Ocular neovascularization is a shared pathogenesis in numerous blinding diseases, including retinal neovascular diseases, such as proliferative diabetic retinopathy (PDR), retinal vein occlusion (RVO), retinopathy of prematurity (ROP), etc. These retinal neovascular diseases are all leading causes of global blindness and have major impacts on human health and quality of life. Although antivasular endothelial growth factor (VEGF) therapy sheds new light on the treatment of retinal neovascular diseases, systemic and local side effects of anti-VEGF therapy cannot be ignored. Furthermore, anti-VEGF agents have no or limited effect on some patients with ocular neovascular diseases. Thus, it is necessary to reveal deeper regulatory mechanisms of retinal neovascularization.

As a commonly recognized animal model used to study retinal neovascular diseases in vivo, the oxygen-induced retinopathy (OIR) model is established by exposing newborn pups to a hyperoxia environment, which has been considered similar to the pathological process of ROP. Through transcriptomics research, we previously identified differentially expressed mRNAs, microRNAs, long noncoding RNAs, circular RNAs, and tRNA-derived small RNAs in the retinal tissues of OIR mouse model. Moreover, novel molecular networks and potential therapeutic targets were discovered by proteomic analysis in the OIR model. However, the mechanism of pathological retinal neovascularization remains unclear.

As the final products of enzymatic processes, metabolites reflect physiological and pathological functions and activities from cells to tissues. Metabolomics analyses are commonly used to identify the pathological process of diseases through identifying networks of metabolic profiles. Recent studies reported that metabolomics revealed novel diagnostic biomarkers in cancer, anxiety disorders, osteoporosis, and ocular diseases. Metabolomics has been utilized in the study of extracellular vesicles, genetic testing, and mechanical investigation of macrophages. Recent studies also revealed the alteration of metabolites in several ocular disorders, such as diabetic retinopathy, central retinal vein occlusion, Vogt-Koyanagi-Harada disease, macular neurodegenerative disease, and...
FIGURE 1. Establishment of OIR mouse model. Flowchart of the current experimental design: untargeted metabolomics analysis (A) and targeted metabolomics analysis of amino acids and their derivatives (B). Representative images of the flat-mounted retinas of OIR mice and control mice (C): the retinas of P12, P13, P17, and P42 mice were immunofluorescent stained by fluorescein-labeled isolectin B4. Scale bars = 500 μm.

MATERIALS AND METHODS

Animal Ethics and the Mouse Model of Oxygen-Induced Retinopathy

C57BL/6J mice (Hunan SJA Laboratory Animal, Changsha, China) were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. This study's experimental procedures were approved by the Institutional Animal Care and Use Committee of Central South University.

The OIR model was induced in mice as previously described by exposing newborn pups to an environment with 75% oxygen at postnatal day 7 (P7), and were returned to a room air environment at P12, whereas control mice have been kept in the room air environment continuously. Retinas of both groups were collected at P17 for untargeted metabolomics analysis, and at P12, P13, P17, and P42 for targeted metabolomics analysis.

To identify animal modeling success, a mouse was randomly chosen from each litter of both groups at P12, P13, P17, and P42. The flat-mounted retinas were immunofluorescent stained by fluorescein-labeled isolectin B4 (Vector Laboratories, Burlingame, CA, USA) and were photographed by the fluorescent microscope DMi4000B (Leica, Wetzlar, Germany).
Sample Collection and Preparation

Retinas of both eyes were frozen and stored at −80°C immediately after the mice were euthanized at each time point. The mixture of retinas from two eyes of each mouse was dissolved in 20 µL of cold methanol/acetoniitrile/H₂O. After ultrasonication and protein precipitation, the mixture was centrifuged for 20 minutes (14,000 g, 4°C) and dried in a vacuum centrifuge. Samples were re-dissolved in 100 µL solvent. To perform liquid-chromatography tandem mass spectrometry (LC-MS/MS) analysis, supernatants were collected. The standard curve was established for targeted metabolomics.

