by inflammation (Fang et al. 2013; Fei et al. 2019; Zhou et al. 2014). For example, we previously showed that glial TLR4 activation markedly exacerbated the brain injury of ICH mice, while inhibiting the TLR4 activation by its antagonist TAK242 significantly alleviated the brain injury via reducing the neuroinflammation (Wang et al. 2013), which was consistent with a later research result that TAK242 protects against acute cerebral ischemia/reperfusion injury via targeting TLR4 signaling (Hua et al. 2015). Given that glial cells, like microglia, have some similar inflammatory profiles to macrophages, indicative of a similar metabolic profiles when they were activated. These results suggest that ICH-induced brain neuroinflammation would also involve the protein succinylation, which might be an important mechanism for the neuroinflammation mediated secondary brain injury after ICH.

Therefore, in this study, we aimed to characterize how the neuroinflammation influenced protein succinylation in TAK242-treated ICH mouse brains and control brains using a high-resolution mass spectrometry-based, quantitative succinylproteomics approach. Bioinformatic analysis showed that TAK242 treatment induced hypersuccinylated and hyposuccinylated proteins in ICH brains were mainly located in mitochondria and cytoplasm, and enriched in many processes. KEGG analysis showed that TAK242 induced downregulation of succinylation was significantly linked to fatty acid metabolism and lysosome. Moreover, a combination analysis of our succinylproteomic data with previously published transcriptome data identified that 7 and 3 succinylated proteins significantly high express in neurons and astrocytes, respectively. In conclusion, our analyses uncover a number of TLR4 signaling affected succinylation processes and pathways in mouse ICH brains and provide new insights for understanding ICH pathophysiological processes.

2 Materials And Methods

2.1 Animals

C57BL/6 mice (male, 8-week-old, 20-24g) were purchased from Byrness Weil Biotech. Ltd. (Chengdu, China). All mice were bred in a temperature-controlled environment and specific-pathogen-free (SPF) conditions with a 12-hour light/dark cycle and free access to water and food. All procedures and animal experiments were approved and conducted in accordance with the Animal Ethics Committee of the Chengdu University of Traditional Chinese Medicine.

2.2 Collagenase-induced mouse model of ICH and TAK242 treatment

The ICH model was conducted according to our previously developed method (Pan et al. 2018). Briefly, mice were immobilized on the stereotaxic apparatus (RWD Life Science Co, Shenzhen, China) after being anesthetized with 3% isoflurane for induction and 1.5% for maintenance. 0.5 µl of 0.075 IU type VII collagenase (Sigma Aldrich) was injected into the left striatum (0.8 mm anterior and 2 mm lateral of bregma, at a depth of 3.5 mm) using a syringe pump (Hamilton, Bonaduz, AG) at a rate of 0.0625 µl/min. The control (sham) group was injected with 0.5 µl saline. During this surgical operation, the animal's body temperature was maintained at 37°C. Unsuccessful ICH models, such as asymptomatic or dead, evaluated by on a behavioral test (Corner task) were excluded from this study.

According to our previous report (Wang et al. 2013; Xiong et al. 2016), TAK242 (CLI-095, MedChemExpress) was formulated with 1% dimethyl sulfoxide (σ) and double-distilled water to a final concentration of 0.4 mg/mL and then injected intraperitoneally at a dose of 3 mg/kg once daily for 3 successive days beginning 6 hours after ICH. Mice were randomly assigned into 3 groups: sham operation group (sham group), ICH + vehicle group (administration of the same volume of solvent used to formulate the TAK242 solution), and ICH + TAK242. The perihematomal and contralateral brain tissues at 3 days after ICH were collected for succinylproteomic analysis.

2.3 Brain sample preparation for LC-MS/MS

The brain samples were grinded by liquid nitrogen and transferred to centrifuge tube with 4 volumes of lysis buffer (8 M urea, 1% Protease Inhibitor Cocktail, 3 µM TSA, 50 mM NAM), and followed by sonication three times on ice. Then, the remaining debris was removed by centrifugation at 12,000 g at 4°C for 10 min to collect the supernatant. The protein concentration was determined with the BCA kit according to the manufacturer's instructions. For digestion, each brain protein sample had a final concentration of 20%, and incubated in ice water for 2 hours. Subsequently, to get the peptides mixes for LC/LC MS analysis, the protein digestion was performed just as previously research described (Guo et al. 2017).

