Exploring the protective mechanism of baicalin in treatment of atherosclerosis using endothelial cells degranulation model and network pharmacology

Conglin Ren (201911010811035@zcmu.edu.cn)
Taizhou Hospital, Shanghai University of Traditional Chinese Medicine
Mingshuang Li
Taizhou Hospital, Shanghai University of Traditional Chinese Medicine

Research Article

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Abstract

Background: Baicalin is widely used in clinical practice. The present study aimed to assess the underlying mechanism of baicalin in treatment of atherosclerosis (AS) with the help of network pharmacology, molecular docking and experimental validation.

Materials and methods: The target genes of baicalin and AS were identified from public databases, and the overlapping results were considered to be baicalin-AS targets. Core target genes of baicalin were obtained through the PPI network and clinical data sets GSE132651. Human aortic endothelial cells (HAECs) were treated with baicalin, followed by Lipopolysaccharide (LPS) to construct a model of endothelial injury. The expression of core targets was examined by real-time qPCR. Flow cytometry was used to detect reactive oxygen species (ROS) level.

Results: NOX4 is likely to be the core target of baicalin for AS, and its expression was inhibited in AS patients compared to controls. Functional enrichment analysis demonstrated that most targets were involved in oxidative stress. Intervention with baicalin increased the expression levels of NOX4 and NOS3 (eNOS). Moreover, baicalin also blocked the LPS-induced ROS generation in HAECs.

Conclusions: Baicalin might ameliorate oxidative stress on endothelial cells via targeting NOX4.

1. Introduction

Atherosclerosis (AS) is the main cause of cardiovascular disease (CVD) and difficult to detect in early stages until lipid-burdened plaque ruptures or erodes, leading to thrombosis and tissue damage[1]. Endothelial cells (ECs) are the first line of defense for vascular health. Both EC activation and infiltration of circulating monocytes are crucial factors in the occurrence and progression of atherosclerosis. Inflammatory EC recruit leukocytes by expressing adhesion molecules. Circulating monocytes infiltrate the damaged endothelium and differentiate into macrophages. Excess lipid load promotes macrophages differentiation into foam cells. This process promotes the release of pro-inflammatory factors and generation of reactive oxygen species (ROS)[2]. Oxidative stress mediated by ROS plays a key role in process of atherosclerosis. ROS contribute to atherosclerosis via mediating inflammation, apoptosis and altered vascular tone. NADPH oxidase (Nox) enzyme complexes are predominant sources of ROS in the vessel wall[3]. Increased expression of Nox1, Nox2 and Nox5 has been reported as contributing to atherosclerosis and vascular diseases[4, 5]. However, Nox4 is the predominant isoform in ECs, which exerts antioxidant effects by increasing endothelial NO release by AKT-dependent phosphorylation[6, 7].

Chinese herbal medicine is the most widely used form of complementary and alternative medicine, which has been practiced for centuries in Asia. In treatment of coronavirus disease 2019, the effects were remarkable[8–11]. Baicalin are representatives of flavonoids, whose multiple pharmacological functions have been studied. Baicalin has been reported to alleviate oxidative stress and inflammation via the NF-κB pathway. It could arrest growth cycle of vascular smooth muscle cells and inhibit intimal proliferation after injury[12].

Network pharmacology, first proposed by Andrewl Hopkins in 2007[13], combines pharmacology with bioinformatics to reveal specific targets for drug interventions in disease process, helping to advance the development of precision medicine[14]. It also can be used to analyze the mechanism of herbal formula, such as BuYangHuanWu decoction[15] and Chaihu Lizhong Tang[16]. In current study, we used multiple public databases to construct pharmacological networks and elucidate the targets of baicalin in treatment of AS. Then, with the help of molecular experiments, an attempt was made to explore the protective mechanism of baicalin in treatment of AS. The flow chart was exhibited in Fig. 1.

2. Materials And Methods

2.1. Predicting target genes of baicalin

We queried the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) for the 2D structures, molecular formulas, canonical SMILES strings and SDF files of baicalin and predicted potential targets in three public databases including Similarity ensemble approach (SEA)[17], Swiss Target Prediction [18]. and Gene Cards [19]. For each database, species was limited to "Homo sapiens" and the top 100 results ranked by predicted score or p-value were retained. In addition, only genes that were simultaneously predicted by two or three databases were screened as high confidence target genes.

2.2. Collection of target genes for AS

Similarly, to identify key genes associated with the development of AS, we searched for the keyword "atherosclerosis" in multiple databases(i.e., DisGeNET [20], Gene Cards and DrugBank [21]) and collected overlapping results as vital pathogenic targets of AS for further study.

2.3. Identification of baicalin-AS targets

The target genes of baicalin were matched with AS-related targets, and overlapping data were considered to be potential targets of baicalin for AS treatment. Venn diagrams were generated in R software using “VennDiagram” package to visualize the intersection genes.

