Activation of protein kinase A signaling and inhibitions of glycine/glutathione biosynthesis involves in the transition from prediabetes to diabetes based on metabolomics data analysis

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Abstract

Metabolomics is expected to identify potential metabolites and related pathways, and further reveal the underlying mechanisms of the transition from prediabetes to diabetes. In this study, a metabolomics-based gas chromatography-mass spectrometry (GC-MS) technique was used for demonstrating the serum metabolic profiles among healthy, prediabetes, and diabetes at fasting state and 2h oral glucose tolerance test (2h OGTT) state. With Ingenuity Pathway Analysis (IPA) tool, the comparative analysis showed no significant differences in the pathway analysis ($P > 0.05$) between prediabetes and diabetes at either fasting state or 2h OGTT state. The self-comparative analysis demonstrated the glycine/glutathione biosynthesis in diabetes were more inhibited than that in prediabetes or healthy control at 2h OGTT state compared with fasting state ($P<0.05$). In addition, the protein kinase A signaling pathway in prediabetes or diabetes was significantly inhibited more than that in healthy control ($P<0.05$). Therefore, the glycine/glutathione biosynthesis and protein kinase A signaling could differentiate the diabetic subjects from the prediabetic and healthy control subjects, and may involve in prediabetes transition to diabetes. This study provided more metabolomics information for the transition from prediabetes to diabetes.

1. Introduction

As one of the fastest growing epidemics worldwide, diabetes is a metabolic disorder characterized by elevated blood glucose concentration and increased insulin resistance leading to serious microvascular and macrovascular complications. Widespread changes in lifestyle and aging of the global population have resulted in an unprecedented rise in the prevalence of diabetes in the world, especially in low and middle income countries [1,2]. China has the largest number of subjects with diabetes, with high morbidity and mortality [3,4]. In 2013, the overall prevalence of diabetes in China was 10.9%, while the prevalence of prediabetes was 35.7% [4,5]. If blood glucose levels are higher than normal but lower than the threshold applied for the diagnosis of diabetes, prediabetes, is considered as a significant risk factor for diabetes and cardiovascular diseases [6]. Prediabetes is a reversible process and early intervention in prediabetic subjects can reduce the risk of diabetes by 40% to 58% [7-9]. Characterization and identification of subjects in the prediabetic state is important for the prevention, management and treatment of diabetes [10]. In recent years, an increasing number of studies have begun to focus on prediabetes transition to diabetes, and the pathogenesis is still unclear [11-13]. Accordingly, it is imperative to determine the potential mechanisms involved in the progression from prediabetes to diabetes.

Diabetes is a systemic metabolic disorder, thus metabolomics technique is an appropriate approach to explore the pathogenesis of prediabetes and diabetes from the systemic metabolism. Increasing numbers of studies have explored the relationship between a wide range of metabolites and diabetes using metabolomics technique [14-16]. Metabolomics is the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modifications [17]. By measuring and mathematically modeling changes in the products of metabolism (low molecular

weight biochemicals including amino acids, sugars, nucleotides, organic acids, and lipids) found in biological fluids and tissues, high-throughput metabolomics technology have provided fresh insights into the pathophysiological pathways and understanding of the disease [18]. As a monitoring tool for metabolism and signaling pathways, metabolomics is expected to identify potential metabolites and to reveal the metabolic changes and the underlying mechanisms of the progression from prediabetes to diabetes.

In this study, a metabolomics-based gas chromatography-mass spectrometry (GC-MS) technique was used for demonstrating the serum metabolic profiles among healthy, prediabetes and diabetes. On the basis of metabolomic data combined with bioinformatics analysis, we aimed to unveil the metabolism and signaling pathways involved in the pathogenesis of prediabetes transition to diabetes.

2. Materials And Methods

2.1. Study subjects

This study is a community-based cross-sectional investigation at Hangxin Hospital, Beijing, China, from July to November 2010. A total of 4000 community subjects underwent the health screening program. According to the diagnosis and classification criteria proposed by the American Diabetes Association (ADA) in 2010 [19], diabetes was defined as someone with (1) a fasting plasma glucose (FPG) level of 7.0 mmol/L or higher, or (2) a 2h oral glucose tolerance test (2h OGTT) level of 11.1 mmol/L or higher, or (3) a glycosylated hemoglobin A1c (HbA1c) concentration of 6.5% or more. If the subject met one or more of these criteria, he/she was categorized as diabetes. Prediabetes was defined as someone with (1) FPG $\geq$ 5.6 mmol/L and < 7.0 mmol/L, or (2) 2h OGTT level of $\geq$ 7.8 mmol/L and < 11.1 mmol/L, respectively, or (3) a HbA1c concentration of $\geq$ 5.7% and < 6.5%, subjects without a prior diagnosis of diabetes. If the subject met one or more of these criteria, he/she was categorized as prediabetes. The inclusion criteria included age level of > 18 and < 75 years, local residents in Beijing, complete data measurements and informed consents. Subjects with cardiovascular and cerebrovascular diseases, mental disorders, gastrointestinal disease, nephropathy, metabolic syndrome, malignant tumors, pregnancy or incomplete recorded information were excluded from this project based on their medical records.

After investigation, a total of 105 subjects (69 males and 36 females) including 35 subjects for diabetes group, 35 subjects for prediabetes group and 35 subjects for healthy control group with complete data were finally enrolled in this study. The study was performed according to the guidelines of the Helsinki Declaration. A standard protocol was designed by Institute of Basic Research in Clinical Medicine and was approved by the Ethics Committee of China Academy of Chinese Medical Sciences. Written informed consent was obtained from all subjects.

2.2. Data measurements and blood sampling

All subjects were asked to fill out a questionnaire focusing on demographic characteristics (age, gender, education, etc.), anthropometrics (height, weight and waist circumference (WC)), medical history, and

health-related behavior under the guidance of physicians. Fasting blood samples and 2h OGTT blood samples were drawn via venipuncture from the study subjects by clinical nurses. After storage for 2 h at 4°C, the blood samples were centrifuged at 3000 rpm for 10 min. The obtained serum was divided into two parts: one part was used for the measurement of FPG, HbA1c, 2h OGTT, total cholesterol (TC), triglyceride (TG), high- and low-density lipoprotein cholesterol (HDL-C, LDL-C), and uric acid (UC) concentrations according to the manufacturers' instructions for the respective commercial test kits. The remaining 100 μL serum was added to 320 μL of methanol, and the mixture was vortexed for 60 s. After centrifugation at 15000 rpm for 10 min at 4°C, the supernatant was stored at -80°C for GC-MS analysis.