2.4 Succinyllysine peptide Enrichment

The tryptic peptides were dissolved in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, pH 8.0) and Affinity Enrichment of succinylpeptides was carried out according to previous reports (Colak et al. 2013). Briefly, total peptides incubated with pre-washed succinyllysine antibody beads (PTM-402, PTM Bio, China) at 4°C overnight with gentle shaking. The bound peptides were eluted from the beads with 0.1% trifluoroacetic acid and the eluted fractions were combined and vacuum-dried. For LC-MS/MS analysis, the resulting peptides were desalted with C18 ZipTips (Millipore) according to the manufacturer's instructions.

2.5 LC-MS/MS Analysis

LC-MS/MS analysis was performed according to previously described protocols (Jin and Wu 2016). The tryptic peptides were dissolved in solution A (0.1% formic acid, 2% acetonitrile) and separated with a gradient from 9–25% solution B (0.1% formic acid in 90% acetonitrile) within 36 min at a constant flowrate of 500 nL/min on an EASY-nLC 1200 UPLC system (Thermo Fisher Scientific). The separated peptides were analyzed in Q Exactive™ HF-X (Thermo Fisher Scientific) with a nano-electrospray ion source. The electrospray voltage was 2.2 kV. The full MS scan resolution was set to 120,000 for a scan range of 350–1600 m/z. The HCD fragmentation was performed at a normalized collision energy (NCE) of 28%. The fragments were detected in the Orbitrap at a resolution of 15,000. Fixed first mass was set as 100 m/z. The automatic gain control (AGC) target was set at 1E5, with an intensity threshold of 5E4 and a maximum injection time of 50 ms.

2.6 Database Search

MaxQuant (v1.6.15.0) was used to retrieve the secondary mass spectral data in this experiment. Trypsin/P was specified as a cleavage enzyme, and up to 4 missing cleavages were allowed. The mass tolerance for precursor ions in the first search and the main search were set as 20 ppm and 4.5 ppm, respectively, and the mass tolerance for fragment ions was set at 0.02 Da. A carbamidomethyl on Cys was set as a fixed modification, and succinylation modification and oxidation on Met were specified as variable modifications. FDR was adjusted to < 1%.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al. 2019) partner repository with the dataset identifier PXD025622.

2.7 Bioinformatics analysis

Bioinformatic analysis was performed using pipeline writing with Perl and R. Statistical analysis of the succinylproteome was performed on logarithmic intensities for those values that were found to be quantified in one experimental condition. The MoMo analysis tool based on the Motif-X algorithm was used to analyze the motif characteristics of the succinyllysine sites. To identify significantly modified succinylpeptides, the three-samples replicates analysis were performed and set a *P*-value cutoff of 0.05. The comparison of relative quantitative value of each modification site between TAK242- and Vehicle-treated ICH brains came to differentially succinyllysine sites using student's *T*-test with a *P*-value < 0.05. Categorical annotation was supplied in the form of Gene Ontology (GO) cellular component, biological process, and molecular function, and Kyoto Encyclopedia of Genes and Genomes (KEGG) for pathway annotation. For each category, a two-tailed Fisher's exact test was employed to test the enrichment of the differentially modified protein against all identified proteins. The category with a corrected *P*-value < 0.05 was considered significant. Motif-x software was used to assess conserved sequence motifs of succinylation.

2.8 Hierarchical Clustering

According to previous reported methods (Bai et al. 2020), hierarchical clustering of differentially expressed (DE) succinylpeptides was performed to determine the differences among ICH groups.

Hierarchical clustering was carried out using the heatmap.2 function in R statistical analysis package (version 3.4.0) (Ihaka and Gentleman 1996). The clustering for heatmap.2 was obtained with Ward's algorithm using Euclidean distance. Groups were clustered and visualized with heat-maps based on the relative expression profiles.