2.4. Functional enrichment analysis of compound-disease targets

To investigate the biological roles or pathways in which intersection genes are involved, we performed Gene Ontology (GO) and KEGG analysis in R software using “clusterProfiler” [22] and “ggplot2” packages[23]. Results of GO and KEGG with statistical significance (adjust p-value < 0.05) were screened and visualized by bubble plots and compound-target-pathway networks, respectively.

2.5. Protein–Protein Interaction (PPI) data and hub gene modules

The STRING database (https://string-db.org/, ver. 10.5)[24, 25] was used to analyze the protein– protein interactions of the intersection targets of the baicalin and AS. The database defines PPIs with confidence

ranges for data scores. Result was exported as a “TSV” format file and imported into Cytoscape[26] to visualize the PPI network. The MCODE plugin was installed in Cytoscape to identified clusters made up of highly related genes in the network[27]. The parameters used in our analysis were default values. Genes in the highest scoring cluster were considered to be hub genes of baicalin for AS treatment

2.6. Molecular Docking

2D structure of baicalin has been obtained in PubMed. And the crystal structures of hub target genes were downloaded from RCSB Protein Data Bank (http://www.pdb.org/). Then, AutoDOCK 1.5.6 software was used to prepare receptors, including deleting water, adding hydrogen and setting docking paraments. “Grid box” was set to maximum to perform the blind docking. A binding energy less than − 5 $kJ•mol^{-1}$ indicates that the ligand can spontaneously bind the receptor. PyMOL was selected to visualize the results of molecular docking.

2.7. Probing the expression of hub genes in endothelial injury model

To examine gene expression patterns of hub genes (up- or down-regulation) in endothelial injury model, we performed clinical dataset analysis on GSE132651, which is a microarray dataset that quantified gene expression of ECs from living subjects. This dataset consisted of 19 samples, including 6 normal controls and 13 subjects with abnormal endothelial function (showing a risk for early AS). We retrieved original matrix files from GEO repository (https://www.ncbi.nlm.nih.gov/geo/) and screened differentially expressed genes (DEGs) between the two groups using “Limma” package [28]. All DEGs were required to meet $|\log2 \text{ fold change (FC)}| > 0.5$ as well as $p\text{-value} < 0.05$.

2.8. Reagents

Baicalin (purity > 98%) was supplied by Yuan-ye biotechnology (Shanghai, China). Lipopolysaccharide (LPS), DMSO, and cell counting kit-8 (CCK-8) were obtained from Meilun biology (Dalian, China). EGM-2 Bullet Kit were obtained from Lonza (#CC-3162, Basel, Switzerland), including endothelial cell basal medium, FBS, VEGF, etc. RNA-quick purification kit was purchased from Yi-shan biotechnology (Shanghai, China). First strand cDNA synthesis kit and SYBR Green master mix were obtained from Thermo Scientific (Shanghai, China). PCR primers were synthesized by Sangon biotechnology (Shanghai, China). DCFDA / H2DCFDA cellular ROS assay kit was obtained from Abcam (Massachusetts, USA).

2.9. Cell culture

Human aortic endothelial cells (HAECs) were purchased from Chinese Tissue Culture Collections (CTCC). Cells were cultured in MEM medium supplemented with 10% FBS, 2 mM L-glutamine, non-essential amino acids, 1000 units/mL penicillin, 100 ug/mL streptomycin, and 0.25 ug/mL amphotericin B, and maintained in a humidified incubator at 37 ℃ with 5% CO2. Cells were pretreated with optimal concentrations of baicalin for 12h, and then stimulated with LPS (1 ug/ml) for 6 hours.

2.10. Cell viability assay

Cytotoxicity of baicalin was tested by CCK-8 assay, according to the manufacturer's instructions. Briefly, to obtain optimal dose of intervention, HAECs were seeded in 96-well culture plates and exposed to gradually increasing concentrations of baicalin for 12 h. The absorbance value of each well was measured at 450 nm using EnSpire multifunctional microplate reader (PerkinElmer, USA). The cell viability of 0 μM served as the control group.

2.11. Quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted from HAECs and its purity was checked using Nanodrop (Thermo Scientific, USA). After that, mRNA was transcribed into the first-strand cDNA and subjected to polymerase chain reaction on 7500 real-time PCR system (Applied Biosystems, USA). We chose GAPDH as endogenous control and compared gene expression based on the 2-△△CT algorithm. The primer sequences used in current study include: GAPDH forward 5'- GTCTCCTCTGACTTCAACAGCG – 3'; reverse 5'-ACCACCCTGTTGCTGTAGCCAA – 3'; NOX4 forward 5'- GCCAGAGTATCACTACCTCCAC – 3'; reverse 5'-GTGACTCCTCAAATGGGCTTCC – 3'; NOS3 forward 5'-GAAGGCGACAATCCTGTATGGC – 3'; reverse 5'-TGTTCGAGGGACACCACGTCAT – 3'.