LC-MS/MS Analyses for Untargeted Metabolomics

The analyses were carried out using a UHPLC (1290 Infinity LC, Agilent Technologies, CA, USA) coupled to a quadrupole time-of-flight (AB Sciex TripleTOF 6600). Using a 2.1 mm × 100 mm ACQUITY UPLC BEH 1.7 µm column (Waters, Ireland), samples were analyzed for HILIC separation. The parameters of the untargeted metabolomics can be referred to in our previous study.29

HPLC-MS/MS Analyses for Targeted Metabolomics

Utilizing a UHPLC (1290 Infinity LC; Agilent Technologies) coupled to a QTRAP (AB Sciex 5500), analyses of this study were performed. The mobile phase contained A = 25 mM CH₃COONH₄ + 0.08% FA in water and B = 0.1% FA in ACN. Samples were in the automatic sampler at 4°C, and the column temperatures were kept at 40°C constantly. The gradients were at a flow rate of 250 µL/min. A 4 µL aliquot of each sample was injected. The gradient was 90% B linearly reduced to 70% in 0–12 minutes, and then was reduced to 50% in 12–18 minutes and reduced to 40% in 18–25 minutes, and kept for 25–30 minutes. Then the B was raised to 90% in 30–30.1 minutes and kept for 30.1–37 minutes.

The quality control (QC) samples were applied to examine and evaluate the repeatability and stability of this system. At the same time, the standard mixture of AA metabolites was set for the correction of chromatographic retention time.

In ESI cationic mode, settings of conditions were as below: Ion Source Gas1 (Gas1) as 40, Ion Source Gas2 (Gas2) as 40, curtain gas (CUR) as 30, source temperature: 500°C, IonSpray Voltage Floating (ISVF) ± 5500 V. Adopt the MRM mode detection ion pair.

Data Processing

For the untargeted metabolomics, using ProteoWizardM-SCvert, the raw MS data (wiff.scan files) were converted to MzXML files before they were imported into freely available XCMS software. In the extracted ion features, only the variables with over 50% of the nonzero measurement values in at least one group were maintained. Through comparing the accuracy m/z value (<25 ppm), and tandem mass spectrometry (MS/MS) spectra with an in-house database (Shanghai Applied Protein Technology Co. Ltd., Shanghai, China) established with available authentic standards, the compound identification of metabolites was conducted.

For targeted metabolomics, the Multiquant software was applied for the extraction of chromatographic peak area and retention time. The AA standards correct retention time was applied to identify the metabolites.31 Together with the biological samples, the quality control samples were processed. Detected metabolites in pooled samples with the coefficient of variation (CV) less than 30% were denoted as reproducible measurements.

Bioinformatics Analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (https://www.kegg.jp/) was conducted to reveal the enriched pathways of the altered metabolites.

Statistical Analysis

For untargeted metabolomics, after normalized to total peak intensity, the processed data were uploaded before importing into SIMCA-P (version 14.1; Umetrics, Umea, Sweden) for multivariate data analysis. To assess the robustness of the model, the 7-fold cross-validation and response permutation testing was applied. In the OPLS-DA model, the variable importance in the projection (VIP) value of each variable was calculated to indicate its contribution to the classification. To measure the significance of each metabolite, metabolites with the VIP value >1 were further applied to Student's t-test at the univariate level, with a P < 0.05 or P < 0.10 as statistically significant. For targeted metabolomics of amino acids and their derivatives, metabolites with P < 0.05 were marked as notably altered metabolites.

RESULTS

Untargeted Metabolomics of Retinal Tissues of OIR Mice and Controls

To evaluate the success of animal modeling of OIR, flat-mounted retinas of P12, P13, P17, and P42 mice of the same litter batches of both OIR model and control groups were immunofluorescent stained by isoelectin B4, and typical pathological neovascular tufts and avascular areas were detected by representative images of OIR group, compared to the normal physiological vasculature of the retina in the control group (Fig. 1C).

To identify the differentially expressed metabolites in OIR retinas, 20 samples of retinas (10 OIR samples and 10 controls) at P17 were assessed for the initial untargeted metabolomics analysis. A total of 5899 cationic peaks and 4697 anionic peaks of metabolites were recorded according to the XCMS records.

