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Original Article

Phosphoproteomics reveals the apoptotic regulation of aspirin in the placenta of preeclampsia-like mice

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Abstract: Preeclampsia (PE) is a severe gestational complication, and dysfunctional placenta plays an essential role in PE pathogenesis. Although low-dose aspirin is currently the most promising prophylactic drug for PE prevention, the exact mechanism of aspirin remains unclear. A previous study reported that treatment with low-dose aspirin could ameliorate PE-like symptoms in lipopolysaccharide (LPS)-induced PE-like mouse model. This study aimed to uncover the potential mechanism of aspirin action in PE through quantitative phosphoproteomics comparison. We established the following four groups: a control (CTRL) group, an LPS-treated (L) group, an LPS + aspirin co-treatment (LA) group, and an aspirin-treated (A) group. A total of 4350 phosphosites and 4170 phosphopeptides from 1866 phosphoproteins were identified in the placenta on embryonic day 13.5. Among the significantly altered phosphoproteins identified, apoptosis-related pathways were significantly regulated in both the L group *vs*. CTRL group and the LA group *vs*. L group comparisons. We demonstrated that apoptosis was increased in the placenta of PE-like mice and was inhibited in the LA group by quantify the apoptosis-positive cells and the protein levels of cleaved caspase 3, 8, and 9. Moreover, the phosphorylation of HSP90β (S254) and GSK3β (Y216) may be a crucial factor in the aspirin-mediated regulation of apoptosis according to protein-protein interaction analysis. This study revealed that apoptosis regulation is a mechanism of aspirin action in PE, particularly in women with over-activated inflammation. The phosphorylation of HSP90β (S254) and GSK3β (Y216) could be the key intervention targets.

Keywords: Preeclampsia, aspirin, phosphoproteomics, apoptosis, HSP90β, GSK3β

Introduction

Preeclampsia (PE) is a pregnancy-specific syndrome that is diagnosed when a pregnant woman presents with increased blood pressure and proteinuria or other maternal organ dysfunctions [1]. When left untreated, pregnant women with PE would have severe complications such as eclampsia, liver rupture, stroke, pulmonary edema, or kidney failure, which can be lethal. Currently, the most effective treatment is delivery, hence the development of an approach to prevent PE is important. From lifestyle improvement, the supplementation of calcium, vitamin C and vitamin E, to the administration of low-dose aspirin, low-molecular-weight heparin, and antioxidants [2], aspirin is the most promising drug for PE prevention [3]. Meta-analyses of randomized controlled trials (RCTs) suggested that in high-risk women, the effect of aspirin for the prevention of PE is optimal when initiated before 16 weeks of gestation [4]. The effective window for certain population makes it necessary to understand the exact mechanism of action of aspirin in the prevention of high-risk PE.

The canonical effect of aspirin is dependent on cyclooxygenase (COX), which inhibits thromboxane A2 (TXA2) production and subsequently reverses the TXA2/prostaglandin I2 (PGI2) imbalance in PE patients, thereby improving placental function by favoring systemic vasodilatation and inhibiting platelet aggregation [5]. Meanwhile, there are COX-independent pathways, such as decreasing tissue factor expression in the endothelium [6], regulating the NF-κB/eNOS pathway in endothelial cell [7], or targeting the transcription factors STOX1 expression in PE-like mice [8]. Previously, we established a PE-like mouse model by injecting lipopolysaccharide (LPS) and found that aspirin

treatment could significantly reverse the PE-like phenotype, such as lower hypertension, proteinuria and ameliorate fetal growth retardation (FGR), improve placental damage, and inhibit the excessive activation of the inflammatory response [9]. In this study, we performed phosphoproteomics research on the placenta on embryonic day (E) 13.5, in the earlier stage of pregnancy after the definitive placenta formed (E12.5), to better observe the placenta development [10]. We aim to determine whether a particular physical process is involved in the aspirin-mediated effect in an LPS-induced PE-like mouse model, further unveiling the mechanism underlying aspirin treatment in PE.

Protein phosphorylation is the most extensive posttranslational modification. This modification can either activate or inactivate biological processes, and it can control nearly all aspects of cellular function [11]. The regulation of protein phosphorylation markedly impacts many cellular events for homeostasis, including metabolism, transcriptional and translational regulation, and cellular signaling [12]. The specific profile of phosphoproteins has been shown to be associated with many diseases, such as diabetes, renal dysfunction, ovarian cancer, and Alzheimer's disease. Phosphoproteomics allows for high-throughput detection of differential phosphosites to reveal the signaling pathways or targets, which are critical in disease pathogenesis and drug treatment [13-15]. Currently, studies performing systematic detection of phosphorylation changes in PE or aspirin mechanism research are limited. Here, we present a quantitative phosphoproteomics approach to identify differentially phosphorylated sites in signaling and response proteins from LPS-induced PE-like mice and in mice treated with aspirin. This study explored the molecular mechanisms of aspirin treatment in PE involving phosphoproteins and signaling networks.

Materials and methods

Animals and grouping

The animal study was approved by the Animal Ethics Committee of Peking University First Hospital. Female CD1 (ICR) mice (8 weeks old) were raised in a specific pathogen-free environment with a fixed 12-h/12-h light/dark cycle, and food and water were available ad libitum. Mating was performed overnight, and noon on the day of plug detection was considered embryonic day 0.5 (E0.5). The mice were randomly divided into four groups: the control (CTRL) group, LPS-treated (L) group, LPS + aspirin co-treatment (LA) group and aspirin-treated (A) group. The detailed methods used in animal experiments and for the identification of PE-like symptoms were described previously [9]. The placentas were collected on E13.5.

Protein extraction and digestion

A portion of each collected placental tissue sample was placed in a 2-ml centrifuge tube with quartz sand and SDT (4% SDS, PF8.0 100 mM TrisHCl, 100 mM dithiothreitol (DTT)) lysis buffer, homogenized with an MP homogenizer (24×2, 6.0 M/S, 60 s, twice), and processed via supersonic lysis (80 W, 10 s working, 15 s intermittent, 10 cycles). The lysates were incubated in boiling water for 15 min. After centrifugation at 14,000×g for 40 min, the supernatant was filtered through a 0.22-μm filter, and collected. The protein concentration was then measured by the bicinchoninic acid method with bovine serum albumin as the standard.

SDS-PAGE was performed as follows: after adding 5× loading buffer and boiling for 5 min, 30 μg of extracted protein from each sample was used for 12.5% SDS-PAGE under a constant current of 14 mA for 90 min. After electrophoresis, Coomassie brilliant blue solution was used for staining.