2.12. Intracellular ROS measurement

Briefly, HAECs pretreated with baicalin for 12 hours were harvested and stained with 20 μM DCFDA in culture medium. After incubation at 37℃ for 30 minutes (protected from light), the cells were washed with 1X buffer and treated with 1 ug/ml LPS for 6 hours. Then, cells were gently pipetted to make single cell suspension and analyzed on flow cytometer (BD Accuri C6, USA). Data processing was carried out on FlowJo software (version 10, USA).

2.13. Statistical analysis

Statistical analysis was performed in GraphPad Prism 7.0 (California, USA). Data were shown as mean ± SEM. The t-test was used for comparisons between two groups; one-way analysis of variance was taken for comparisons between three or more groups. Result with p-value < 0.05 was considered significant.

3. Results

3.1. Collection target genes of baicalin

The skeletal formula of baicalin was created using ACD/ChemSketch software (version 12.0, Advanced Chemistry Development, Toronto, Canada) (Fig. 2a). We collated target genes predicted by SEA, Swiss Target Prediction and Gene Cards databases for these two compounds. Based on the screening criteria mentioned in “methods”, we obtained 120 targets with high confidence for baicalin.

3.2. Identification of AS-related target genes

By integrating disease targets derived from the DisGeNET, Gene Cards, and DrugBank databases, we ultimately retained 497 genes that appeared in at least two databases. Admittedly, the pathophysiology of AS is complicated, with an extensive number of genes involved and interacting with each other.

3.3. Baicalin -AS target genes

We obtained intersection data by matching target genes of baicalin with AS-related target genes. As shown in Venn diagrams (Fig. 2b and TableS1), 45 baicalin-AS target genes were generated, which were potential targets of baicalin for AS treatment.

3.4. Functional enrichment analysis of baicalin-AS targets

The potential biological processes and signaling pathways involved in the therapeutic application of baicalin were probed by functional enrichment analysis. Top 10 GO terms of baicalin-AS targets, ordered by adjust p-value, were shown in Fig. 3a. Interestingly, the top three were closely related to oxidative stress, including responses to oxidative stress, cellular responses to oxidative stress, and metabolic processes of reactive oxygen species (ROS). Baicalin has already reported to alleviates oxidative stress and inflammation via Nrf2 and MAPK Signaling Pathway. Therefore, the finding in our study was consistent with the pharmacological mechanism of baicalin studied in the literatures. KEGG pathway analysis showed that DEGs primarily participated in IL-17 signaling pathway, Human cytomegalovirus infection, Fluid shear stress and atherosclerosis and TNF signaling pathway. Details were presented in Fig. 3b. The enrichment analysis results revealed that most of the therapeutic targets were associated with inflammation, suggesting that baicalin may against AS through anti-inflammatory effects. The correlations of compounds, target genes, and signaling pathways were manually sorted in Excel and then entered into Cytoscape software to construct visible interaction networks Fig. 3C.

3.5. Protein–Protein Interaction Network Construction

Totally, 45 target genes were imported into STRING for building a PPI network containing 44 nodes and 542 edges (Fig. 4A). The hub target genes were more likely to play a vital role in anti-AS containing STAT3, IL6, TNF, AKT1, NOX4, CASPAS3, MMP9, PTGS2, HIF1 and IL1B.

3.6. Verification of Ligand–Receptor Interaction

The five hub target genes, STAT3, IL6, TNF, MMP9 and NOX4, were molecularly docked with baicalin (Table 1). It was confirmed that baicalin had a high binding activity with the hub target genes, among which binding with NOX4 was the strongest. Thus, it was speculated that NOX4 might be an essential target of baicalin in the treatment of AS (Fig. 4B).

Table 1
Results of molecular docking of hub target genes and baicalin.

The target gene	Key components	Binding energies (kcal/mol)
NOX4	Baicalin	-9.31
IL6	Baicalin	-5.26
STAT3	Baicalin	-5.73
TNF	Baicalin	-6.21
MMP9	Baicalin	-5.48

3.7. NOX4 expression was down-regulated in endothelial injury condition

In terms of the GSE132651 dataset, a total of 151 DEGs (78 up-regulated and 73 down-regulated genes) were obtained between AS and control samples according to the thresholds we set. Next, we assessed the expression of hub genes in GSE132651 based on probe intensity, and found that Only NOX4 showed differences, with expression was markedly suppressed in the AS samples (mean, 176.8) than in controls (mean, 285.1) ($p < 0.05$) (Fig. 4C).

3.8. The cytotoxic effect of baicalin in HAECs

The dose-dependent cytotoxic effects of baicalin on HAECs was detected by CCK-8 assay. Figure 5a showed that baicalin had no significant cytotoxicity on the HAECs within 30uM. As the results, we took a safe concentration of 30uM for baicalin in following experiments

3.9. Baicalin increased NOX4 and eNOS expression

ECs are the first line of cardiovascular defense. We stimulated HAECs with LPS to establish EC dysfunction model[29, 30]. As shown in Fig. 5B, the mRNA expression of NOX4 was decreased under LPS stimulation, while baicalein increased its expression. It suggested that baicalin may exert therapeutic effects by targeting NOX4. eNOS interacted with L-arginine to promote the synthesis of nitric oxide. Abnormal function of eNOS to produce NO efficiently will increase oxidative stress. Baicalin can elevate eNOS level, thereby increasing nitric oxide bioavailability and alleviating oxidative stress.