Blood sample (6mL) was randomly divided into six parts and extracted identically. These six samples were injected continuously to verify the repeatability of the sample preparation method. 20 μL are extracted from each blood sample to produce a mixed quality control (QC) sample and 100μL aliquot is extracted from this mixed sample by the same method. The mixed sample is used to provide a representative "mean" sample containing all the analytes encountered during the analysis and to verify the stability of the GC-MS system.

2.3. GC-MS analysis

GC-MS analysis was performed using GCMS-QP2010 Plus (Shimadzu, Kyoto) and capillary column (Rxi-50, 30 m×0.25 mm, 0.25 ft m).With helium as carrier gas, the rate was 1.0 mL/min. The oven temperature varied from 60 ~ 80℃ at 5℃/min, then from 80 ~ 90℃ at 2℃/min (keep for 3 min), from 90 ~ 150℃ at 10℃/min (keep for 1 min), from 150 ~ 220℃ at 1℃/min, and from 220 ~ 290℃ at 10℃/min. The injector and interface temperature were maintained at 250℃.The mass spectrum in electron impact mode was generated at 70 eV. The ion source temperature was maintained at 250℃.One sample of 1μL was injected with a split mode injection (split ratio□60:1).Based on the linear retention index (RI) and the comparison of MS data with reference compounds, these components were preliminarily identified. \The linear retention indices of all components were determined by homologous n-alkanes (C_{10}–C_{40}).These components were identified by comparing with the mass spectra of NIST05 and NIST05S.[20]

2.4. Data processing and statistical analysis

The number of components in different samples was selected according to the retention time of the common peaks. The retention time and peak areas of GC-MS were obtained in one table. The table is then used as input data for multivariate statistical analysis. A multivariate statistical analysis, including unsupervised principal component analysis (PCA) and partial least-squares-discriminant analysis (PLS-DA), was used for metabolic profiles using SIMCA-P 11.0 statistical package (Umetrics AB, Umeå, Sweden).SAS 9.1.3 Statistical package (order No.195557) for statistical analysis. Chi-square test was used in attribute data analysis. The measured data were normally distributed. Analysis of variance was used for the comparison between groups. *P* values < 0.05 were set as significant for all of the statistical tests.

2.5. Pathway analysis

The analysis of bio-functions and canonical pathways for the candidate metabolites were conducted by using the Ingenuity Pathway Analysis system (IPA, Ingenuity® Systems, http://www.ingenuity.com), to gain insight into the typical metabolic alterations associated with the biomarkers and the mechanisms related to the transition from prediabetes to diabetes.

3. Results

3.1. Baseline characteristics of the study subjects

The clinical and biochemical characteristics of the subjects are shown in Table 1. The subjects with prediabetes or diabetes tended to have significantly higher age, weight, body mass index (BMI), WC and FPG compared with the healthy control subjects ($P < 0.05$). The subjects with diabetes had significantly higher HbA1c, FPG and 2h OGTT than the subjects with prediabetes ($P < 0.05$), and there was no significant difference in HbA1c, FPG and 2h OGTT between the subjects with prediabetes and healthy control subjects. Moreover, gender, TC, TG, HDL-C, LDL-C and UA were not significantly different among the three groups.

3.2. Testing with the GC-MS method

Six samples from random blood samples were injected continuously to assess their repeatability. According to the different chemical polarity and m/z value, five kinds of commonly used injection extract ion chromatograms (EICs)were screened. The relative standard deviation (RSDs)of peak area is 4.13% ~ 13.13%, and the relative standard deviation of retention time is 0.04% ~ 0.98%.

The stability of the method in large-scale sample analysis is proved by the test of pooled QC samples. PCA results showed that QC samples were closely clustered. In addition, the peak area, retention time and quality accuracy of the five EICs selected from the five QC samples also showed good system stability. The RSDs of the five peaks were 4.94% ~ 14.88%, 0.03% ~ 1.10%, and 0.14 $x10^{-4}$% ~ 0.76 $x10^{-4}$%, respectively. The results showed that the large scale sample analysis has no significant effect on the reliability of the data.[20]

3.3. Identification of the differential metabolites

Typical base peak chromatograms (BPCs) of serum samples were obtained from diabetes, prediabetes and healthy control. Multiple pattern recognition methods of PLS-DA were adopted on the basis of the metabolic changes in these subjects as revealed by BPCs. These methods facilitated the classification of the metabolic phenotypes and helped to identify the differential metabolites.

As shown in Table 2, 8 differential metabolites were identified at fasting state and 14 differential metabolites were identified at 2h OGTT state in the subjects with prediabetes. As shown in Table 3, 14 differential metabolites were identified at fasting state and 16 differential metabolites were identified at 2h OGTT state in the subjects with diabetes.

3.4. Pathways analysis

With IPA analysis, as shown in Figure 1, the common pathways in both prediabetes and diabetes at fasting state were bupropion degradation, glycine biosynthesis I and glycine biosynthesis III ($P < 0.05$). As shown in Figure 2, the pathways in both prediabetes and diabetes at 2h OGTT state were glycine biosynthesis III, L-dopachrome biosynthesis, growth hormone signaling, maturity onset diabetes of young signaling, and bupropion degradation ($P < 0.05$).

As illustrated in Figure 1 and 2, there were two pathways for differential metabolites in both prediabetes and diabetes at fasting state and 2h OGTT state: glycine biosynthesis III and bupropion degradation. However, the comparative analysis between prediabetes and diabetes at either fasting state or 2h OGTT state showed no significant differences of metabolism pathways were found ($P > 0.05$).