Protein digestion with filter aided sample preparation (FASP) was performed as follows: DTT was added to the protein solution to reach 100 mM, and the mixture was boiled for 5 min and cooled to room temperature. Each sample was mixed with 200 μl of UA buffer (8 M urea, 150 mM Tris-HCl, pH 8.0), loaded in a microcon filtration devices (Millipore, with a molecular weight cut off (MWCO) of 10 kD), and centrifuged at 14,000×g for 15 min. A total of 100 μl of IAA buffer (50 mM iodoacetamide in UA) was added to each sample, and the mixture was shaken at 600 rpm for 1 min. After 30 min of incubation in the dark at room temperature, the mixtures were centrifuged at 14,000×g for 15 min, and the filtrate was discarded. Then, 100 μl of UA buffer was added, and the mixture was centrifuged for 15 min at 14,000×g; these

Table 1. Sample set of quantitative phosphoproteomic analysis

Lable	114	115	116	117
Sample 1	CTRL-1	L-1	LA-1	A-1
Sample 2	CTRL-2	L-2	LA-2	A-2
Sample 3	CTRL-3	L-3	LA-3	A-3

114, 115, 116, 117: the mass of four different reporters in the iTRAQ labeling. CTRL: the control group; L: LPS-treated group; LA: LPS + aspirin co-treatment group; A: aspirin-treated group.

steps were repeated twice. A total of 100 μl of 10-fold diluted dissolution buffer was added, and the mixture was centrifuged at 14,000×g for 15 min; these steps were also repeated twice. Then, the filtrate was discarded. Next, 40 μl of trypsin buffer was added, and the mixture was shaken at 600 rpm for 1 min and incubated at 37°C for 16-18 h. The samples were placed a in new collection tube and centrifuged at 14,000×g for 15 min, and the filtrate was discarded. Then, 40 μl of 10-fold diluted dissolution buffer was added, the mixture was centrifuged at 14,000×g for 15 min, and the filtrate was collected. A C18 cartridge was used to desalt the enriched peptides. After the peptides were lyophilized, they were reconstituted by adding 40 μl of dissolution buffer. The concentration of the collected peptides was estimated based on their OD at 280 nm.

Phosphoproteomic analysis

The phosphoproteomic analysis in the study used liquid chromatography-tandem mass spectrometry (LC-MS/MS) enriched by titanium dioxide (TiO2) affinity chromatography.

For four-plex iTRAQ labeling, one hundred micrograms of digested peptides from each sample was subjected to AB Sciex iTRAQ labeling (**Table 1**). Four-plex iTRAQ labeling was performed according to the manufacturer's instructions.

To enrich the phosphopeptide, the labeled peptide solutions were lyophilized and then dissolved in 1× DHB buffer (3% DHB, 80% CAN, and 0.1% TFA). TiO2 beads were added to the solution, the mixture was shaken for 40 min and centrifuged, and the supernatant was removed. The beads were packed into plastic tips, washed three times with washing buffer 1 (20% acetic acid, 300 mM octane sulfonic acid sodium salt and 20 mg/ml DHB) and washed three times with washing buffer 2 (70% water and 30% ACN). The enriched phosphopeptides were eluted using freshly prepared ABC buffer (50 mM ammonium phosphate, pH 10.5) and were collected and concentrated under a vacuum [16]. The phosphopeptides were dissolved in 30 μl of 0.1% FA, and 20 μl was used for mass spectrometry (MS) analysis.

The phosphopeptides were subjected to capillary liquid chromatography-tandem mass spectrometry using a two-dimensional EASY-nLC-1000 system coupled to a Q Exactive Hybridv Mass Spectrometer (Thermo Scientific). A precolumn (20 mm×100 μm; 5 μm-C18) and an analytical column (250 mm×75 μm; 3 μm-C18) were used (Thermo Fisher Scientific) with mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in 84% ACN). The phosphopeptides were separated at a flow rate of 250 nL/min using the following gradient: 0-55% mobile phase B from 0-220 min, 55-100% mobile phase B from 220-228 min, and 100% mobile phase B from 228-240 min. Data-dependent mass spectra were acquired for 240 min. The full MS surveys were collected over a mass-to-charge ratio (m/z) range of 300-1,800, with the resolution set to 70,000 at m/z 200.

Database search and quantification

Mascot 2.2 (Matrix Science, Boston, MA, USA) and Proteome Discoverer 1.4 (Thermo Fisher Scientific) software were used to identify and quantify phosphoproteins [17]. The identified phosphopeptides were mapped to UniProt sequences and named according to the canonical UniProt sequence. The database used in this research was UniProt_Mouse_84433_20180102.fasta (including 84433 items, downloaded on January 2, 2018).

The raw files were searched with Mascot through Proteome Discover, and the selection parameters were set as follows: tryptic peptides with two missed cleavages, peptide mass tolerance of ± 20 ppm, and fragment mass tolerance = 0.1 Da. Proteome Discoverer 1.4 software was used for quantitative analysis on the peak intensity of each expected iTRAQ reporter ion from each analyzed fragmentation spectrum. The quantification result was the ratio of the signal intensity value of the label on the

sample to that on the reference sample. The parameters for the quantitative analyses were as follows: peptide false discovery rate ≤ 0.01, reject all quantified values if not all quantification channels are present, normalize to protein median, normalize all peptide ratios to the median protein ratio, and median protein ratio should be one after normalization. Differentially phosphorylated peptides were defined as peptides with a fold change between two groups above 1.2 or below 0.83 and with a *p* value less than 0.05.

This peptide score is based on the cumulative binomial probability that an observed match is a random event. The value of the phosphorscore heavily depends on the included data, and scores above 50 indicate a good peptide spectral match. For each phosphorylation site, the phosphorylation site score is an estimate of the probability, and probabilities above 75% indicate that the site is truly phosphorylated [18].

Bioinformatics analysis

GO is a standardized functional classification system that provides a series of dynamically updated standardized data and describes the properties of genes and gene products in organisms in three ways: biological process, molecular function, and cellular component. GO analysis of a differentially phosphorylated protein dataset can immediately reveal the functional enrichment associated with treatment. The process used to perform the Gene Ontology (GO) annotation of the target protein set using Blast2GO can be generalized into four steps: BLAST, mapping, annotation, and annotation augmentation [19]. The target protein set was aligned with the appropriate protein sequence database using the localized sequence alignment tool NCBI BLAST+ (ncbi-blast-2.2.28+-win32.exe) and retained to satisfy E-value ≤ 1e-3. The top 10 alignment sequences were subsequently analyzed. The Blast2GO command line was used to extract the GO entries associated with the eligible alignment sequences in the target protein set (www.geneontology.org). In the annotation process, the Blast2GO command line annotated the GO entries extracted from the process of mapping to the target by comprehensively considering the similarity of the target protein sequence and the alignment sequence, the reliability of the GO entry source, and the structure of GO with a directional acyclic graph.

Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) is a taxonomic system of genes and their products in the KEGG database. Orthologous genes and their products with similar functions on the same pathway are grouped and assigned the same KO (or K) tag. When the KEGG pathways in the target protein set are annotated, the target protein sequence is classified with KO by comparing the KEGG GENES database using KEGG Automatic Annotation Server (KAAS) software, and information about the pathway associated with the target protein sequence according to the KO classification is automatically obtained.

In the enrichment analysis of the GO or KEGG pathway annotation of the target protein set, Fisher's exact test was used to compare each GO term or KEGG pathway in the target protein set to the overall protein set to evaluate the significance of protein enrichment associated with a particular GO term or KEGG pathway.

Search Tool for the Retrieval of Interacting Genes (STRING, http://string-db.org/) was used to construct a protein-protein interaction (PPI) network and to conduct module analysis.

Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining

Apoptosis was detected by the TUNEL assay. Placenta tissues were fixed in 4% formaldehyde for 24 h, then dehydrated and embedded in paraffin and cut into 5-μm slices. Slices were dewaxed, rehydrated, and treated with 20 μg/mL proteinase K for 15 min at 37°C. Samples were then incubated with 50 μl TUNEL reaction mixture (Roche, Mannheim, Germany) for 1 h at 37°C in a wet-box. Then 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) was applied for nuclear staining. The sections were observed, and images were randomly taken under a fluorescence microscope (Olympus, Tokyo, Japan). Six fields were examined for each sample, and the degree of apoptosis was represented by the percentage of the TUNEL-positive nuclei divided by the total number of DAPI-stained nuclei. The number of nuclei was counted by ImageJ software (National Institutes of Health, Bethesda, USA).