3.10. Baicalin inhibited ROS release in HAECs

GO enrichment analysis revealed that baicalin were closely related to oxidative stress. Excessive intracellular ROS cause endothelial dysfunction and oxidative stress injury. To assess whether baicalein were involved in regulation of ROS in HAECs, we used DCFH-DA staining to measure intracellular ROS levels. Notably, the results of flow analysis showed that baicalein significantly reduced intracellular ROS levels.

4. Discussion

Chinese medicine is widely used in clinical treatment in Asia. It has gradually shown its advantages owing to multi-target characteristics and positive therapeutic effects. However, the application of TCM is hampered by complex composition of natural herbs and lack of scientific understanding about clear therapeutic mechanisms. With the flourishing development of bioinformatics and network pharmacology, it is possible to elucidate active ingredients and intervention mechanisms of Chinese herbal medicine. In this study, network pharmacology was applied to predict the putative targets of baicalin for AS treatment. Baicalin, one of main effective components of Scutellaria baicalensis Georgi, has been proved to protect against AS by inhibiting inflammation, blocking the proliferation of vascular smooth muscle cells, and promoting cholesterol efflux from macrophages[31–34]. However, the molecular mechanism of baicalin treatment of AS remain diverse, and several molecular pathways are under intense investigation.

Oxidative modifications of lipids have been detected in vascular lesions and the degree of oxidation correlates with the severity of disease, suggesting a role of oxidative stress in atherogenesis[35]. The vascular isoforms of the NADPH oxidase (NOX) family of proteins have been shown to be important mediators of this. NOX1 and NOX2 have been shown to be deleterious to vascular disease development, particularly in the context of atherosclerosis. By contrast, NOX4, the main NADPH oxidase isoform in the endothelium, protects against atherosclerosis in several murine models independently[36–38]. NOX4 mainly released H2O2 to stimulate endothelial NOS expression and NO formation. It can also protect AS through mediating homeostatic correction of endoplasmic reticulum stress[39, 40]. eNOS promotes the formation of NO in the vascular endothelium. ROS are spatially close to eNOS and can directly provoke the uncoupling of eNOS, reducing NO bioavailability and activating other redox-sensitive systems[41]. Oxidative stress was considered as the major contributor to eNOS uncoupling and endothelial dysfunction. It was also known that ROS impair endothelial NO-mediated coronary micro-vessel dilation[42].

Endothelial cells serve as the first line of defense for AS. Its damage is one of the earliest manifestations of AS. We selected the clinical dataset, GSE132651, to explore the expression of target genes in response to endothelial injury. NOX4, as the only gene with statistically significant differences, was down-regulated in endothelial injury condition in clinical dataset GSE132651. Our results precisely indicated that baicalin were highly correlated with oxidative stress. GO results showed that the targets of baicalin-AS target genes could be enriched in oxidative stress. Given previous literature demonstrating that NOX4 was closely related to oxidative stress. Through RT-qPCR, we found that baicalin could raise the NOX4 and eNOS expression. Meanwhile, it also reduced intracellular ROS levels. Therefore, we conclude that baicalin may exert its pharmacological function by targeting NOX4 to reduce oxidative stress.

Conclusion

In conclusion, the pharmacological mechanism by which baicalin treated AS was investigated by combining network pharmacology prediction and experimental validation. We demonstrated that baicalin

might affect AS mainly by upregulated NOX4 expression. Moreover, oxidative stress plays an important regulatory role in the mechanism of baicalin in AS treatment. Baicalin might ameliorate oxidative stress on endothelial cells via targeting NOX4.

Some limitations about this study should be pointed out. Firstly, in order to obtain comprehensive compound-related targets, we used three different databases systematically. However, due to different computer algorithms of each database, there may be some discrepancies. Secondly, our conclusions were only verified in cellular experiments. Animal experiments were not currently performed. In subsequent studies, Nox4-/- mice should be presented to scientifically and rigorously demonstrate the in-depth mechanism of flavonoids in the mitigation of AS.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The dataset analyzed during the current study are available in the GEO repository, GSE132651. Other data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

The authors declare no conflicts of interest in this work.

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Authors' contributions

Mingshuang Li and Conglin Ren contributed to the experimental design and financial support for this study. Mingshuang Li contributed to carry out the experimental design and data analysis and wrote the paper. Conglin Ren performed the molecule docking analysis.

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Not applicable.