2.9 Weighted Gene Correlation Network Analysis (WGCNA)

The analysis was carried out with the WGCNA R package (Langfelder and Horvath 2008). Pearson correlation matrix (with direction, i.e., for building signed correlation network) was calculated using the brain samples (Sham, TAK242 + ICH and Vehicle + ICH), and an adjacency matrix was calculated by raising the correlation matrix to a power of 16 using the scale-free topology criterion (Zhang and Horvath 2005) with modifications. Correlation clusters were defined by hybrid dynamic tree-cutting method (Langfelder and Horvath 2008) with the minimum height for merging modules at 0.2 and manually merged if necessary. For each cluster, a consensus trend was calculated based on the first principal component (i.e., eigengene). Succinylproteins were assigned to the most correlated cluster with a cutoff of Pearson correlation coefficient (R) at least 0.7.

2.10 Protein-Protein Interaction (PPI) Network Analysis

Succinylproteins in each Cluster were superimposed onto a composite PPI database by combining STRING (v10) (Szklarczyk et al. 2015), BioPlex (Huttlin et al. 2017), and In Web_IM (Li et al. 2017). The details of the PPI network analysis were according to previous reported methods (Bai et al. 2020).

2.11 Integrative analysis of succinylproteome data and transcriptome data

Integrative analysis was performed by comparing the succinylproteome data with a previously published transcriptome data (Zhang et al. 2014). FPKM (fragments per kilobase of transcript per million mapped reads) was used as a proxy for mRNA abundance. Based on this transcriptome data, four most studied neural cells (neuron, astrocyte, microglia, and endothelial cell) in stroke were focused, and the relatively high expression genes of neural cells were defined in this analysis as that the gene expression in one of the four neural cell types is higher than all the other three cellular types, that is the ratio > 1.0 , when the ratio > 10 define as specificity high expression in this study. Then, we matched these differentially succinyllysine sites with the transcripts to localize the cellular information of protein succinylations.

3 Results

3.1 Succinylproteome profiling analysis of intracerebral hemorrhage brain tissues

TLR4-mediated inflammation is the major contribution to the secondary brain injury after ICH (Fang et al. 2013), and we previously showed that inhibiting TLR4 signaling by its antagonist TAK242 significantly reduced the brain injury via downregulation of inflammation response (Wang et al. 2013). Additionally, considering that protein succinylation is closely related to inflammation. Then, we aimed to investigate how neuroinflammation affects protein succinylation in the perihematomal brain tissue by TAK242 treatment of ICH. Brain tissues were collected from mice with ICH treated with TAK242 or vehicle for 3 consecutive days and from Sham group also as control.

Label free LC-MS/MS was performed to detect the succinylproteome of brain tissues with the anti-succinyllysine antibody. The resulting MS/MS data was processed by MaxQuant search engine (v.1.6.15.0), and the mouse SwissProt database (17045 entries) concatenated with reverse decoy database were searched by tandem mass spectra. The accuracy of identification of the levels of spectrum, peptide, and protein is set as FDR < 1% (Fig. 1A). Then, the quality control of succinylproteomics data was confirmed by the peptide lengths distribution (Fig. 1B) and the first-order mass error of spectra (Fig. 1C). PCA analysis identified better quantitative repeatability among brain samples (Fig. 1D). Motif analysis showed that 4 conserved sequences surrounding lysine succinyllysine sites, and a significant abundance in the frequency of Ala, Gly, Ile and Val adjacent to succinylated Lysine residues were identified in the succinylproteome (Fig. 1E, F).