The pathways in the three groups at 2h OGTT state compared with fasting state were shown in Figure 3. Protein kinase A signaling pathway showed statistical significance in healthy (2h OGTT state vs. fasting state) ($P < 0.05$), and no statistical significances in both prediabetes and diabetes (2h OGTT state vs. fasting state) ($P > 0.05$); glycine biosynthesis I and glutathione biosynthesis pathways showed much more statistical significance in diabetes (2h OGTT state vs. fasting state) than in prediabetes and healthy (2h OGTT state vs. fasting state) ($P < 0.05$). The metabolite changes in the three pathways with statistical significances in the healthy, prediabetes and diabetes at 2h OGTT state compared with fasting state were shown in Figure 4. In the upper part of glycine biosynthesis I pathway, L-serine showed fold change, but no significant changes (from -1.242 to -1.267) in healthy, prediabetes and diabetes (2h OGTT state vs. fasting state). In the glutathione biosynthesis, interestingly, glycine only shown in the diabetes, and the results suggested the pathway was inhibited. In the protein kinase A signaling, the results showed the pathway was inhibited in healthy, or otherwise, the pathway was activated in prediabetes and diabetes.

4. Discussion

Diabetes is the result of prediabetes progression. Despite a considerable amount of studies being collected and analyzed regarding diabetes, the molecular mechanisms of prediabetes transition to diabetes is still unknown [21]. Deciphering the biomarkers and mechanisms of prediabetes transition to diabetes is vital to preventing disease progression. In the present study, the serum metabolite changes of prediabetes and diabetes based on serum metabolic profiles were identified using GC-MS technique.

Pathway analysis showed no significant differences were found between prediabetes and diabetes at either fasting state or 2h OGTT state. Meanwhile, the glycine biosynthesis III and bupropion degradation were two common metabolism pathways in both prediabetes and diabetes at fasting state and 2h OGTT state. The bupropion with naltrexone is a combination therapy for obesity and obesity with diabetes, and the combination could significantly improve the lipid metabolism, glucose metabolism and insulin resistance [22-25]. Glycine is the proteinogenic amino acid of lowest molecular weight, harboring a

hydrogen atom as a side-chain [26]. In the present study, glycine was found down-regulated in serum in both prediabetes and diabetes. It has been confirmed that glycine pathway is associated with diabetes, and the decline in glycine levels is involved in the pathogenesis of glucose intolerance, insulin resistance and diabetes [27-29].

The two pathways, including glycine biosynthesis I and glutathione biosynthesis, are crucial for prediabetes transition to diabetes in this study. We found glycine/glutathione biosynthesis levels in diabetes were more activated than that in prediabetes or healthy control at 2h OGTT state compared with fasting state. Likewise, researchers found the top-ranking metabolites with insulin resistance were in the glycine biosynthesis and glutathione biosynthesis pathways [30-32]. The reduced glycine levels has been considered as the most robust and consistent amino acid markers for prediabetes and incident diabetes [33]. The glutathione, often referred to the master antioxidant, participates not only in antioxidant defense systems, but also many metabolic processes [34]. There is increasing evidence that dysregulation of glutathione synthesis contributes to the pathogenesis of insulin resistance and incident diabetes [35,36]. Nevertheless, there has been no study on the relationship between the pathways and prediabetes transition to diabetes.

Protein kinase A is a multi-unit protein kinase that mediates signal transduction of G-protein-coupled receptors through its activation by adenyl cyclase-mediated cAMP [37]. The cAMP- dependent protein kinase A pathway, known to promote cell growth and delay apoptosis [38], could regulate glucose homeostasis at multiple levels including insulin and glucagon secretion, glucose uptake, glycogen synthesis and breakdown, gluconeogenesis and neural control of glucose homeostasis [39]. Therefore, the glycine/glutathione biosynthesis and protein kinase A signaling may all involve in prediabetes transition to diabetes. As illustrated in Figure 3 and 4, compared with fasting state, the characterization of diabetes at 2h OGTT state was the protein kinase A signaling not activated and glycine/glutathione biosynthesis activated. The characterization of prediabetes at 2h OGTT state was the protein kinase A signaling not activated and glycine/glutathione biosynthesis not activated. The characterization of healthy control at 2h OGTT state was the protein kinase A signaling activated and glycine/glutathione biosynthesis not activated.

This study provided a good template for determining the differential metabolites of subclinical disease status and a better understanding of prediabetes progression based on metabolomics. There are, however, several limitations of this study. The subjects in this study were all office workers in Beijing, and the prevalence of prediabetes and diabetes may be higher than that in a rural area. Further studies with a larger sample size and more detailed information collection are needed.

Declarations

Author Contributions: Conceptualization, Ning Zhao, Miao Jiang, Weini Chen and Aiping Lu; Data curation, Junxuan Zhu, Yumin Guo and Xuyan Niu; Formal analysis, Zhan Gu; Funding acquisition, Aiping

Lu; Investigation, Ning Zhao, Miao Jiang, Weini Chen, Biao Liu, Junxuan Zhu and Yumin Guo; Methodology, Li Li, Junping Zhan and Hongwei Kong; Project administration, Chi Zhang, Junping Zhan and Cheng Lu; Resources, Hongwei Kong; Software, Xiaojuan He; Supervision, Cheng Lu and Aiping Lu; Validation, Li Li, Chi Zhang and Xuyan Niu; Visualization, Xiaojuan He, Shiping Cheng; Writing – original draft, Ning Zhao and Zhan Gu; Writing – review & editing, Hongwei Kong, Cheng Lu and Aiping Lu. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Tables

Table 1. Baseline characteristics of the study subjects.

Variables	Healthy control	Prediabetes	Diabetes	*P* value
Age (years)	48.49±9.58	55.09±8.50[a]	55.46±8.44[b]	0.0073
Gender (M/F)	19/16	25/10	25/10	0.2215
Weight (kg)	65.16±10.09	73.40±8.94[a]	74.33±11.37[b]	0.0020
BMI (kg/m^2)	23.43±2.71	25.44±2.10[a]	25.91±2.83[b]	0.0011
WC (cm)	81.80±7.28	89.57±6.37[a]	90.21±8.57[b]	<0.0001
HbA1c (%)	5.45±0.35	5.59±0.36	6.24±0.55[bc]	<0.0001
FPG (mmol/L)	5.28±0.22	6.24±0.39[a]	7.10±0.56[bc]	<0.0001
2h OGTT (mmol/L)	6.19±1.09	6.97±1.78	10.91±3.17[bc]	<0.0001
TC (mmol/L)	5.22±0.90	5.31±0.81	5.40±0.90	0.9131
TG (mmol/L)	1.79±0.91	2.09±1.23	2.18±1.33	0.5065
HDL-C (mmol/L)	1.19±0.21	1.14±0.30	1.14±0.26	0.3456
LDL-C (mmol/L)	3.04±0.79	2.97±0.65	2.93±0.67	0.7274
UA (μmol/L)	336.0±88.56	366.3±92.15	383.8±111.96	0.1181

[a] $P < 0.05$ (vs. healthy control); [b] $P < 0.05$ (vs. healthy control); [c] $P < 0.05$ (vs. prediabetes).