As the principal component analysis (PCA) showed, in cationic and anionic modes, the QC samples gathered together closely (Figs. 2A, 2B), which indicated that the experiments have good repeatability. To determine the differences in metabolic profiles, OPLS-DA score plots were conducted to identify the metabolomic profile differences between these two groups. As demonstrated in Figures 2C and 2D, remarkable separations of the OIR group and control group were recognized in both cationic and anionic modes. Then, the permutation analysis of the OPLS-DA model was performed, and results indicated that the OPLS-DA model fitting was valid and stable in both ion modes (Figs. 2E, 2F).

Volcano Plot analysis has been applied to screen potential alteration of the metabolites following the criteria of fold change (FC) > 1.5 and P < 0.05. Identified altered metabolites with the criteria between these two groups (red points) under cationic mode (Fig. 3A) and anionic mode (Fig. 3B).
Subsequently, the significantly changed metabolites under the criteria of VIP > 1 and \( P < 0.1 \) were selected and finally recognized after compound identification with the database. Under the cationic mode, 58 altered metabolites (52 of them met \( P < 0.05 \)) were recognized significantly different (Fig. 4, Table 1). Particularly, between the two groups, there were 14 altered amino acids and their derivatives. On the other hand, 49 metabolites (45 of them...
metabolites with an abundance fold change greater than 1.5 and adjusted p-value less than 0.05) were significantly altered under the anionic mode, including 12 amino acids and their derivatives (Fig. 5, Table 2).

**Involved Pathways of Changed Metabolites in OIR Retinas**

The KEGG analyses were carried out to discover the involvement of signaling pathways of those altered metabolites.

As Figure 6 demonstrated, some essential pathways have been detected. The top 10 enriched pathways are: (1) ATP-binding cassette (ABC) transporters; (2) central carbon metabolism in cancer; (3) alanine, aspartate, and glutamate metabolism; (4) choline metabolism in cancer; (5) synaptic vesicle cycle; (6) protein digestion and absorption; (7) nicotine addiction; (8) taste transduction; (9) aminocarboxyl-tRNA biosynthesis; and (10) glycerophospholipid metabolism.
FIGURE 4. Heatmap of the differential metabolites under the cationic ion mode of untargeted metabolomics. The blue color represents low relative level of each metabolite, and the red color represents high relative level of each metabolite. Similarly hereinafter in Figure 5 and Figure 7.
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>VIP</th>
<th>Fold Change</th>
<th>P Value</th>
<th>m/z</th>
<th>rt(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betaine</td>
<td>8.95366</td>
<td>2.34901</td>
<td>0.00000</td>
<td>118.08524</td>
<td>750.7</td>
</tr>
<tr>
<td>4-Guanidinobutyric acid</td>
<td>3.26583</td>
<td>4.17917</td>
<td>0.00000</td>
<td>146.09125</td>
<td>673.567</td>
</tr>
<tr>
<td>Dimethylglycine</td>
<td>1.91824</td>
<td>2.88435</td>
<td>0.00000</td>
<td>104.06937</td>
<td>564.983</td>
</tr>
<tr>
<td>1-Stearoyl-sn-glycerol 3-phosphocholine</td>
<td>8.94919</td>
<td>2.40241</td>
<td>0.00000</td>
<td>524.36977</td>
<td>336.257</td>
</tr>
<tr>
<td>3-Aminobutanoic acid</td>
<td>1.37205</td>
<td>6.39603</td>
<td>0.00000</td>
<td>104.0694</td>
<td>628.794</td>
</tr>
<tr>
<td>Cellobose</td>
<td>1.20499</td>
<td>2.78840</td>
<td>0.00000</td>
<td>160.11902</td>
<td>749.718</td>
</tr>
<tr>
<td>Xanthine</td>
<td>2.17359</td>
<td>2.30053</td>
<td>0.00000</td>
<td>153.03982</td>
<td>395.371</td>
</tr>
<tr>
<td>(3-Carboxypropyl)trimethylammonium cation</td>
<td>5.70891</td>
<td>1.78635</td>
<td>0.00000</td>
<td>146.1169</td>
<td>741.139</td>
</tr>
<tr>
<td>Methyl acetocetate</td>
<td>1.04695</td>
<td>2.50466</td>
<td>0.00000</td>
<td>137.06985</td>
<td>548.367</td>
</tr>
<tr>
<td>L-Carnitine</td>
<td>1.52509</td>
<td>2.36630</td>
<td>0.00000</td>
<td>137.06985</td>
<td>548.367</td>
</tr>
<tr>
<td>1-O-(cis-9-Octadecenyl)-2-O-acetyl-sn-glycero-3-phosphocholine</td>
<td>1.13325</td>
<td>2.17326</td>
<td>0.00000</td>
<td>550.38401</td>
<td>331.269</td>
</tr>
<tr>
<td>1-Methylnicotinamide</td>
<td>1.50346</td>
<td>2.85537</td>
<td>0.00000</td>
<td>137.06985</td>
<td>548.367</td>
</tr>
<tr>
<td>Uracil</td>
<td>4.94330</td>
<td>4.23729</td>
<td>0.00000</td>
<td>137.06985</td>
<td>548.367</td>
</tr>
<tr>
<td>Allantoin</td>
<td>1.91593</td>
<td>3.57525</td>
<td>0.00000</td>
<td>146.09125</td>
<td>673.567</td>
</tr>
<tr>
<td>L-Citrulline</td>
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<td>1.85888</td>
<td>0.00000</td>
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<td>548.367</td>
</tr>
<tr>
<td>D-Pipeolicinic acid</td>
<td>1.10437</td>
<td>2.71995</td>
<td>0.00000</td>
<td>137.06985</td>
<td>548.367</td>
</tr>
</tbody>
</table>