In this study, we identified a concentration of approximately 6700 succinyllysine events and quantified approximately 3500 sites (Fig. 1A). Approximately 36% of succinylproteins in control (Sham) brains were identified with one succinyllysine site, 16.7% with two succinyllysine sites, 11.1% with three succinyllysine sites, and 36.2% with more than three succinyllysine sites, even approximately 10% with more than 10 succinyllysine sites (Fig. 2A), which were comparable to the percentage of succinylproteins in TAK242- or Vehicle- treated ICH brains (Fig. 2A-C). Venn diagram showed that more than 90% of succinyllysine sites (Fig. 2D) and succinylated proteins (Fig. 2E) were identical among the sham, TAK242- and Vehicle- treatment ICH brains. Conversely, 20 succinyllysine sites on 4 succinylated proteins were found to be succinylated exclusively in TAK242-treated ICH but not control and Vehicle-treated brains (Fig. 2D, E), and 44 succinyllysine sites on 13 succinylated proteins were exclusively found in Vehicle-treated but not control and TAK242-treated ICH brains (Fig. 2D, E).

3.2 TAK242 treatment obviously changed the inflammation-associated protein succinylation after ICH

To explore succinylproteome dynamics after TAK242 treatment, we used a stepwise pipeline (Fig. 3A) to define 139 differentially expressed (DE) succinyllysine sites (FDR < 20%, 121 proteins, Supplementary Table 1) in 40 pathways, adapted the weighted gene correlation network analysis (WGCNA) (Langfelder and Horvath 2008) to cluster these DE succinyllysine sites, and interpreted the clusters with protein-protein interaction (PPI) network. Most of these succinyllysine sites were identified into 4 clusters from this analysis (Fig. 3B) except a few numbers of succinyllysine sites (n = 2) (Supplementary Table 2). Cluster 1 (n = 37) displays an increase in the downregulated protein succinylation caused by ICH after

TAK242 treatment, including Ccdc51_K159su, Hapln1_K105su and Nefm_K842su (Fig. 3B). These succinylated events are enriched in 24 pathways: β -Alanine metabolism, propanoate metabolism, gap junction, synaptic vesicle cycle, necroptosis, cGMP-PKG signaling pathway, etc. (Fig. 3C). Cluster 2 (n = 30) shows a marked further decrease in the ICH-associated downregulated protein succinylation after TAK242 treatment, including Atp1a3_K349su and Nipsnap2_K276su (Fig. 3B). Cluster 2 is enriched in 16 pathways, especially the following three pathways: mineral absorption, thyroid hormone synthesis and aminoacyl-tRNA biosynthesis (Fig. 3C). In contrast, Cluster 3 (n = 31) shows a marked further increase in the ICH-associated upregulated protein succinylation after TAK242 treatment (Fig. 3B), identifying 12 pathways, such as lipoic acid metabolism, synthesis and degradation of ketone bodies, butanoate metabolism, and valine, leucine and isoleucine degradation (Fig. 3C). Finally, we found that Cluster 4 shows an increase in ICH-associated downregulated protein succinylation, followed by a notable increase after TAK242 treatment (Fig. 3B); and most of these succinylated proteins were enriched metabolism, such as oxidative phosphorylation (OXPHO), fatty acid degradation, glyoxylate and dicarboxylate metabolism, and ferroptosis, etc. (Fig. 3C). Furthermore, we integrated the DE succinylproteins in each Cluster pattern with the PPI network to define 11 functional modules, providing evidence of PPI to support these enrichment pathways (Fig. 3D).

3.3 Analysis of succinylproteomic data of ICH-associated brain tissues treated with TAK2424

Next, we analyzed the succinylproteomic data between the TAK242- and Vehicle-treated ICH brains to identify how the TLR4-mediated inflammation affects protein succinylation changes in the perihematoma brain tissue by TAK242 treatment of ICH. Firstly, we found that 29 succinyllysine sites of 28 succinylated proteins were increased, and 24 succinyllysine sites on 23 succinylated proteins were downregulated when compared TAK242-treated ICH brains to Vehicle-treated groups (Fig. 4A). GO analysis using A.GO.TOOL (Scholz et al. 2015) showed that, compared to the brain proteome, the brain succinylproteome was overrepresented for GO localization categories of myelin sheath, respiratory chain complex II, succinate dehydrogenase complex, fumarate reductase complex, golgi membrane, T-tubule, plasma membrane, synapse, neuronal cell body membrane, postsynaptic density, etc. (Fig. 4B). Then, we further analyzed the subcellular localization of these significantly changed succinylproteins, and we found that approximately 48% hyposuccinylated proteins were located in the mitochondria and followed by 30.4% in cytoplasm (Fig. 4C). The percentage of succinylproteins located in mitochondria was decreased to about 32% in TAK242-treated ICH brains with concomitant increasing in the percentage of plasma membrane and nucleus to 17.9% and 7.1%, respectively; and peroxisome, cytoskeleton and cytoplasm, nucleus were additionally identified (Fig. 4D).