Abbreviations: BMI, body mass index; WC, waist circumference; HbA1c, hemoglobin A1c; FPG, fasting plasma glucose; OGTT, oral glucose tolerance test; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; UA, uric acid.

Table 2. Differential metabolites at fasting state and 2h OGTT state in prediabetes.

ID	Symbol	Expr Fold Change
Fasting State		
C05984	2-hydroxytric acid	1.144
C00187	cholestero	-1.117
C00031	D-glucose	1.147
C00031	D-glucose	1.179
C00031	D-glucose	1.193
C01582	galactose	1.125
C00037	glycine	-1.217
C00249	palmitic acid	1.088
C01530	stearic acid	1.070
C00086	urea	1.175
2h OGTT State		
C05984	2-hydroxytric acid	1.145
C00219	arachidonic acid	1.170
C00031	D-glucose	1.194
C00031	D-glucose	1.179
C01582	galactose	1.132
C00116	glycerol	1.270
C00037	glycine	-1.210
C00082	L-tyrosine	1.153
C01432	lactic acid	1.145
C00712	oleic acid	1.195
C00249	palmitic acid	1.114
C16435	poly-L-prol	1.107
C00794	sorbitol	1.178
C01530	stearic acid	1.111
C00086	urea	1.170

Table 3. Differential metabolites at fasting state and 2h OGTT state in diabetes.

ID	Symbol	Expr Fold Change
Fasting State		
C01089	(R)-3-hydroxytyric acid	1.813
C05984	2-hydroxytyric acid	1.514
C00031	D-glucose	1.292
C00031	D-glucose	1.334
C00031	D-glucose	1.371
C01796	erythrose	-1.443
C01582	galactose	1.307
C00116	glycerol	1.431
C00037	glycine	-1.156
C00082	L-tyrosine	1.208
C01595	linoleic acid	1.188
C00712	oleic acid	1.439
C00249	palmitic acid	1.226
C01530	stearic acid	1.129
C00086	urea	1.181
C00366	uric acid	1.241
2h OGTT State		
C01089	(R)-3-hydroxybutyric acid	1.507
C05984	2-hydroxybutyric acid	1.580
C00187	cholesterol	1.073
C00031	D-glucose	1.668
C00031	D-glucose	2.139
C00031	D-glucose	1.913
C01796	erythrose	-1.412
C01582	galactose	1.844
C00116	glycerol	1.382
C00037	glycine	-1.207

C00082	L-tyrosine	1.295
C01432	lactic acid	1.218
C01595	linoleic acid	1.091
C00712	oleic acid	1.264
C00249	palmitic acid	1.121
C01530	stearic acid	1.098
C00086	urea	1.180
C00366	uric acid	1.247

Figures

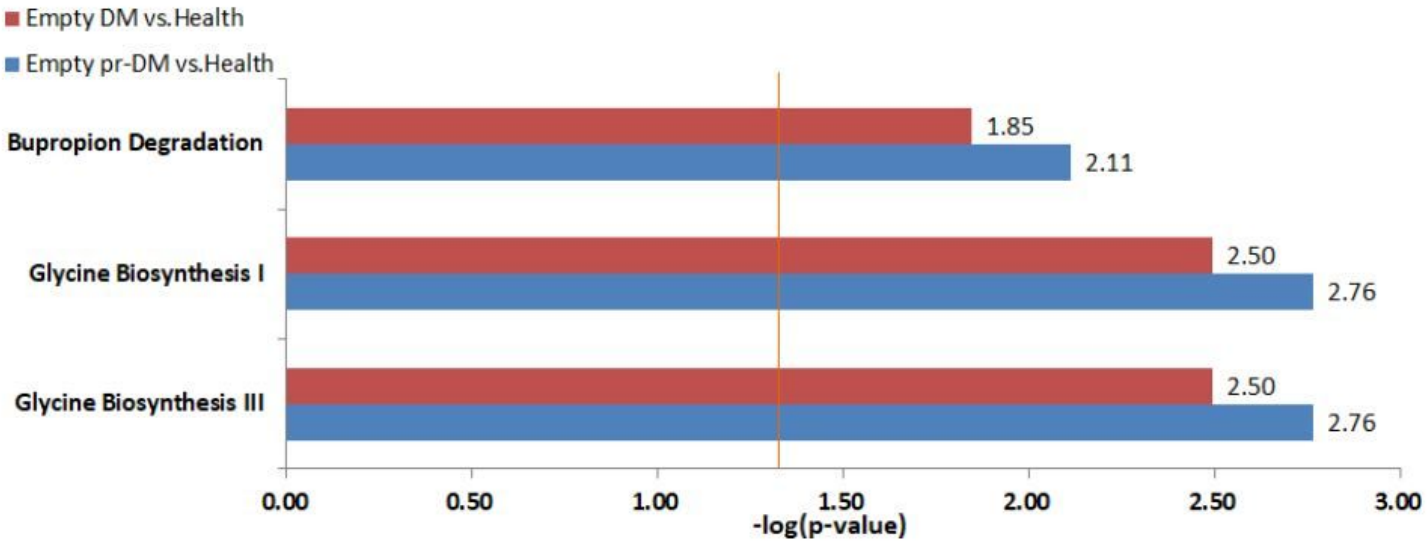
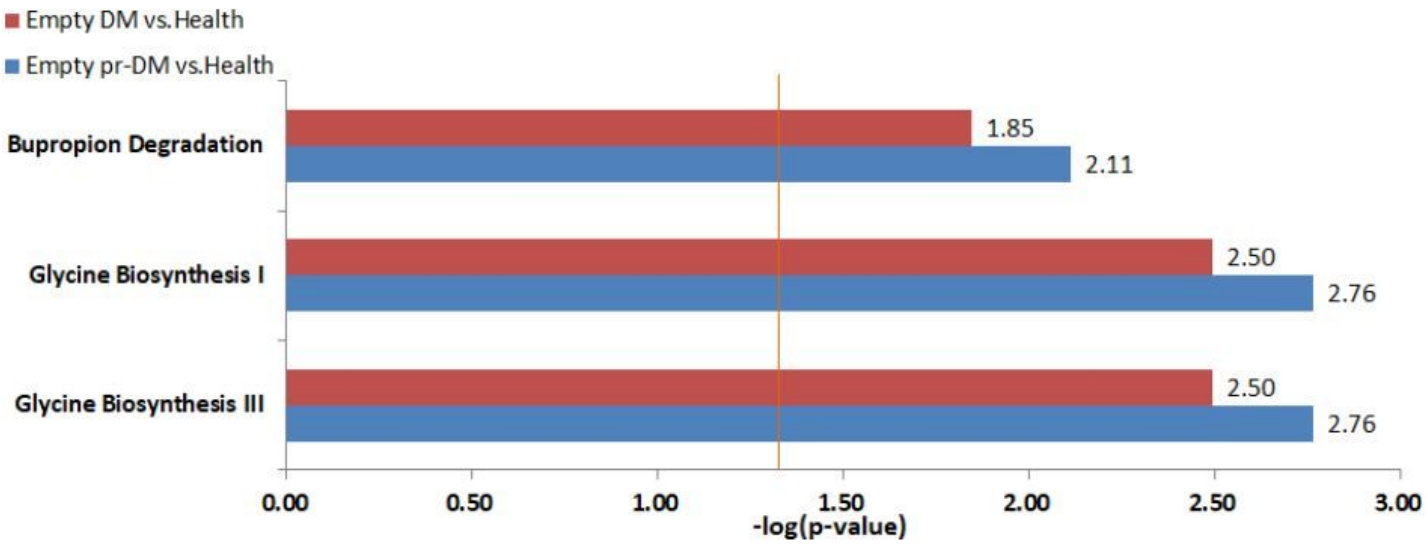