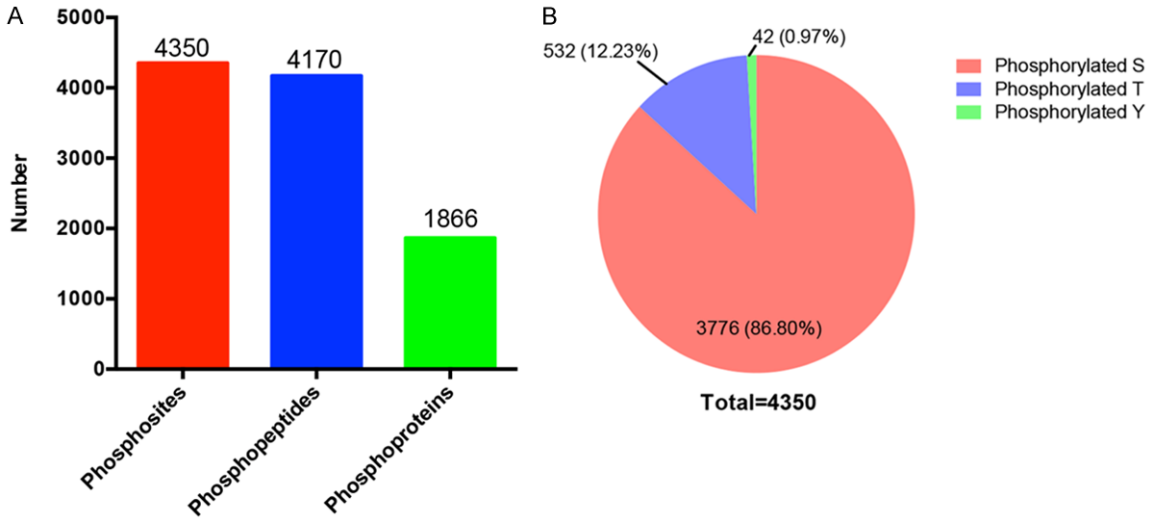

Figure 1. The character of phosphorylated identification in all groups. A. The identification and quantification of phosphosites, phosphopeptides, and phosphoproteins. B. The percentage of identified phosphorylated S, T, and Y sites. S: serine, T: threonine, Y: tyrosine.

Western blotting

Protein was extracted from maternal placenta tissues with radio-immunoprecipitation assay (RIPA) lysis buffer. A 30-μg aliquot of protein was used for electrophoresis in 12% polyacrylamide gels and transferred to a polyvinylidene fluoride membrane. The membrane was blocked in 5% albumin from bovine serum at room temperature for 1 h and then incubated at 4°C overnight, with rabbit anti-mouse primary antibodies against caspase 3, cleaved caspase 3, caspase 8, cleaved caspase 8, caspase 9, cleaved caspase 9 (Cell Signaling Technology, Beverly, MA, USA; 1:1000), HSP90β, HSP90β (S254), GSK3β, and GSK3β (Y216) (Abcam, Cambridge, UK; 1:1000). The membranes were washed 3 times at room temperature for 10 min each, incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA; 1:1000) for 1 h and washed an additional 3 times for 10 min each. Band visualization was achieved using an enhanced chemiluminescence western blotting analysis system. The resulting images were analyzed with ImageJ software (National Institutes of Health, Bethesda, USA), and the data were standardized to β-actin.

Statistical analysis

Quantitative data were expressed as the mean ± standard error of the mean. Differences were statistically significant when $P<0.05$. All data were analyzed with SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). The statistical significance of the results was evaluated by Student's t-test when only two groups were compared and by one-way analysis of variance (ANOVA) when multiple groups were compared.

Results

Phosphoproteomics profiling and quantification in all groups

We showed that aspirin could ameliorate hypertension, proteinuria, and FGR in LPS-induced PE-like mice in a previous study [9] (Table S1). We applied an MS-based iTRAQ quantitative phosphoproteomics method to analyze the changes in phosphorylation of the placenta on E13.5 in the CTRL, L, LA, and A. four groups.

In total, 4,350 phosphosites were identified with quantitative information. The phosphosites corresponded to 4,170 phosphopeptides and 1,866 phosphoproteins (**Figure 1A**). Among the identified phosphosites, the percentages of phosphorylated serine sites, threonine sites, and tyrosine sites were 86.80%, 12.23%, and 0.97%, respectively (**Figure 1B**).

Differentially phosphorylated peptides in response to PE-like changes and aspirin treatment

The numbers of differentially phosphorylated peptides between pairs of groups are shown in

Table 2. The numbers of differentially alerted phosphorylated peptides between pairs of groups

Comparisons	All	Upregulated	Downregulated
L *vs.* CTRL	198	103	95
LA *vs.* L	107	50	57
LA *vs.* A	211	99	112
A *vs.* CTRL	140	68	72

CTRL: the control group; L: LPS-treated group; LA: LPS + aspirin co-treatment group; A: aspirin-treated group.

Table 2. In the L and CTRL group comparison, there were 198 phosphorylated peptides with significant changes, including 103 upregulated and 95 downregulated peptides. In the comparison of the LA group and the A group, a total of 107 phosphorylated peptides were changed, including 50 upregulated and 57 downregulated.

GO analysis of differentially phosphorylated proteins in response to PE-like changes and aspirin treatment

To investigate the substantive changes induced by the PE-like stimulation and by aspirin treatment, we focused on the comparisons between the L group and the CTRL group and between the LA group and the L group. The top 20 significantly enriched GO terms in the L *vs.* CTRL group comparison included the following biological processes: the positive regulation of cellular protein localization, spindle organization, the positive regulation of apoptotic signaling pathway and many others (**Figure 2A**). In the comparison between the LA group and the L group, the biological processes involving the negative regulation of the apoptotic signaling pathway, RNA splicing, regulation of growth, and many others were significantly changed (**Figure 2B**).

In the L *vs.* CTRL group comparison, the positive regulation of apoptotic signaling pathway (GO: 2001235) and the positive regulation of apoptotic process (GO: 0043065) were significantly overrepresented (both $P<0.01$; enrichment factor is 0.26 and 0.18, respectively). In the LA *vs.* L group comparison, the negative regulation of the apoptotic signaling pathway (GO: 2001234) and the regulation of the apoptotic signaling pathway (GO: 2001233) were significantly overrepresented (both $P<0.01$; enrichment factor is 0.26 and 0.17, respectively). Therefore, we speculated that the apoptotic process is involved in the pathogenic mechanism in LPS-induced PE-like mice and plays an essential role in aspirin treatment.

KEGG pathway analysis of regulated phosphoproteins

To identify potential signaling pathways involved in the effects of PE-like stimuli and aspirin treatment on the placenta, we performed a search with the identified phosphoproteins in the KEGG database. After analyzing the differentially phosphorylated proteins between the L group and the CTRL group, the pathways of necroptosis, RNA transport, and antigen processing and presentation were significantly altered (**Figure 3A**). When the LA group was compared to the L group, the phagosome, peroxisome, gap junction, choline metabolism in cancer, phosphonate, and phosphinate metabolism pathways were found to be significantly enriched (**Figure 3B**).