3.4 Analysis and annotation of differentially succinylated proteins in ICH brains after TAK2424 treatment

We found that ICH-associated hyposuccinylation was comparable with hypersuccinylation in succinylproteins carrying one or two succinyllysine sites per protein after TAK242 treatment when compared to the Vehicle treatment (Fig. 5A). GO cellular component enrichment analysis showed that both hyposuccinylation and hypersuccinylation datasets were significantly enriched for plasma membrane, integral component of membrane and cell periphery (Fig. 5B). Only the hypersuccinylation, but not hyposuccinylation, was significantly linked to structures (e.g., synaptic membrane, presynaptic membrane, presynaptic active zone membrane, presynaptic active zone, postsynaptic specialization, postsynaptic density, and postsynapse), metabolism and its relative enzymes (e.g., succinate dehydrogenase complex, respiratory chain complex II, fumarate reductase complex, and Atpase complex, Atpase dependent transmembrane transport complex), somatodendritic compartment, myelin sheath, cytoplasmic ribonucleoprotein granule, contractile actin filament bundle, actomyosin, etc. (Fig. 5B); whereas hyposuccinylation, but not hypersuccinylation, was enriched for membranes, including vacuolar membrane, trans-golgi network membrane, side of membrane, organelle membrane, membrane, lytic vacuole membrane, lysosomal membrane, golgi membrane, coated vesicle membrane, and clathrin-coated vesicle membrane, coated vesicle and Clathrin-coated vesicle (Fig. 5B). In consistent with the GO localization enrichment results, we found that most of the hyposuccinylated proteins were enriched for metabolism (e.g., sterol metabolic process, secondary alcohol metabolic process, monocarboxylic acid metabolic process, lipid oxidation, cholesterol metabolic process, cellular lipid catabolic process) and transportation (e.g., regulation of anion transport, positive regulation of ion transport, inorganic anion transport, anion transport, anion transmembrane transport) (Fig. 5C). While, only hypersuccinylated proteins were mostly enriched for GO categories linked to synapse working (trans-synaptic signaling, synaptic vesicle transport, synaptic vesicle localization, synapse organization, regulation of synaptic vesicle transport and anterograde trans-synaptic signaling) and vesicle functions (e.g., vesicle organization, vesicle docking, regulation of secretion by cell, regulation of secretion, anion transport) (Fig. 5C). The GO molecular function enrichment analysis showed that both hyposuccinylation and hypersuccinylation datasets were significantly enriched for protein kinase binding and kinase binding (Fig. 5D). However, only hypersuccinylation, but not hyposuccinylation, was significantly linked to succinate dehydrogenase activity, steroid hormone binding, nitric-oxide synthase binding, G-protein coupled receptor binding, chaperone binding, exopeptidase activity, dopamine receptor binding, cytoskeletal protein binding, etc. (Fig. 5D); whereas hyposuccinylation, but not hypersuccinylation, was enriched for symporter activity, solute: cation symporter activity, clathrin binding, carbohydrate binding, C-acyltransferase activity, anion transmembrane transporter activity, and Acetyl-coa c-acyltransferase activity (Fig. 5D). Additionally, the KEGG analysis of these significantly changed succinylated proteins showed that hypersuccinylation was enriched for carbon metabolism, synaptic vesicle cycle, citrate cycle (TCA cycle), carbohydrate digestion and absorption, arginine biosynthesis, SNARE interactions in vesicular transport, etc. (Fig. 6A), while hyposuccinylation was significantly related to fatty acid elongation, fatty acid degradation, fatty acid metabolism, and lysosome. (Fig. 6B).