**Table 1.** Significantly Altered Metabolites by Untargeted Metabolomics Under Cationic Mode

**Metabolomics Analysis Targeting Amino Acids and their Derivatives**

To confirm the reliability of the untargeted metabolomics and explore the metabolomics profiles in key time points of the OIR model, we performed metabolomics analysis targeting amino acids and their derivatives in retinas of OIR mice and room air controls at P12, P13, P17, and P42. The assessed amino acids and their derivatives include 31 metabolites (Fig. 7, Table 3). No significant change was
found in all of the amino acids and their derivatives at P12 (see Fig. 7A). However, in the early time of hypoxia at P13, 15 metabolites were significantly altered ($P < 0.05$). Among them, eight were increased and seven were decreased, whereas the ranges of fold change were not large (see Fig. 7B). At P17, 19 metabolites were significantly changed in OIR retinas ($P < 0.05$; see Fig. 7C). According to the absolute quantification experiments, only one metabolite (creatinine) was significantly decreased, whereas 18 metabolites were significantly increased in retinal tissues of OIR mice. Aminoadipic acid ranked first among the altered amino acids and their derivatives ($FC = 3.888804$, $P = 0.000000$). Moreover, at the adult time (P42), there are 13 amino acids and their derivatives significantly changed, including only one upregulated metabolite and 12 downregulated metabolites (see Fig. 7D).
**DISCUSSION**

This study primarily screened the metabolomic profile changes in the retinal tissues of OIR mice through untargeted metabolomics analysis and identified a variety of altered metabolites involving in retinal neovascularization (Tables 1, 2). Particularly, numerous amino acids and their derivatives were detected to be significantly altered. Thus, a targeted metabolomics study focusing on amino acids and their derivatives was made to confirm whether those metabolites were significantly changed. As shown in Figure 7 and Table 3, at the time point of P12, when the OIR mice were just removed from the hyperoxia environment, all of the assessed amino acids and their derivatives were not significantly altered. However, 15 and 19 amino acids and their derivatives were found to be significantly changed at P13 and P17, respectively. These findings indicate that the metabolomic alteration of amino acids and their derivatives coincided with the development of retinal angiogenesis. Interestingly, the metabolomic alteration was also found at P42 in the OIR retinas, which suggested that hypoxia-induced angiogenesis could have a long-term impact on the profile of metabolites, it may last even when retinal neovascularization has regressed.