Among the pathways we obtained in the L *vs.* CTRL group and LA *vs.* L group comparisons, the necroptosis (map04217, $P<0.05$, rich factor is 0.33) and phagosome (map04145, $P<0.05$, rich factor is 0.3) pathways were known to be associated with the apoptosis process and were significantly enriched. After the comprehensive analysis of the KEGG and GO results, we found that the apoptosis signaling pathway might be highly important for the LPS-induced PE-like changes and for the aspirin action.

Apoptosis was increased in E13.5 placenta from PE-like mice and attenuated in the aspirin-treated group

GO and KEGG analysis strongly suggested that several apoptosis-related pathways were significantly altered both in the L group compared to the CTRL group and in the LA group compared to the L group. TUNEL assay was used to labeling the in-situ DNA strand breaks, which is a classic feature of apoptotic cells. In this study, we found that the TUNEL-positive labeled (green) nuclei in the placental labyrinth were obvious increased in the L group and decreased in the LA group (**Figure 4A**). The percentage of TUNEL-positive nuclei was significantly increased in the L group, as 3.3-fold as the CTRL group ($P<0.05$). In the LA group, the percentage was significantly decreased compared to the L group ($P<0.05$); however, it was signifi-

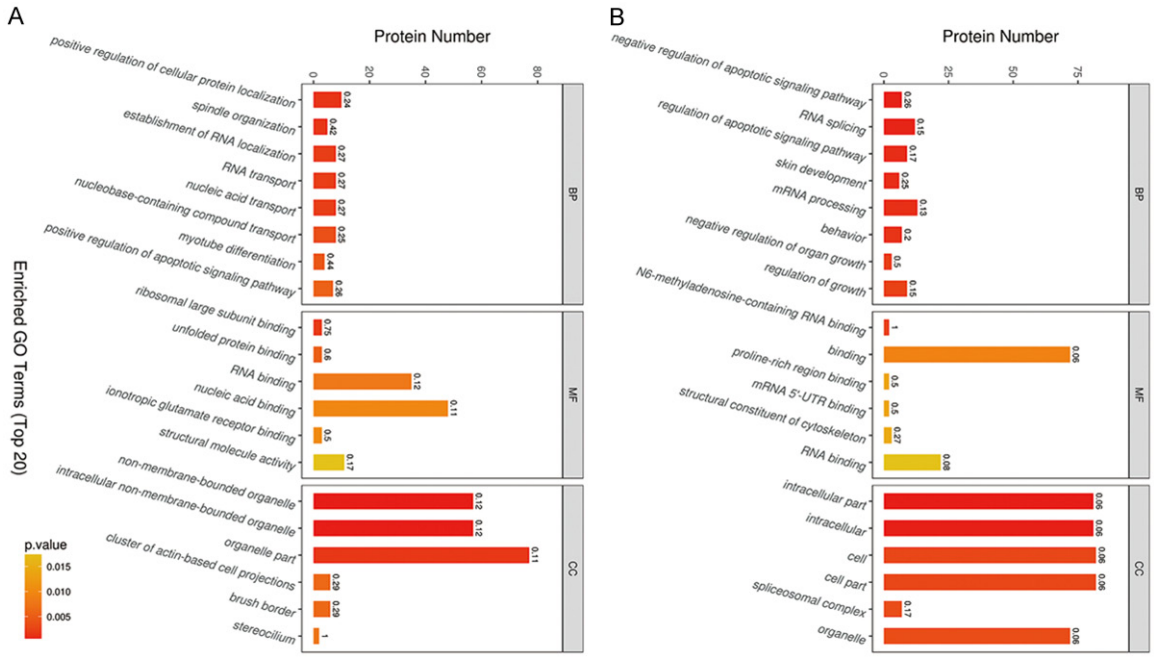

Figure 2. Gene ontology analysis of the response of differentially phosphorylated proteins response to preeclampsia-like change and aspirin treatment. A. L group *vs.* CTRL group, the positive regulation of apoptotic signaling pathway was significantly enriched. B. LA group *vs.* L group, the negative regulation of apoptotic signaling pathway was significantly enriched. The numbers to the right of the tab show the enrichment factor. The enrichment factor indicates the proportion of differentially expressed phosphoproteins corresponding to a certain GO functional class to the number of proteins identified in that GO functional class.

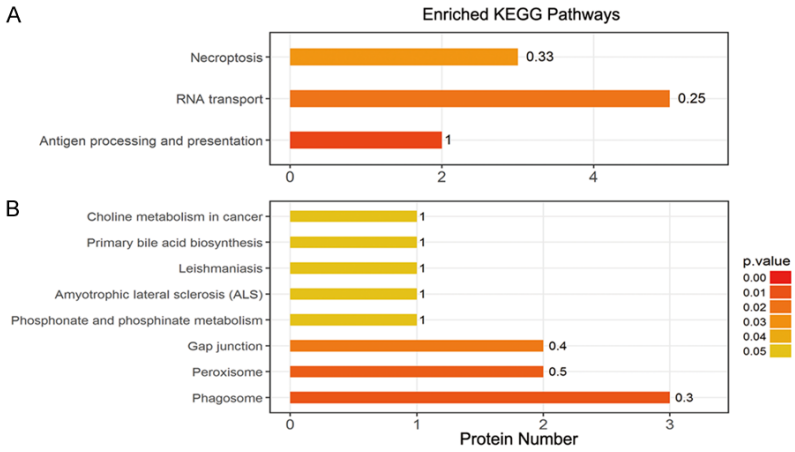

Figure 3. Enrichment analysis of KEGG pathways of phosphorylated proteins differentially expressed in response to preeclampsia-like change and aspirin treatment. A. L group *vs.* CTRL group, necroptosis pathway was significantly enriched. B. LA group *vs.* L group, phagosome pathway was significantly enriched. The numbers to the right of the tab show the enrichment factor.

cantly higher than the CTRL group (P<0.05) (**Figure 4B**).

Also, we detected the cleaved levels of protein caspase 3, caspase 8, and caspase 9 by western blotting; increases in these activated proteins can indicate the enhancement of apoptosis. In this study, the ratios of cleaved caspase 9/caspase 9, cleaved caspase 8/caspase 8, and cleaved caspase 3/caspase 3 were significantly increased up to 1.8-, 1.4-, and 1.6-fold, respectively, in the L group compared to the CTRL group (P<0.05); and were significantly decreased in the LA group compared to the L group (P<0.05). Moreover, these ratios were not significantly different between the CTRL group and the LA group (P>0.05) (**Figure 5**).