3.5 Combined transcriptome and succinylproteome analysis in ICH brains treated by TAK242

Furthermore, we performed a combination analysis of a previously reported RNA-seq data of neural cells (Zhang et al. 2014) with our succinylproteome data to reveal the cellular information of ICH-associated significantly changed protein succinylations after TAK242 treatment. Given that neurons, astrocytes, microglia, and endothelial cells are the four kinds of most studied neural cell types in stroke research, therefore, we chose the four kinds of neural cells for this joint analysis. The relatively high expression genes of neural cells were defined as that the gene expression in one of the four neural cell types is higher than all the other three cellular types.

After matching the relatively high expression genes of neural cells with the obviously changed succinylproteomic data, we found that most of the succinylproteins were relatively high expressions in neurons, astrocytes and endothelial cells (Fig. 7A-C). Furthermore, we found that 37% of hypersuccinylated proteins were identified in neurons, 22% in astrocytes, 16% in endothelial cells, and 7% in microglia (Fig. 7B); while, the percentage of hyposuccinylated proteins was decreased to 32% in neurons with concomitant increasing in the percentage of endothelial cells (Fig. 7C). As the specificity expression level of these proteins in nerve cells varies greatly, for example, the ratios of the matched proteins in neurons and astrocyte exceeds 5. Therefore, we further analyzed the information of ratio distribution specificity of these matched succinylated proteins. The pie charts show that the specificity ratios of 7 proteins (Ina, Atp1a3, Cntn1, Vsnl1, Dnm1, Cend1 and Stxbp1) in neurons exceed 10 than other neural cells, and 5 proteins (Mapt, Uchl1, Snca, Slc25a22, Stx1b) exceed 5 (Fig. 7D). For the astrocytes, there were also 3 proteins' specificity ratio exceed 10, including Atp1a2, Slc1a3, and Slc6a11, and 1 protein (Acsf6) exceed 5 (Fig. 7E). In contrast, the specificity ratios of all the matched succinylated proteins in the microglia (Fig. 7F) and endothelial cells (Fig. 7G) were under 3. Moreover, we found that the specificity ratios of most of these succinylated proteins in the four neural cells varied from 1 to 3 (Fig. 7D-G), especially for endothelial cells because of 86% protein specificity ratios were under 2 (Fig. 7G).

4 Discussion

TLR 4 signaling contributes to brain injury after ICH and promotes the accumulation of the protein succinylation, which has been shown to regulate metabolic processes, and immunity and inflammation. In this study, we are the first to investigate the contribution of TLR4 signaling in protein succinylation in ICH brains using a high-resolution LC-MS/MS-based quantitative succinylproteomic approach. The results showed that the TAK242 treated ICH brains underwent significant changes in protein succinylation. These significantly changed succinylated proteins were mainly distributed into mitochondria and cytoplasm, and may participate in the metabolic processes, neuronal functions and so on. KEGG pathway analysis showed that TAK242 treatment induced downregulation of protein succinylations were mainly enriched in fatty acid metabolism, which was previously little recognized. Further integration of our succinylproteomics data with previously published transcriptome data showed that some specificity high expression succinylated proteins (ratio > 10) were found in neurons and astrocytes after ICH treated with TAK242, suggesting that these proteins' succinylation may be involved in TLR4 signaling-related regulation of biological functions of neurons and astrocytes. The mouse brain

succinylproteome dataset generated from this work provides a useful resource for future investigations into the roles of TLR4-mediated inflammation in protein succinylation in brain injury after ICH.