Author information

1: Taizhou Hospital, Shanghai University of Traditional Chinese Medicine, Taizhou, Zhejiang, China

2Taizhou Hospital of Traditional Chinese Medicine, Taizhou, Zhejiang, China

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Figures

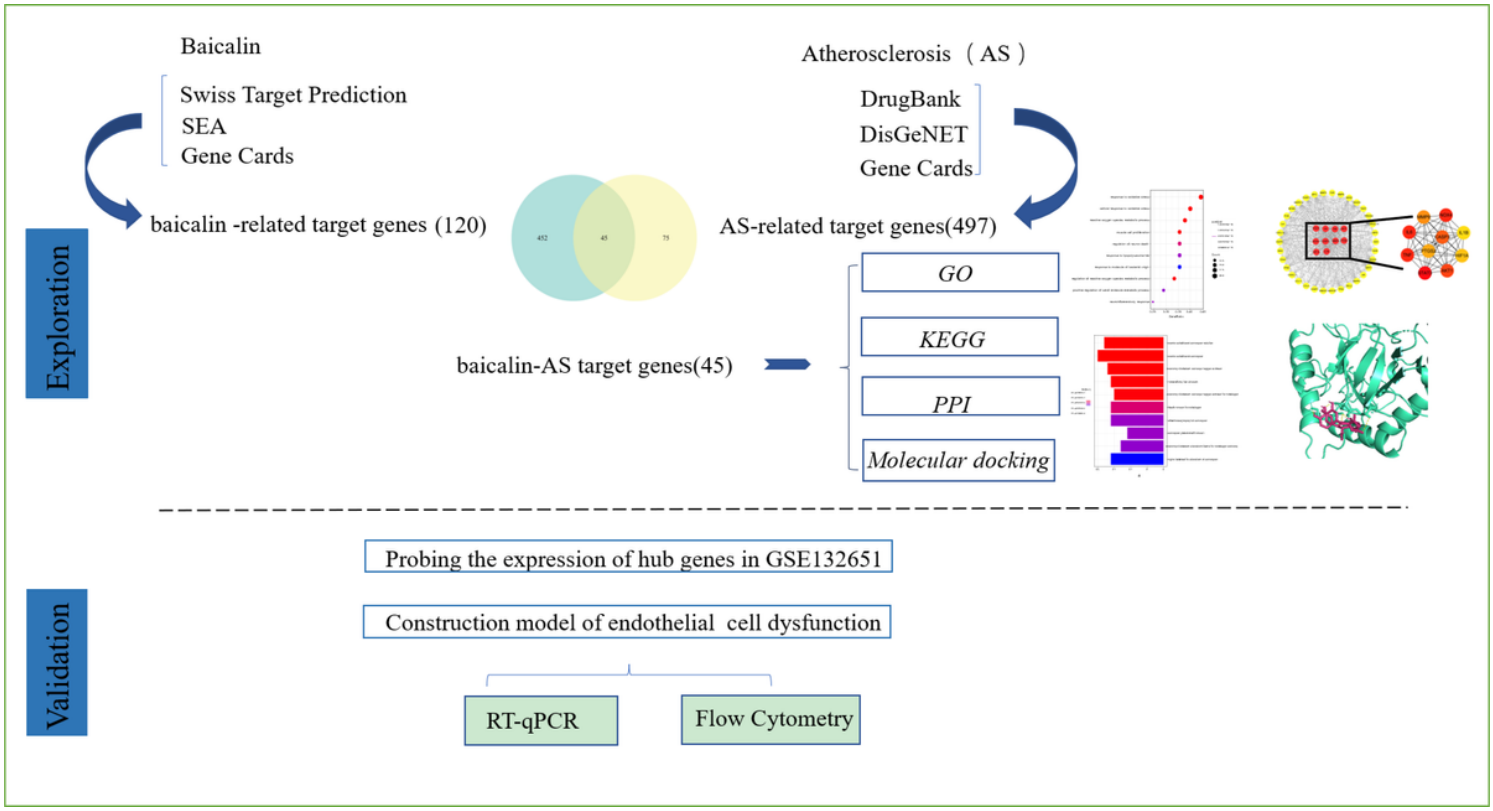

Figure 1

The flow chart of this study. AS: atherosclerosis.