Phosphoprotein-phosphoprotein interaction networks of apoptotic process related phosphoproteins

A network of the known and predicted interactions of differentially phosphorylated proteins

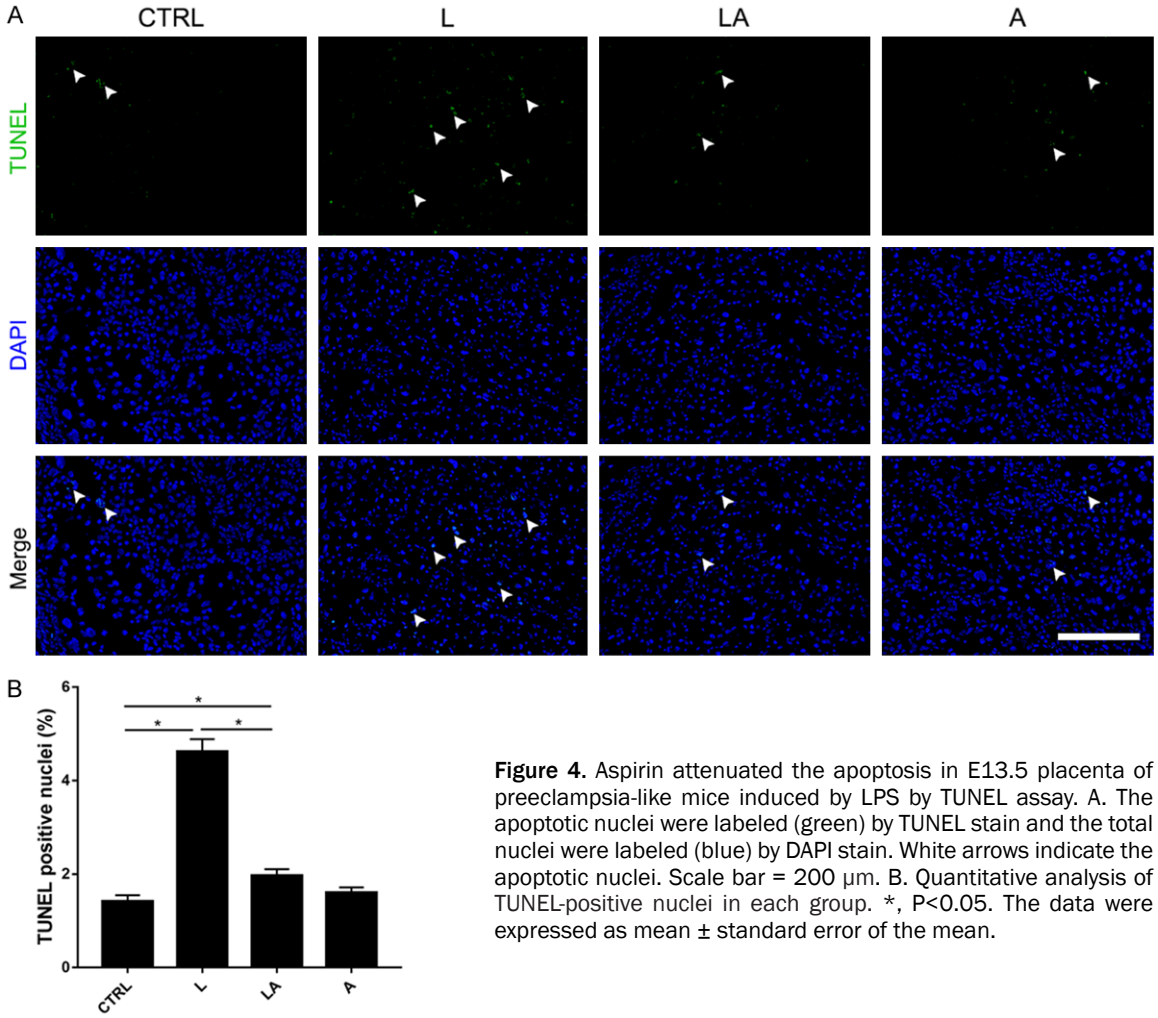

Figure 4. Aspirin attenuated the apoptosis in E13.5 placenta of preeclampsia-like mice induced by LPS by TUNEL assay. A. The apoptotic nuclei were labeled (green) by TUNEL stain and the total nuclei were labeled (blue) by DAPI stain. White arrows indicate the apoptotic nuclei. Scale bar = 200 μm. B. Quantitative analysis of TUNEL-positive nuclei in each group. *, P<0.05. The data were expressed as mean ± standard error of the mean.

(P<0.05) in either the L group *vs*. CTRL group comparison or the LA group *vs*. L group comparison, according to the STRING database, is shown in **Figure 6**. To determine which phosphoproteins significantly altered play essential roles in regulating the apoptotic process in response to PE-like stimuli and aspirin treatment, a map of all detected phosphoproteins (Table S2) involved in the apoptosis pathway and significantly related to components of the apoptotic pathway in GO and KEGG analysis was constructed. The map of these interactions revealed several outstanding findings. Phosphoproteins such as heat shock protein 90β (HSP90β), glycogen synthase kinase 3β (GSK3β), mitogen activated protein kinase 8 (MAPK8), and ribosomal protein S6 (RPS6) had an increased number of interacting partners, which suggests that these phosphoproteins could play key roles in the response mechanism to aspirin in PE-like mice. In particular, among the evaluated phosphoproteins, HSP-90β binds to the most interacting partners, which suggests that it might be a hub protein with a vital role in the aspirin-mediated regulation of apoptosis.

The phosphorylation levels of HSP90β (S254) and GSK3β (Y216) normalized to the total protein level

The PPI network revealed that the phosphorylation of several remarkable proteins might play a pivotal role in the apoptosis regulation of aspirin action in PE-like mice. Moreover, the phosphoproteomics MS analysis indicated that the serine at site 254 of HSP90β was obviously changed among the groups. The quantification of the phosphorylated peptides revealed three main patterns of the phosphorylation of serine 254 on HSP90β with different peptide-spectrum matches (PSMs): increased in the L group