PTMs have been recognized as an extension of metabolic regulation of cell life activities. For example, the TCA metabolite succinate could promote inflammation may via increasing protein succinylation (Tannahill et al. 2013). However, to date, the protein succinylation regulated by inflammation in ICH brains is little known to us although the roles of inflammation in contributing brain injury after ICH has largely been known. Therefore, in this study, we aimed to investigate the protein succinylations affected by TLR4 signaling activation in ICH-induced brain injury. Using quantitative succinylproteomic analysis, we first revealed a concentration of approximately 6700 succinyllysine events and quantified approximately 3500 sites. Then, 139 DE succinyllysine sites in 40 pathways were defined and identified into 4 clusters by WGCNA. Among them, Cluster 1 displays that TAK242 treatment increased the downregulated protein succinylation caused by ICH and these succinylated proteins are enriched metabolism, gap junction, synaptic vesicle cycle, necroptosis, cGMP-PKG signaling pathway, etc.. The succinylated proteins in Cluster 4 were upregulated by ICH but decreased after TAK242 treatment. Interestingly, most of these succinylated proteins were enriched metabolism, such as oxidative phosphorylation (OXPHO), fatty acid degradation, glyoxylate and dicarboxylate metabolism, and ferroptosis, etc.. These results suggest that TLR4-mediated inflammation might be involved in metabolic processes after ICH via protein succinylation, which was an important addition to the study field that inflammation and metabolism (Corcoran and O'Neill 2016; Yin et al. 2016; Killen et al. 2019). Although some studies have already showed that LPS stimulation could promote protein succinylation (Mills and O'Neill 2014; Tannahill et al. 2013; Wang et al. 2017), our study provides a useful resource for details of inflammation mediated protein succinylation.

Furthermore, we compared the TAK242- and Vehicle treated ICH brains to obtain the information about roles of TLR4 signaling in protein succinylation after ICH. Our data showed that 29 succinyllysine sites of 28 succinylated proteins were increased, and 24 succinyllysine sites on 23 succinylated proteins were downregulated in TAK242 groups when compared Vehicle group, suggesting that TAK242 treatment could significantly change the protein succinylation. Then, GO and KEGG analysis of these DE succinylated proteins indicated that TLR4 signaling-associated hypersuccinylation and hyposuccinylation can almost independently affect biological processes after ICH. In particular, the KEGG pathway analysis showed that the TAK242 reduced succinylated proteins were mainly involved in the elongation, degradation and metabolism of fatty acid, indicating that fatty acid metabolism may play an important role in involving brain injury and that might be closely correlated with inflammation after ICH.

Additionally, as different neural cells respond differently to the brain injury after ICH, accordingly, we made a conjoint analysis of a previously published transcriptome with our succinylproteome data to insight into which kind of neural cell is the major response cell to protein succinylation regulated by TLR4 signaling. The results showed that the protein succinylation preferentially occurred in neurons, endothelial cells and astrocytes after ICH treated by TAK242. Furthermore, we found that seven succinylated proteins (Ina, Atp1a3, Cntn1, Vsnl1, Dnm1, Cend1 and Stxbp1) were significantly high

expression in neurons than other three neural cells, and three succinylated proteins (Atp1a2, Slc1a3, and Slc6a11) were markedly high expression in astrocytes than other three neural cells, these results strongly suggest that these proteins' succinylation might be important to regulate neuronal and astrocytic functions in response to TAK242-treated ICH brains. However, follow-up studies will be needed to further define the roles of these succinylated proteins after ICH.

In summary, our study has first investigated the protein succinylation profiles regulated by TLR4 signaling after ICH and the TLR4 signaling-associated protein succinylation changes uncovered in this study indicate that the changed protein succinylation may be a downstream mechanism of inflammation that contributes to brain injury. The identified differentially protein succinylation in TAK242-treated ICH brains provides new molecular and system-level insights into ICH pathogenesis and offers novel insight into the damage mechanisms of inflammation-related brain injury after ICH.

5 Declarations

Conflict of Interest statement

The authors declare no competing interests.

Ethical Approval

The animal protocol was approved by the Animal Care and Ethics Committee of the Chengdu University of Traditional Chinese Medicine, whose standards meet the Animal Care guidelines of the NIH, USA.

Reviewer account details to access this dataset PXD025622

Username: reviewer_pxd025622@ebi.ac.uk

Password: zzCwENms

Author contributions

SGY and XYX designed the research studies and wrote the manuscript. YJL, YRY, and CYT conducted the experiments and analyzed the data. SHY, XXZ, JY, and ZQZ analyzed the data.