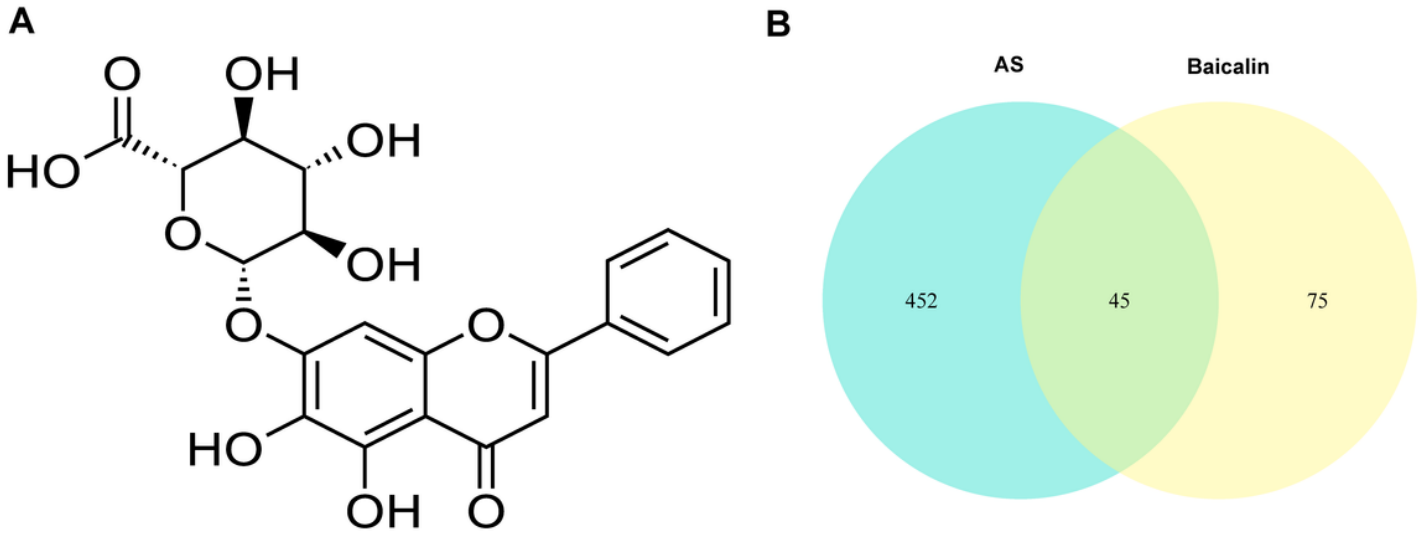

Figure 2

Identification of compound-disease target genes. **(A)** The chemical structure of baicalin. **(B)** Venn diagram of baicalin-AS target genes.