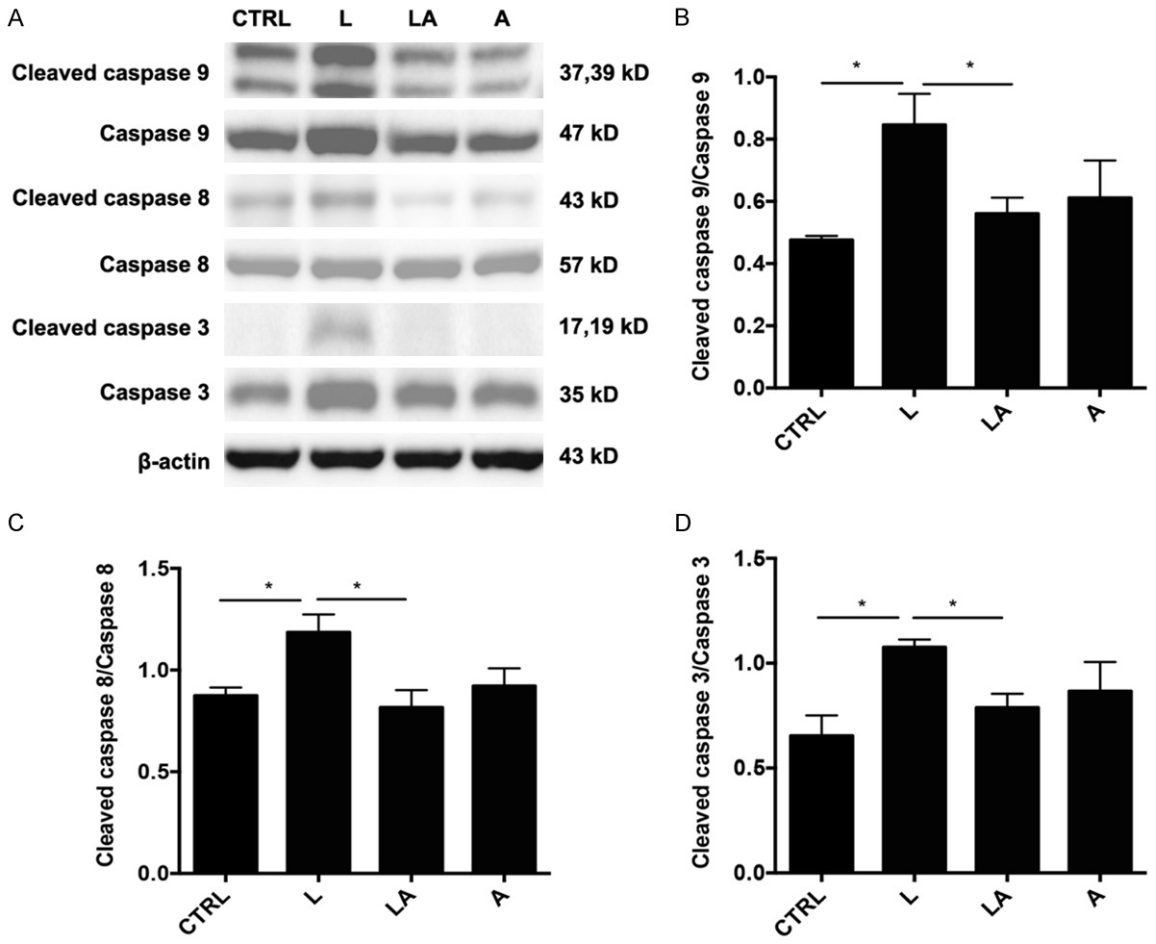

Figure 5. Aspirin attenuated the apoptosis in E13.5 placenta of preeclampsia-like mice induced by LPS by western blotting detection. (A) The protein expression of cleaved caspase 9, 8, and 3 and their correspondent caspases. The quantitative analysis of relative protein levels of cleaved caspase 9 (B), 8 (C), and 3 (D) to their corresponding caspases of each group. *, P<0.05. The data were expressed as mean ± standard error of the mean.

compared to the CTRL group and decreased in the LA group compared to the L group (except when the PSM was 292) (**Figure 7A**). Additionally, after detecting the level of HSP-90β phosphorylation (S254) by western blotting and normalizing this result to the total protein level of HSP90β, the ratio of HSP90β (S254)/HSP90β was found to be significantly increased up to 1.5-fold in the L group compared with the CTRL group. The ratio was significantly decreased in the LA group compared to the L group (P<0.05), and there was no significant difference between the LA group and the CTRL group (P>0.05) (**Figure 7B**). GSK3β (Y216) is another phosphoprotein located in the center of the network, which may have an important role. The quantification of tyrosine 216 phosphorylation on GSK3β yielded two main results based on PSMs: the phosphopeptide was decreased in the L group compared to the CTRL group and increased in the LA group compared to the L group (**Figure 8A**). Western blotting showed that the ratio of GSK-3β (Y216)/GSK3β was significantly decreased in the L group compared to the CTRL group (P<0.05) and increased in the LA group compared to the L group (P<0.05) (**Figure 8B**).

Discussion

PE is a severe gestational complication and has multiple risk factors and pathogenic pathways. The molecular mechanisms underlying this disease include placental ischemia, immune imbalance, oxidative stress, inflammation, and others [20]. The dysfunctional trophoblast invasion plays a central role in the development of PE [21]. Apoptosis, a form of programmed

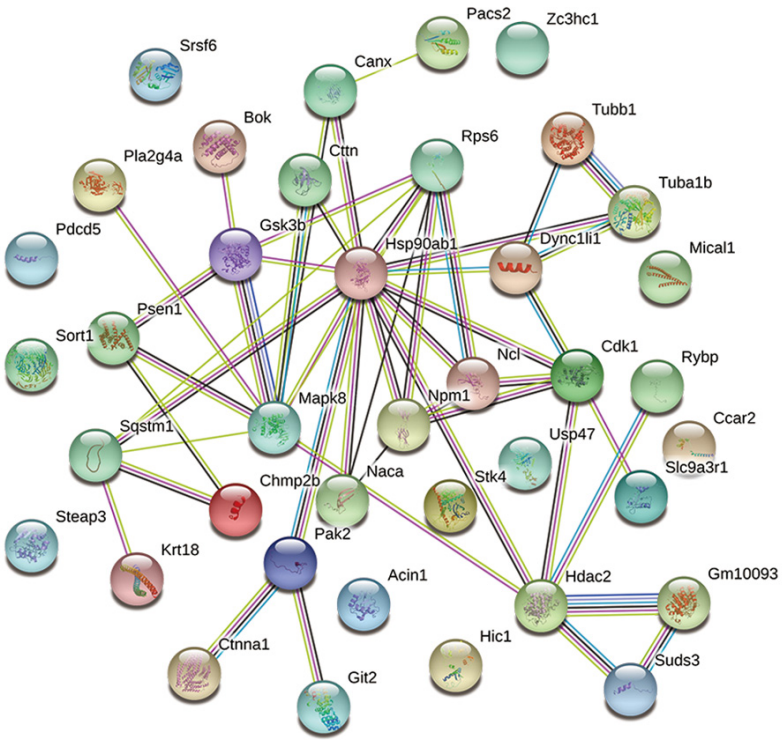

Figure 6. Protein-protein interaction network of the phosphorylated proteins involved in the apoptotic pathway differentially altered either in the L *vs.* CTRL group or the LA *vs.* L group comparisons. The network nodes (circles) are phosphoproteins and the edges show known or predicted functional associations (threshold: 0.4, medium confidence interval). Colored lines between the phosphoproteins indicate the various types of interaction evidence (light blue line: database evidence; purple line: experimental evidence; green line: neighborhood evidence; red line: fusion evidence; blue line: co-occurrence; black line: co-expression; yellow line: text mining evidence). HSP90β, GSK3β, MAPK8, and RPS6 have more interacting proteins.

cell death, plays an essential role in the physiologic and pathophysiologic mechanisms of the placenta, is a basic physiologic phenomenon in trophoblasts, endothelial cells, and stromal cells [22]. Appropriate apoptosis is vital for the invasion of the placenta to ensure sufficient placental blood supply, is believed to be important for healthy placental growth and development [23].

Our phosphoproteomics research found that the phosphoproteins involved in the regulation of the apoptotic signaling pathway were significantly altered, indicating that apoptosis was enhanced in the PE-like mouse placenta. TUNEL assay revealed that the apoptosis-positive cells in the placental labyrinth were significantly increased in the mice with PE-like symptoms. Further, this was identified at the molecular level by western blotting analysis, which showed that cleaved caspase 3, caspase 8, and caspase 9 were significantly increased. The main apoptosis pathways are the extrinsic and intrinsic pathways and the perforin/granzyme pathway. Each pathway requires specific trigger to begin a series of molecular events and activates an initiator caspase (8, 9, or 10), which in turn activates the executioner caspase (3, 6, or 7) (granzyme A works in a caspase-independent manner). Our finding that apoptosis was upregulated in the PE placenta is in accordance with previous studies. Several studies have reported that apoptosis is notably exaggerated in PE and intrauterine growth restriction and that apoptosis is a possible target for PE prevention and treatment [24, 25]. In the PE-affected placental tissues, the apoptosis regulator B-cell lymphoma 2 (BCL2)/BCL2-associated X (BAX) ratio was significant lower than those from term-matched normal pregnancies, and was located in the syncytiotrophoblast [26]. Our study demonstrated that an enhancement of apoptosis in the development of the placenta in PE-like mice, which may be likely a cause of dysfunctional placenta invasion.