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Figures

Figure 1

Analysis of succinylproteomic data of ICH brains. (A) Overview of protein and succinylation identification. (B and C) Data quality control of peptide length distribution (B) and peptide mass tolerance distribution (C). (D) PCA analysis shows the dispersion degree of succinylproteome between Sham, TAK242- and Vehicle-treated ICH brains. (E) Amino acid sequence properties of succinyllysine sites. The heat map shows significant position-specific under-representation or over-representation of amino acids flanking the succinyllysine sites. (F) Succinylation motifs and the conservation of succinyllysine sites. The height of each letter corresponds to the frequency of that amino acid residue at that position. The central K refers to the succinylated Lys.

Figure 2

Quantitative succinylproteomics analysis of ICH brains. (A-C) Pie charts showing the proportions of singly and multiply succinylated proteins with the indicated number of succinyllysine sites per protein in Sham (A), Vehicle- (B) and TAK242-treated ICH (C) brains. (D-E) Venn diagram comparison of succinyllysine sites (D) and succinylated proteins (E) in Sham, TAK242- and Vehicle-treated ICH brains.

Figure 3

Whole-succinylproteome profiling of ICH-related brain tissues reveals TAK242 treatment-dependent succinyllysine sites and/or pathways. (A) Bioinformatics pipeline for identifying DE succinyllysine sites, followed by clustering and module analyses. (B) Four major clusters (Cluster 1 – Cluster 4) of DE succinyllysine sites defined by the WGCNA program. Each line represents one succinyllysine site. The intensity of each protein is log₂Z score transformed (mean-centered and scaled by standard deviation). The line color indicates the degree of pattern correlation between each succinyllysine site and eigenvalue of the cluster. (C) Enriched pathways in the Cluster succinyllysine sites by Fisher's exact test. Significantly enriched pathways (FDR < 20%) are shown. (D) Detected PPI modules in Clusters.

Figure 4

Analysis of succinylproteome data in TAK242-treated ICH brains. (A) The heat map shows the differentially succinyllysine sites in TAK242- and Vehicle-treated brains. (B) GO localization categories enriched in the succinylproteome compared to the proteome with Benjamini-Hochberg false discovery rate (FDR)-corrected $P < 0.0001$. (C and D) The subcellular localization of hyposuccinylated (C) or hypersuccinylated (D) proteins in TAK242-treated ICH brains when compared to Vehicle-treated brains.

Figure 5

GO analysis of differentially succinylated proteins in TAK242-treated ICH brain. (A) Distribution of TAK242-treated ICH-associated hyposuccinylated or hypersuccinylated proteins carrying the indicated number of in vivo succinyllysine sites per protein. (B to D) GO cellular component (B), biological process (C), and molecular function (D) categories enriched in ICH-associated hyposuccinylated or

hypersuccinylated proteins after TAK242 treatment when compared to Vehicle treatment. Significant enrichment is shown by Benjamini-Hochberg FDR-corrected $P < 0.05$ (outside the dashed line).

Figure 6

KEGG pathway analysis of differentially succinylated proteins in TAK242-treated ICH brain. (A and B) KEGG pathway analysis of ICH-associated hypersuccinylated (A) or hyposuccinylated (B) proteins after TAK242 treatment when compared to Vehicle treatment.

Figure 7

Combined analysis of succinylproteomic and transcriptomics data. (A-C) The pie charts show the major cellular distribution of the whole differentially ICH-associated succinyllysine sites (A), significantly upregulated (B) and downregulated succinyllysine sites (C) after TAK242 treatment when compared to Vehicle treatment, respectively. Matching the significantly changes proteins ($P < 0.05$ by Student's test) with the relatively high expression genes of neural cells (the gene expression in one of the four neural cell types is higher than all the other cellular types) from the published transcriptome data (Zhang et al. 2014). (D-G) The pie charts show the ratio distribution of modified succinylated proteins specificity high expression in neurons (D), astrocytes (E), microglia (F), and endothelial cells (G).

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