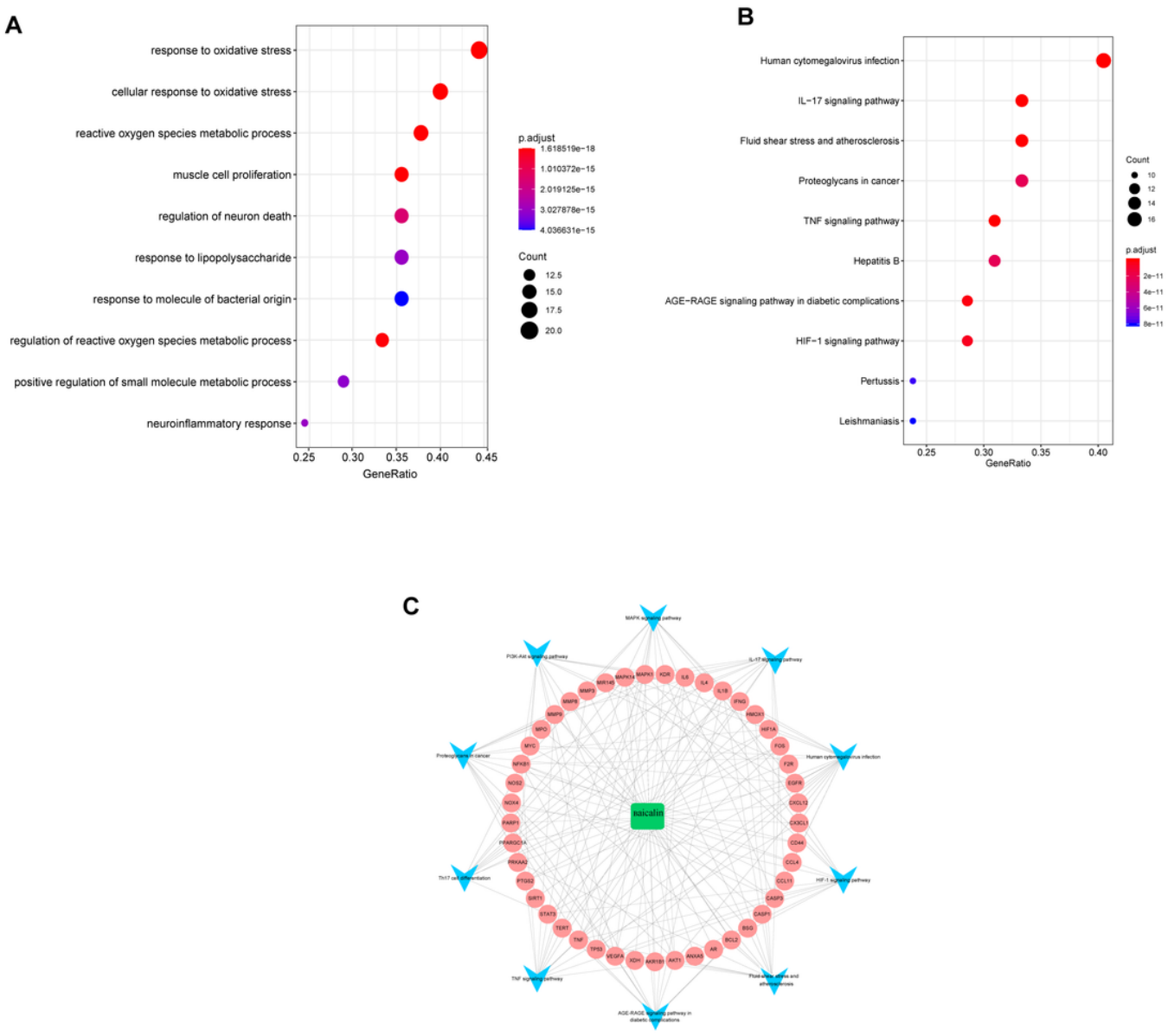

Figure 3

Functional enrichment analysis of baicalin-AS target genes. **(A)** Top 10 terms of GO enrichment analysis for baicalin-AS target genes. **(B)** Top 10 terms of KEGG enrichment analysis for baicalin-AS target genes. **(C)** Compound-target-pathway network. Green squares represent compounds and pink circles represent targets. The blue V shapes represent the pathways.

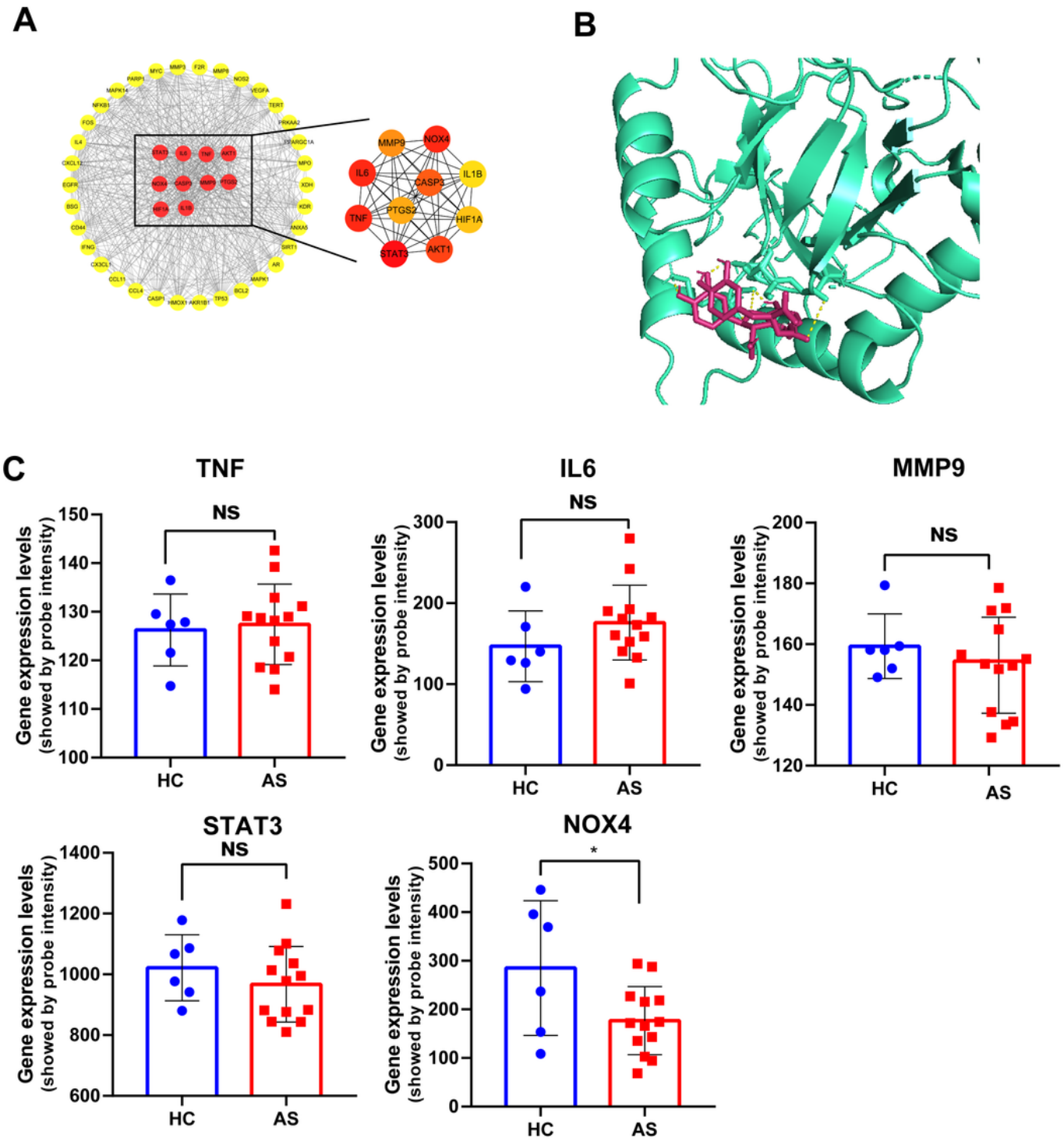

Figure 4

NOX4 was the core target gene of baicalin. **(A)** PPI network topological analysis. Based on gene intersection, this study acquired altogether 44 protein nodes and 10 hub target genes from topological analysis. **(B)** Simulation of protein-ligand docking for baicalin and NOX4. **(C)** Hub genes expression in GSE132651 based on probe intensity. (*$P < 0.05$)