Figure 1

The pathways in both prediabetes and diabetes at fasting state. The pathways included bupropion degradation, glycine biosynthesis I and glycine biosynthesis III ($P < 0.05$).



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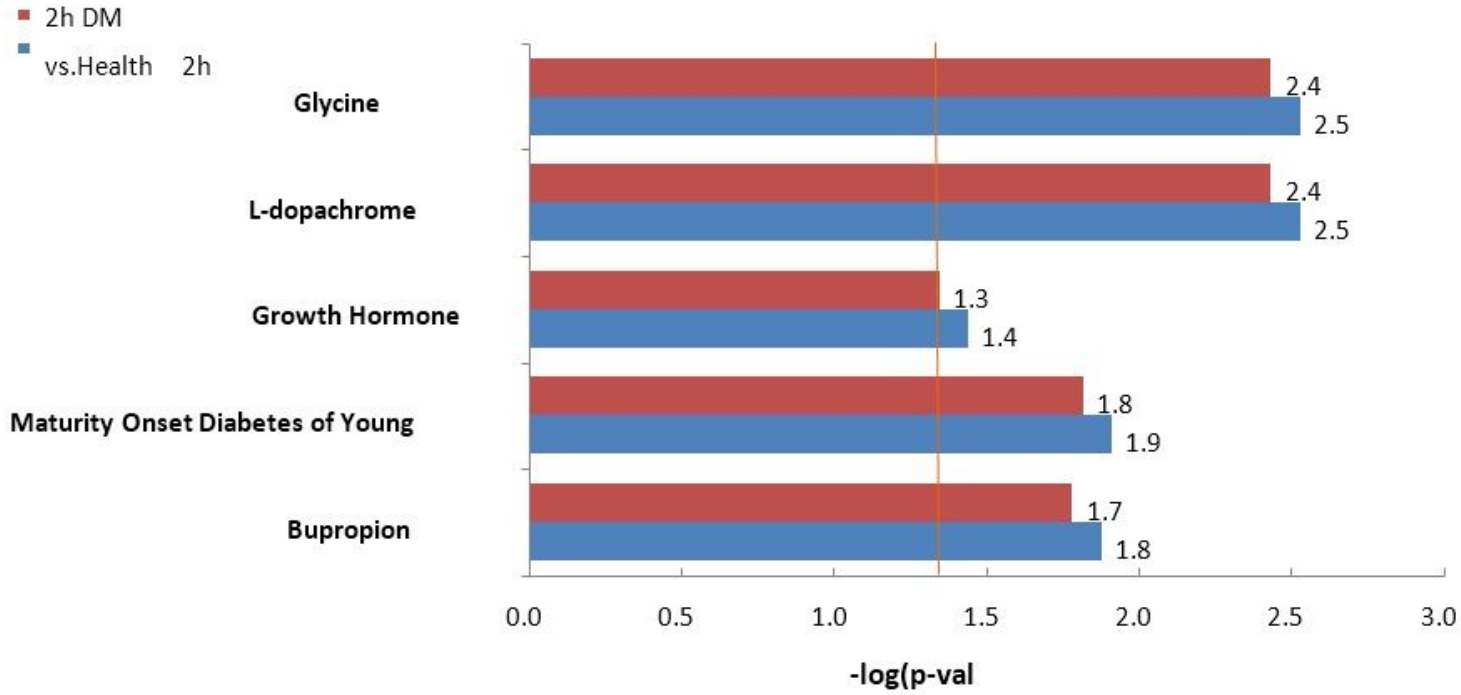
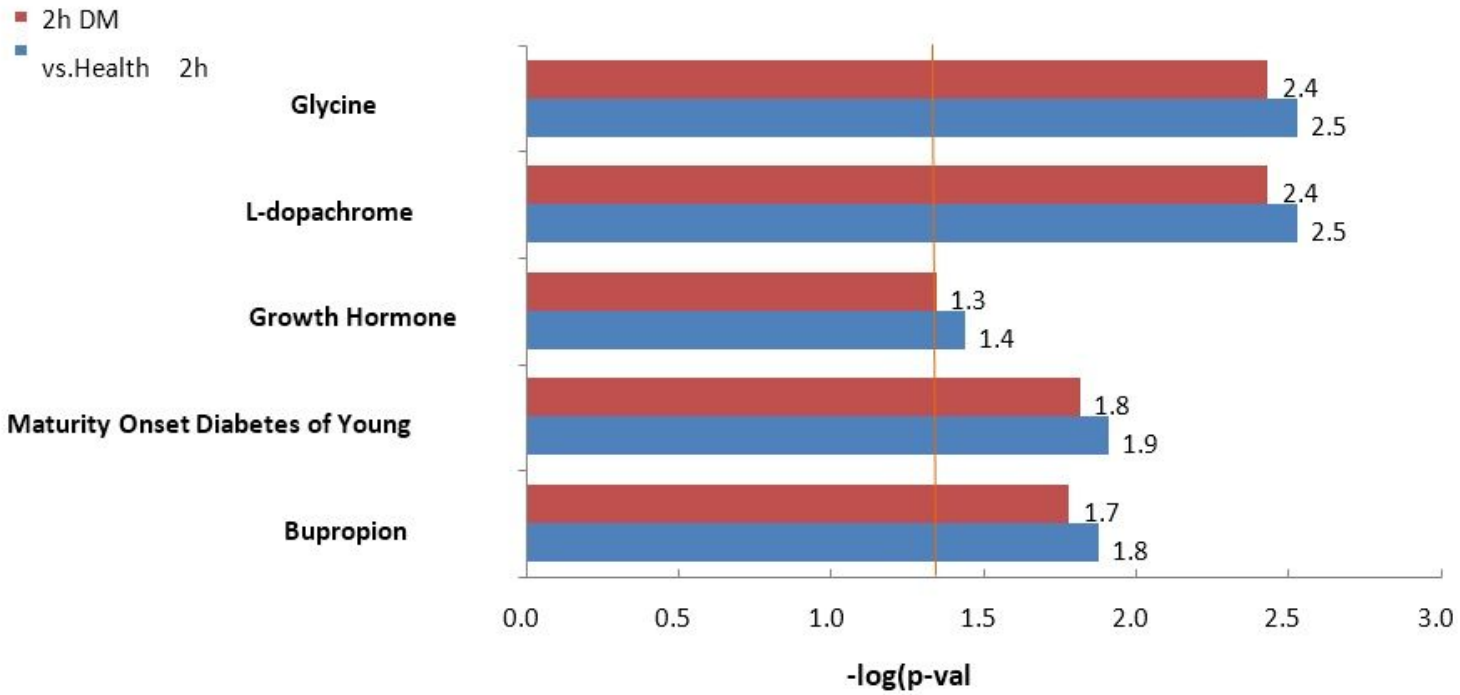