Aspirin is currently the most widely prescribed treatment in the prevention of cardiovascular disease (CVD) and is a promising prophylactic drug for PE prevention [27]. However, aspirin treatment as a strategy to prevent CVD remains under debate, and the results of three large RCTs published recently suggest that aspirin should be used cautiously in the primary prevention of CVD [28]. Many clinical studies investigating the effect of aspirin on PE prevention have also been performed; however, among the methods used to screen high-risk populations prescribed aspirin, the dose and the initial time

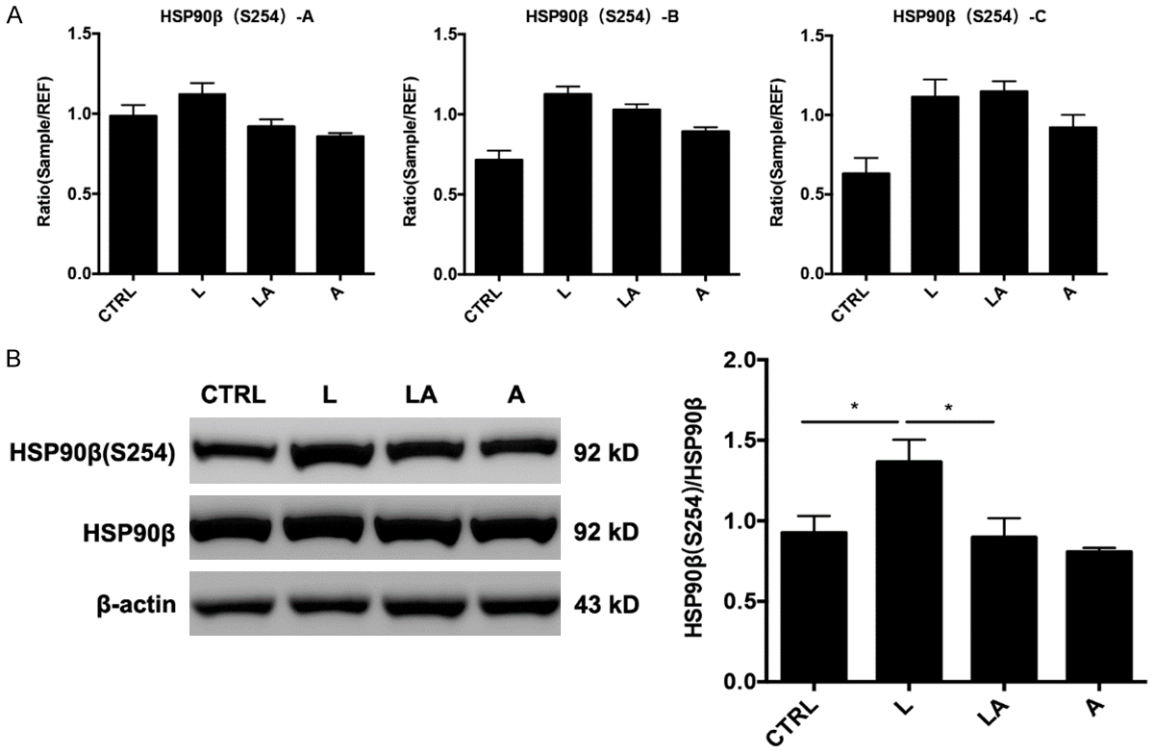

Figure 7. HSP90β phosphorylation (S254) increased in the placenta of preeclampsia-like mice and decreased in aspirin-treated group. A. The levels of HSP90β (S254) phosphorylation based on MS analysis in phosphoproteomics. HSP90β (S254)-A, -B, -C, the same serine sites in peptides with different peptide-spectrum matches, which are 263, 422, and 292, respectively. Ratio: the intensity of the fragmented tag in a sample to the intensity of the fragmented tag in the reference (REF) sample. B. Western blotting analysis of total and phosphorylated (S254) level of HSP90β. *, $P<0.05$. The data were expressed as mean ± standard error of the mean.

are different among studies, and not all high-risk patients have the prevention effect. Most importantly, PE is a multi-factorial and multi-pathogenic disease, and the effect and mechanism may be different under various maternal conditions [29]. Therefore, the biological mechanism of aspirin in PE prevention and whether it is related to the certain maternal conditions have great significance. Studies using both in vivo and in vitro experiments have found that aspirin exerts promising effects in PE prevention by regulating many signaling pathways. Low-dose aspirin can significantly reduce sFlt1 production in JEG3 cells after exposure to hypoxia and had an anti-inflammatory effect on a PE-like mouse model in previous studies [9, 30].

For the first time, we found that aspirin can alleviate the exaggerated apoptosis in PE with in vivo experiments using high-throughput technology. Our phosphoproteomics results showed that aspirin can suppress the upregulated apoptosis in the LPS-induced PE mouse model and can downregulate the cleaved caspases involved in the apoptotic process. Bose P and his colleagues found that aspirin can regulate apoptosis in BeWo cells and placental villi cultured in sera from women with failed in vitro fertilization by assessing DNA laddering, cytokeratin 18 neoepitope formation, and Bcl-2 and caspase 7 expression [31]. Another study also showed a significant decrease in the apoptotic index in PE serum-induced trophoblast cells by aspirin treatment at an appropriate dosage [32]. The PE-like mouse model induced by LPS has excessive activation of inflammation, with the increase of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), and aspirin can reverse these changes. TNF-α can bind to its receptor to activate the apoptotic pathway, and aspirin may improve the development of the placenta through the regulation of apoptotic function in the presence of excessive inflammatory response. It is worth noting that aspirin also has a proapoptotic effect on both