Amino acids and their derivatives are the constituent units of proteins and the most basic substances in life activities.
They play an important role in metabolism, nerve transmission, and liposome transport. Amino acids also participated in the pathogenesis of angiogenesis-related diseases. Liesche et al. demonstrated that $^1$H-Fluoroethyl-tyrosine uptake was mediated by amino acid transporters in glioblastomas and could be associated with tumor neovascularization. Plasminogen kringle 5 exerted an anti-angiogenic effect when 5 acidic amino acids in the NH2 terminal were replaced by serine residues. Macrophages are angiogenic cells that contribute to diverse functions in ocular neovascular diseases, and M2 macrophages have been demonstrated to enhance pathological neovascular tufts in OIR retinas. Metabolic networks, including amino acids and their derivatives, were involved in macrophage polarization, which may identify novel pharmacologic control targets for both M1 and M2 macrophage phenotypes.

For instance, based on the targeted metabolomics analysis, arginine was upregulated in the retinal tissues of OIR model compared to the controls (see Table 3). Arginine is the precursor of angiogenesis modulator nitric oxide (NO), and arginine deiminase (ADI) was reported to attenuate lipopolysaccharide-induced NO synthesis. ADI inhibited angiogenesis and tumor growth, suppression of angiogenesis by ADI could be reversed by exogenous arginine surplus. It has been reported that the combination of arginine restricted diet can improve the visual acuity of patients with choroidal neovascularization (CNV) after the intravitreal injection of ranibizumab. L-arginine contributed additional effects on exercise-induced angiogenesis possibly by enhancing VEGF expression in the hind-leg muscles and heart. On the other hand, the enhancement of NO synthesis pathway is characteristic of pro-inflammatory M1-like macrophages, whereas the expression of arginase characterizes M2 macrophages, and arginase hydrolyzes arginine to ornithine and urea while limiting the synthesis of NO derived from arginine. Elms et al. indicated that diabetes induced arginase 1 upregulation and was associated with diabetes-induced impairment of retinal blood flow via vascular endothelial cell dysfunction. Arginase 1 was involved in diabetes-induced senescence of retinal endothelial cells, and inhibiting its activity could be a possible therapeutic method in treating diabetic retinopathy by prevention of premature senescence. Therefore, amino acids, such as arginine, might be involved in the pathogenesis of
angiogenesis-related diseases via regulating macrophage polarization as well as VEGF expression. Nevertheless, further investigations are necessary to reveal the roles and mechanisms of amino acids in ROP and other retinal neovascular diseases.

We recently reported the plasma metabolomic profile of treatment-requiring ROP by assessing clinical samples, combined with the results of the present study, multiple metabolites were significantly changed in the plasma of patients with treatment-requiring ROP as well as the retinal tissues of OIR mice in the present study. For example, caffeine was downregulated in both studies, which supported that caffeine acts as a protective role in the prevention and therapeutics of ROP in previous studies. Moreover, similar to the targeted metabolomics results of the present study, numerous amino acids and their derivatives were significantly altered in the plasma metabolomic profiles of treatment-requiring ROP. This phenomenon indicated the important involvement of amino acid metabolism in retinal neovascularization. Besides, according to the KEGG analyses, some of the pathways involved in the current study were the...
same as the enriched pathways of the altered plasma metabolites in treatment-requiring ROP infants, such as “ABC transporters,” “protein digestion and absorption,” “aminoacyl-tRNA biosynthesis,” etc. It has been reported that deficiency of amino acid promotes tumor angiogenesis via the pathway of GCN2/ATF4.53 Similarly, a recent study indicated that the restriction of amino acid induces angiogenesis through the pathway of GCN2/ATF4, and regulates the production of VEGF and H.54 Moreover, amino acid deprivation induced VEGF expression level in human retinal pigmented epithelial cell line.55 Therefore, amino acids and their related pathways might play important roles in retinal neovascularization, and further studies are necessary to reveal the functions and mechanisms of amino acid metabolism and those cross-involved pathways in retinal neovascularization.

In conclusion, this study identified metabolomic profile changes in the retinal tissues of OIR mice. By bioinformatics analysis, “ABC transporters,” “central carbon metabolism in cancer,” and “alanine, aspartate, and glutamate metabolism” were the most enriched KEGG pathways associated with the altered metabolites. Through the screening of the untargeted metabolomics along with the targeted metabolomics analysis, a large number of altered amino acids and their derivatives were detected. These results indicated that metabolites, especially amino acids and their derivatives, together with the involved metabolic pathways, might be involved during the pathological process of retinal neovascular diseases.

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