Figure 2

The pathways in both prediabetes and diabetes at 2h OGTT state. The pathways included glycine biosynthesis III, L-dopachrome biosynthesis, growth hormone signaling, maturity onset diabetes of young signaling, and bupropion degradation ($P < 0.05$).



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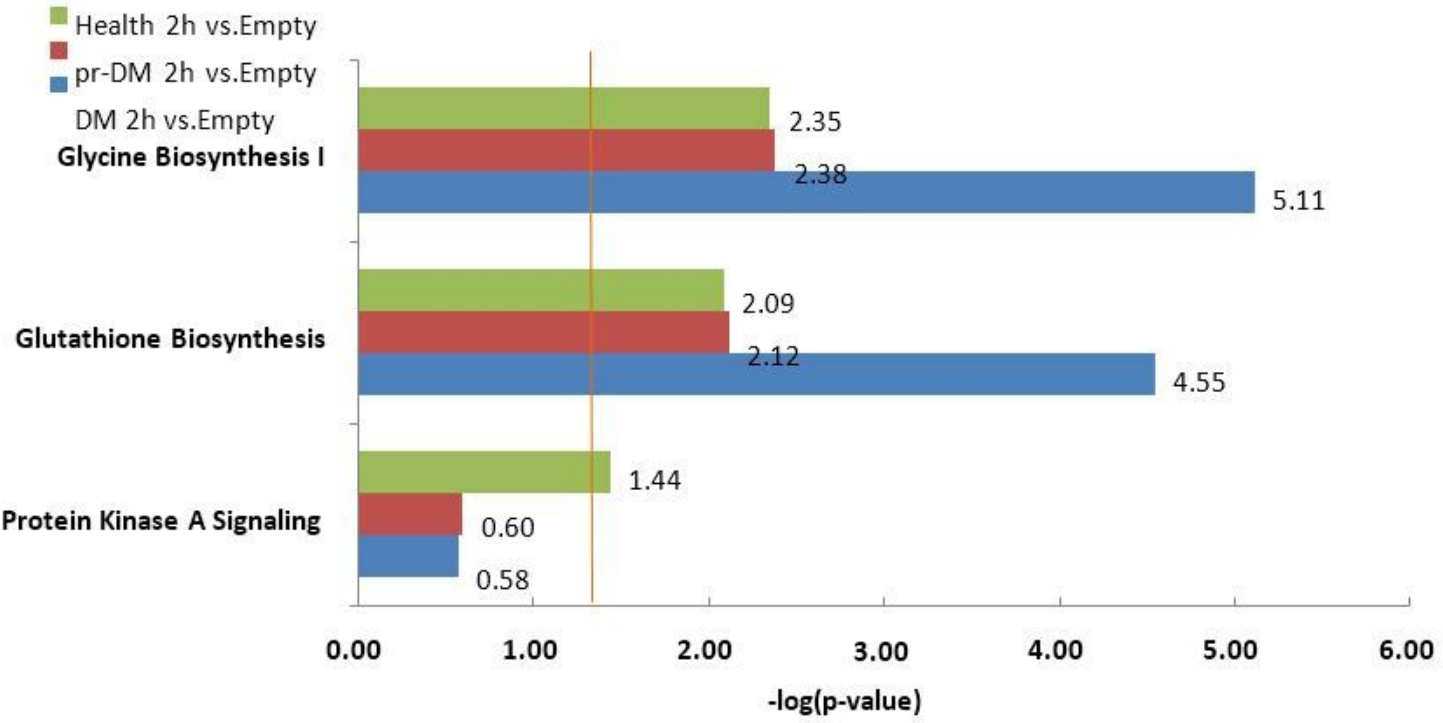
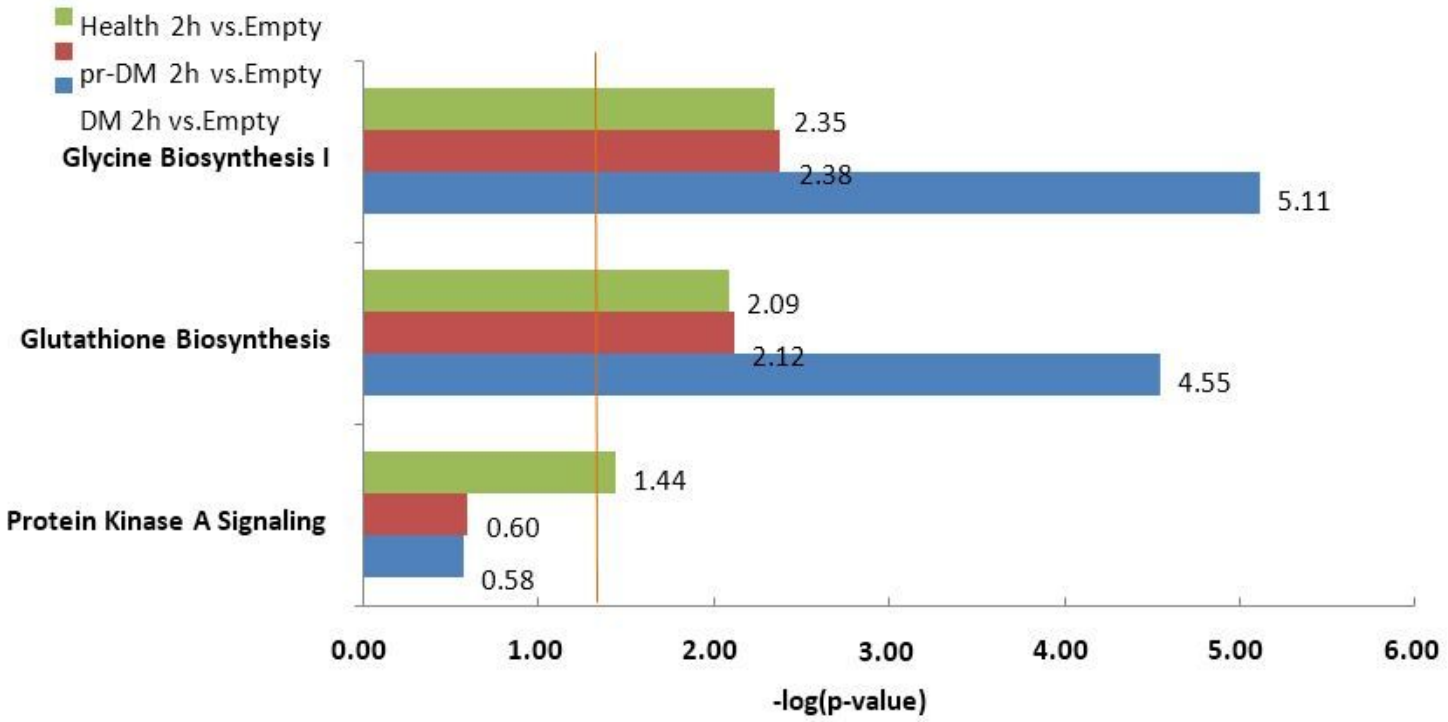


Figure 3

The pathways in the three groups at 2h OGTT state compared with fasting state. Protein kinase A signaling pathway showed statistical significance in healthy (2h OGTT state vs. fasting state) ($P < 0.05$), and no statistical significances in both prediabetes and diabetes (2h OGTT state vs. fasting state) ($P > 0.05$); glycine biosynthesis I and glutathione biosynthesis pathways showed statistical significance in diabetes (2h OGTT state vs. fasting state) than in prediabetes and healthy (2h OGTT state vs. fasting state) ($P < 0.05$).