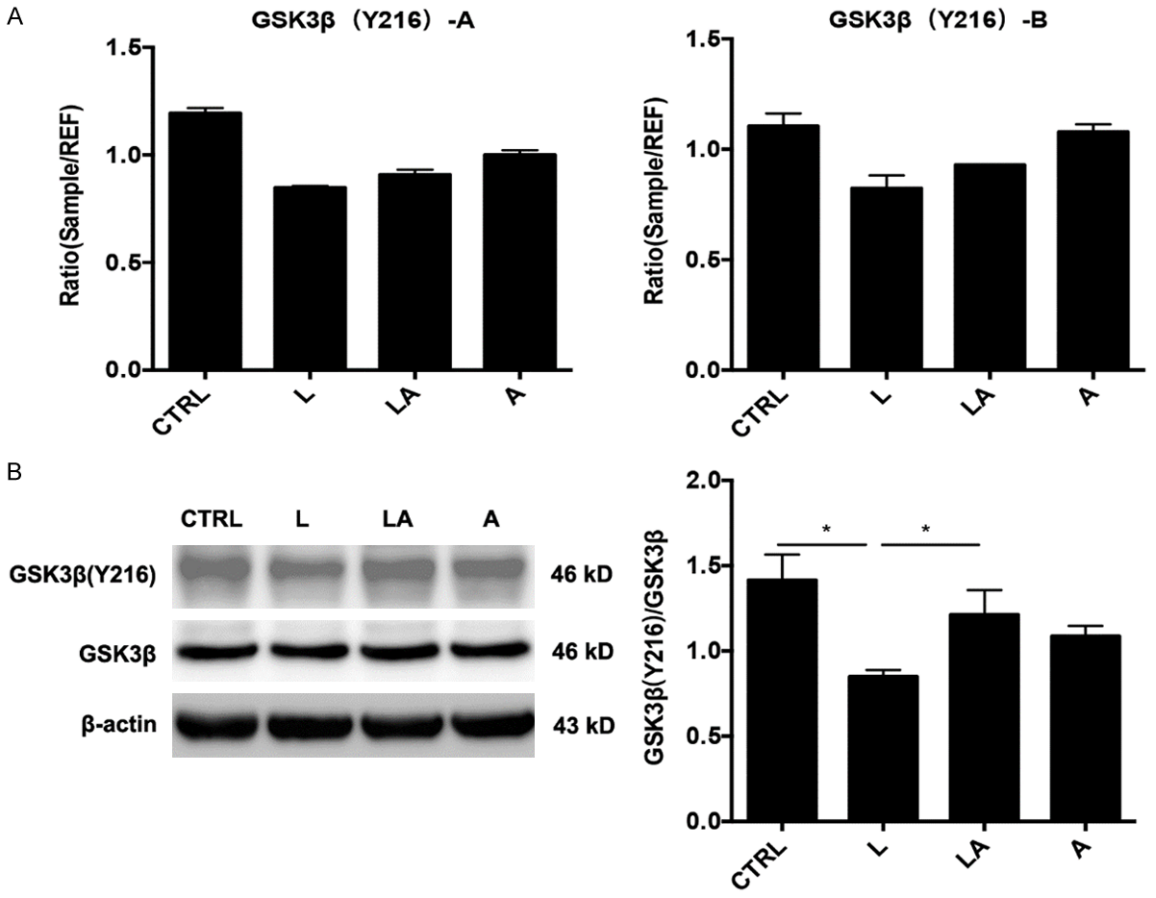

Figure 8. GSK3β phosphorylation (Y216) decreased in the placenta of preeclampsia-like mice and increased in aspirin-treated group. A. The levels of GSK3β (Y216) phosphorylation based on MS analysis in phosphoproteomics. GSK3β (Y216)-A, -B: the same tyrosine sites in peptides with different peptide-spectrum matches, which are 203, and 20 respectively. Ratio: the intensity of the fragmented tag in a sample to the intensity of the fragmented tag in the reference (REF) sample. B. Western blotting analysis of total and phosphorylated (Y216) level of GSK3β. *, $P<0.05$. The data were expressed as mean ± standard error of the mean.

intrinsic and extrinsic apoptotic pathways, which is contrary to our findings [33]. However, the proapoptotic effect of aspirin was mostly shown in cancer research, under a pathological state with excessive cell proliferation. Nevertheless, in PE, the trophoblast invasion of the placenta is insufficient; thus, the exact mechanism of action of aspirin in PE should be different from that in cancer with different context.

After we established that aspirin could alleviate apoptosis in the placenta of a PE-like mouse model, we wanted to determine the targets of aspirin action. We constructed a PPI network with the significantly altered phosphoproteins in the placenta of PE-like mice (from the L group *vs.* CTRL group and the LA group *vs.* L group comparisons) and with apoptosis-related components determined by the GO and KEGG analyses. As a result, we found that the phosphorylation of the HSP90β protein at serine 254 and of the GSK3β protein at tyrosine 216 might play an essential role in aspirin treatment-mediated apoptosis regulation. We further assessed total protein and phosphoprotein expression by western blot analysis of HSP90β and GSK3β, and the results supported the results of the bioinformatics analysis.

HSP90 is a chaperone, and its cochaperones orchestrate crucial physiological processes, such as cell survival, cell cycle control, hormone signaling, and apoptosis [34]. A study by Negroni L and his colleagues showed that HSP90β is phosphorylated on serine 254 upon 5-fluorocytosine treatment in colon cancer cells and suggested that this phosphorylation may play a role in apoptosis in their gene thera-

py model [35]. Kurokawa M and his colleagues reported that the hypophosphorylation of HSP90β at serine 226 and serine 255 is a strong inhibitor of apoptosome formation in tyrosine kinase-induced leukemias and that the phosphorylation status of HSP90β significantly impacts its ability to inhibit apoptosis [36]. Combined with these findings, our study found that the phosphorylation of HSP90β (S254) was significantly increased in the L group and decreased in the LA group, indicating HSP90β (S254) phosphorylation may play an important role in the apoptosis regulation by aspirin.

A study reported that GSK3β likely to regulate mitochondrial protein import through the HSP-90β phosphorylation [37]. HSP90 inhibition can induce apoptosis by downregulating AKT/GSK3β/β-catenin signaling, which involves the phosphorylation of GSK3β at serine 9 [38]. There might be interactions between phosphorylation of HSP90β and GSK3β in apoptosis regulation. GSK3β is an essential component in the Wnt/β-catenin signaling pathway, which plays a vital role in the early development of trophoblasts, and it acts as a cellular sensor and has a paradoxical function in the regulation of apoptosis, promoting intrinsic apoptotic signaling and inhibiting extrinsic apoptotic signaling. The differential phosphorylation of GSK3β at different sites can activate or inhibit the function of GSK3β; with serine 9/21 phosphorylation, the function is inhibited, and with tyrosine 216 phosphorylation, the function is enhanced [39]. In our study, we identified that the phosphorylation of GSK3β (Y216) is downregulated in the placenta of PE-like mice with increased apoptosis and upregulated after aspirin treatment with suppressed apoptosis. Therefore, phosphorylation of GSK3β (Y216) may also play an important role in the apoptotic regulation of aspirin in the placenta of PE-like mice, in a manner that interacts with HSP90β or independently, and its exact regulatory mechanism needs to be clarified with further research.

The phosphorylation of HSP90β (S254) and GSK3β (Y216) is essential in the regulation of apoptosis based on our results, and intervention methods such as medicine and micro RNAs targeting important protein might have great significance in PE prevention or treatment [40, 41]. Notably, the two proteins were involved in the regulation of inflammation [42, 43], which is excessively activated in our LPS-induced PE-like mice and exists in a specific subset of the high-risk population as well. In our research, we established the model at E7.5 and intervened after 4 hours, mimicking the prescription before 16 weeks. The present study provides us clues to the mechanism of action of aspirin in PE with high inflammation activation [44], and reveals potential mediated phosphoproteins.

However, our study has a few limitations. We only provide a potential pathway or targets that can be mediated by aspirin, with no precise regulatory pathway. Additionally, the lack of an overall protein expression profile may cause us to miss additional functions that aspirin can affect. Still, apoptosis regulation indeed is present, and we have validated the total protein expression of HSP90β and GSK3β by western blotting, which shows no significant difference between groups. Many studies are still needed to explore the specific pathway underlying the effect of aspirin on apoptosis and to verify causal and interacting relationships.

Conclusions

In the present study, we found that aspirin treatment can alleviate the upregulated apoptosis in the placenta of LPS-induced PE-like mice, and this effect may be related to the regulation of HSP90β (S254) and GSK3β (Y216) protein phosphorylation. This study describes the potential mechanism of aspirin-mediated apoptosis regulation in the placenta in PE prevention and treatment. It suggests that kinases and phosphatases regulating the phosphorylation of HSP90β (S254) and GSK3β (Y216) may be potential therapeutic targets. These results are meaningful for further studies to determine the exact mechanism of action of aspirin in PE and to better understand the improved clinical prescription of aspirin for PE prevention in the high-risk pregnant women with over-activated inflammation.

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Disclosure of conflict of interest

None.

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