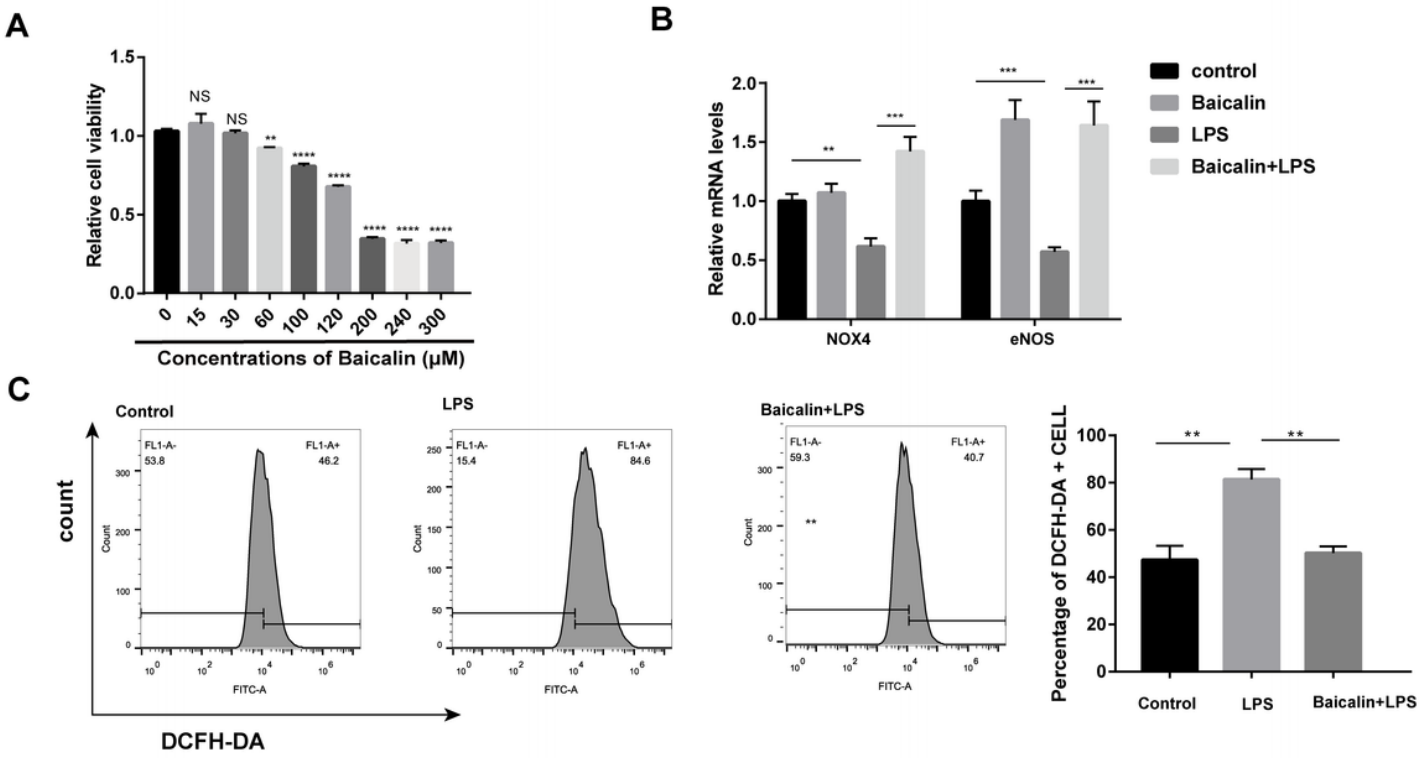

Figure 5

Baicalin increased the expression levels of NOX4 and blocked the LPS-induced ROS generation. **(A)** Effect of different concentrations of baicalin on viability of HAECs assessed by CCK8. **(B)** Baicalin modulated mRNA expression level of NOX4 and eNOS. GAPDH was used as the control. **(C)** Baicalin inhibited ROS release. HAECs were exposed to baicalin for 12 hours and stained with 20 μM DCFDA for 30 mins, followed by 1 ug/ml LPS for 6 hours. ROS levels were detected by flow cytometry analysis. Data were shown as mean ±SEM. Experiments were repeated thrice (n = 3, $^{**}P < 0.01$, $^{***}P < 0.001$, ***** $p<0.0001$).

Supplementary Files

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- targets1.docx