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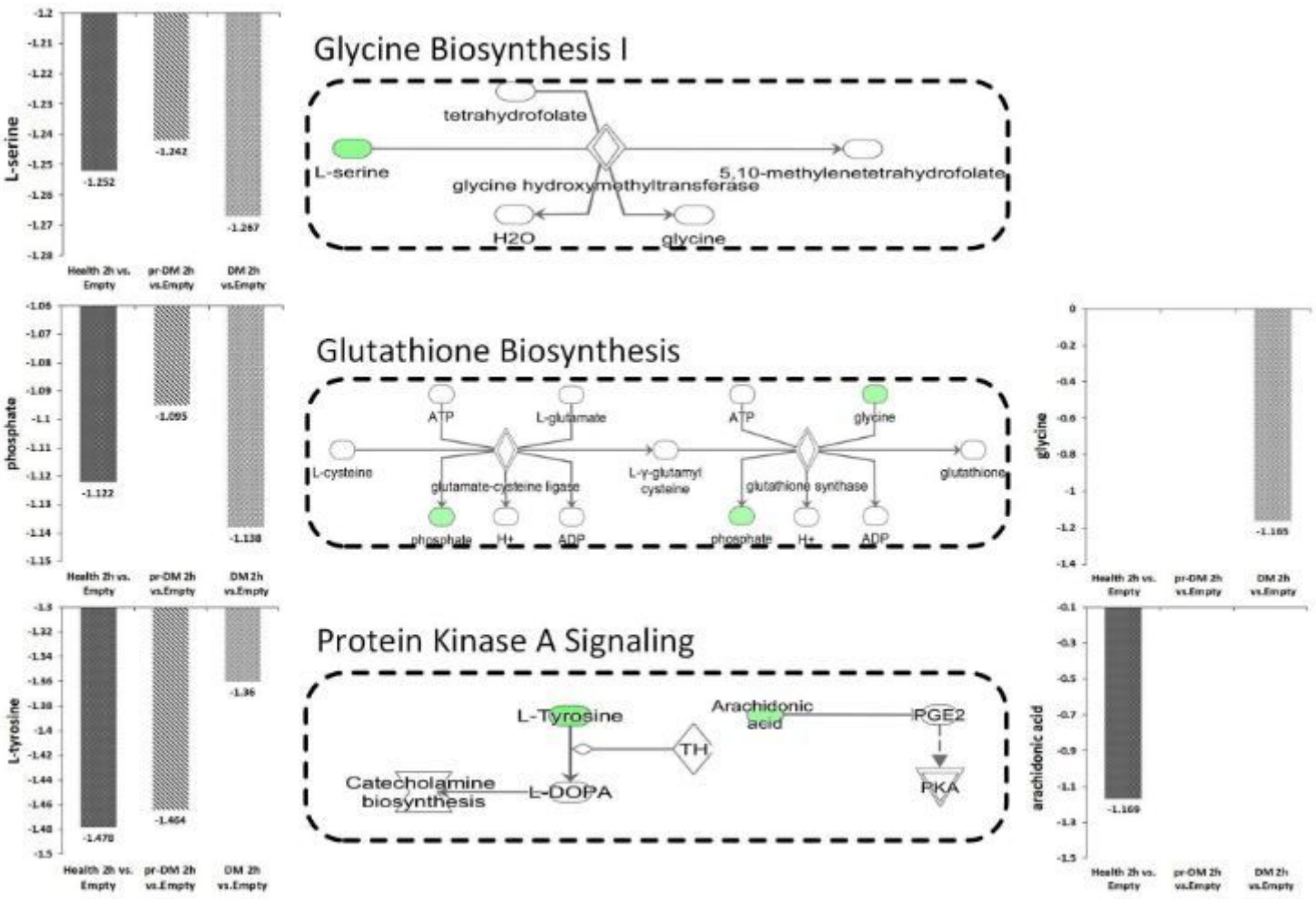
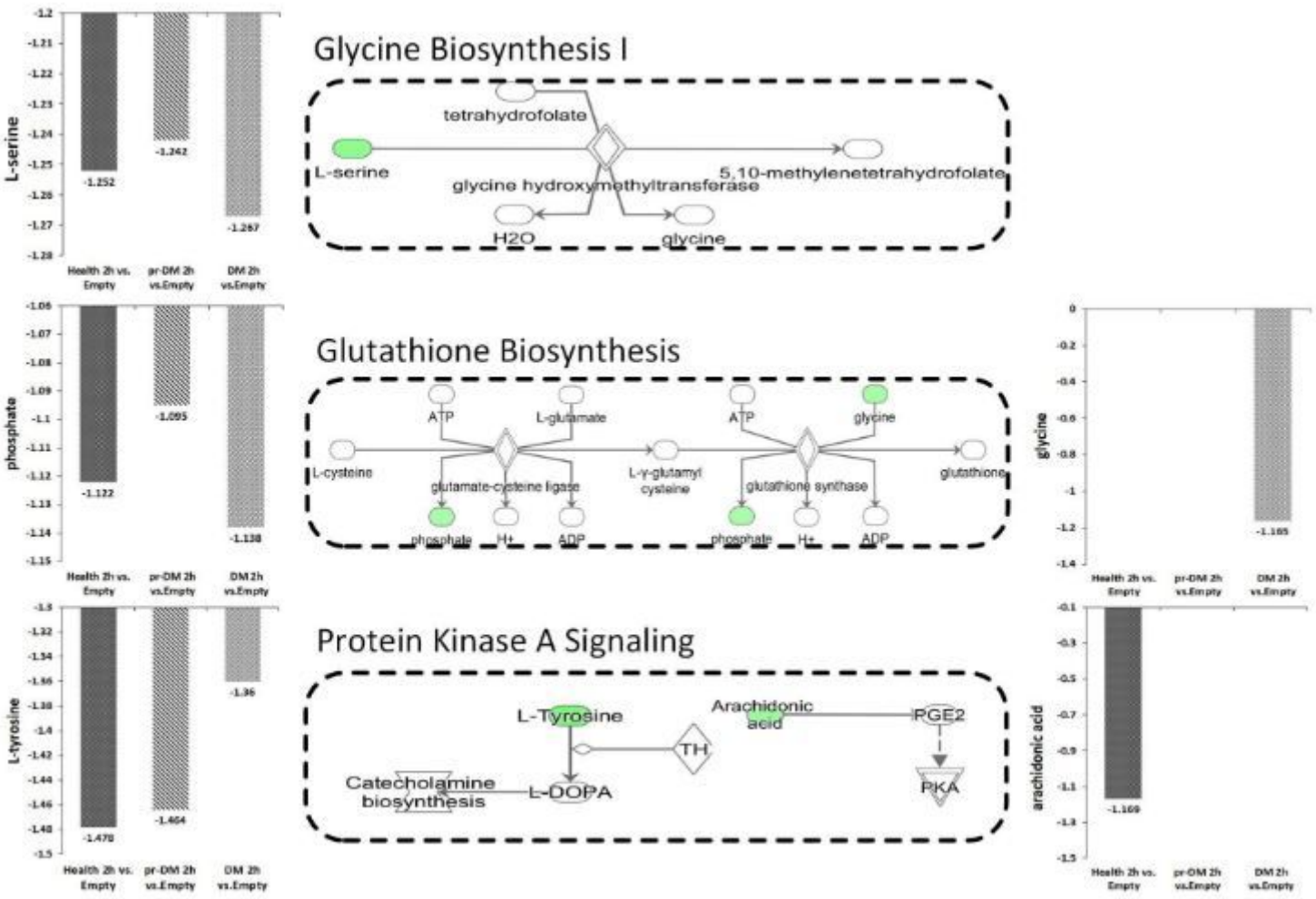


Figure 4

The metabolite changes in the three pathways with statistical significances in the healthy, prediabetes and diabetes at 2h OGTT state compared with fasting state. In the glycine biosynthesis I pathway (upper), L-serine showed fold change, but no significant changes (from -1.242 to -1.267) in healthy, prediabetes and diabetes (2h OGTT state vs. fasting state). In the glutathione biosynthesis (middle), interestingly, glycine only in diabetes, the results suggested the pathway was inhibited in diabetes. In the protein kinase A signaling (lower), the results showed the pathway was inhibited in healthy, or otherwise, the pathway was activated in prediabetes and diabetes.



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