Proteomic Analysis of the Protective Effect of Early Heat Exposure Against Chronic Heat Stress in Broilers

Darae Kang
Animal biotechnology
Kwan Seob Shim (ksshim@jbnu.ac.kr)
Department of Animal Biotechnology, Jeonbuk National University, Jeonju 54896, Korea https://orcid.org/0000-0002-4996-3700

Research

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Abstract

Background

The increasing trend of global warming has affected the livestock industry through the heat stress caused to the animals. Among them, poultry are the most susceptible to heat stress, which results in serious production problems. Therefore, a better understanding of the mechanisms related to the thermal reactions and thermal resistance in poultry would be helpful toward resolving the production issues. In this study, whole proteome analysis was carried out to identify differentially expressed proteins in the liver tissue of broilers under chronic heat stress (35℃/24 h, each day between the broiler ages of 21–35 days). Additionally, the effect of early heat exposure (40℃/24 h, for 1 day only on chicks at 5 days of age) was determined.

Results

In total, 277 differentially expressed proteins due to chronic heat stress were identified (132 downregulated and 145 upregulated). Of those, 95 proteins were regulated by early heat exposure (42 downregulated and 53 upregulated during chronic heat stress). Of the 95 proteins, 8 were related to actin metabolism. According to the KEGG analysis, the proteins were mainly involved in pathways for carbon metabolism and carbohydrate metabolism. Under chronic heat stress, the proteins involved in carbohydrate metabolism were expressed in such a way as to promote the metabolism of carbohydrates, which is the natural means to reduce body temperature but may well induce cell damage.

Conclusion

Early heat exposure reduced the heat stress-induced expression changes of select proteins, indicating the adaptability of the animal to chronic heat stress. The determination of the differentially expressed proteins in the liver proteome under chronic heat stress and early heat exposure suggests that the liver of broilers has various physiological mechanisms for regulating homeostasis to aid heat resistance.

Background

The poultry industry is influenced by a number of factors, including the climate, environment, breeding, specification conditions, and heat stress. Poultry are particularly sensitive to high temperatures because they lack the biological properties to release body heat; for example, they have no sweat glands and are covered with feathers (1). Heat stress leads consistently to reductions in feed consumption, feed efficiency, growth rate, and reproduction, thereby severely affecting the health of animals and impairing their immune functions (2). It can cause the dysfunction of organs and death, consequently leading to economic losses. Studies on broilers under high temperature stress have observed that their feed intake and growth rate are decreased (1), their respiration rates are increased (3), and the weights of organs such as the thymus, spleen, and bursa of Fabricius are reduced (4). The published studies on heat stress in poultry have mainly focused on the behavior, productivity, and blood biochemistry levels of the animals but not on the thermal reactions and mechanisms of thermal resistance.

Some studies have reported on the effects of early heat stress on chicks. Early heat was applied at either the pre- or post-hatching stages or at both stages combined to determine the effects of these various treatments in order to investigate whether the adaptability of chicks to heat stress could be improved through their earlier experience of a high temperature. The heat treatment before hatching (16–18 days of incubation) and at both hatching stages

combined (16–18 days of incubation and in 3-day-old chicks) did not induce heat resistance in the chicks at 42 days, whereas the post-hatching heat exposure (3-day-old chicks) contributed to heat resistance (5). Early heat-treated birds exhibited significantly reduced mortality and improved feed efficiency (6), and responded similarly to birds adapted to heat stress (7). These results indicated the protective effects of early heat exposure against heat stress later in life.

The techniques for studying genomics and proteomics are developing rapidly. Proteomic studies of heat stress effects have been mainly carried out on a number of plants and on animals such as dairy cows, beef cattle, pigs, and chickens. Proteins related to nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated oxidative stress were identified in dairy cows under summer heat stress, and several biomarkers (including mitogen-activated protein kinase kinase 1 (MAP2K1), glutathione *S*-transferase mu 1 (GSTM1), and stress-induced phosphoprotein 1 (STIP1)) were identified in adipose tissue in response to heat stress (8). In heat-stressed pigs, there was an increase in the expression of heat-shock proteins that protect cells by degrading denatured proteins; inflammatory cytokines, such as glucose-regulated protein 94 (GRP94) and Serpin family A member 3 (SERPINA3); and glutathione peroxidase and glutathione *S*-transferase, which are related to immune responses (9). Thus, numerous proteins and regulatory functions related to heat stress have been and are still being studied through proteomic analysis. Through studies of the whole proteome of various organs, the effects of heat stress can be confirmed at the overall metabolic level rather than at fragmented parts, making a detailed understanding of the heat effects possible.

In this study, we conducted a proteomic analysis of broiler liver tissue under chronic heat stress, with/without early heat exposure, to identify the effects of early heat exposure in poultry. We found that the proteins that were differentially expressed in response to chronic heat stress were intended to maintain homeostasis in response to high temperatures, and the proteins of which the chronic heat stress-induced expression changes were re-normalized through the early heat exposure were considered to be less affected by heat. Our results suggest that proteins can be changed through early heat exposure to increase their resistance to chronic heat stress.

Methods

Animals and heat exposure conditions

In total, 114 Ross chickens (1 day old) were purchased from Dongwoo Hatchery (Iksan, Korea). The chicks were weighed and randomly grouped into 3 groups of 48 chicks each without significant weight differences. Each group was further divided into 12 chicks per pen to represent 4 test replications. All 1-day-old chicks were initially raised at 34℃ and the temperature was reduced by 2℃ weekly until it reached 24℃. The humidity was maintained at 57% ± 3% throughout. The chicks had access to water and feed *ad libitum*. The following were the heat treatment groups: Group CC, raised at a suitable temperature without heat exposure; Group CH, chronic heat exposure at 35℃ for 24 h each day between the ages of 21 and 35 days; and Group HH, early heat exposure at 40℃ for 24 h at 5 days of age and chronic heat exposure at 35℃ for 24 h each day between the ages of 21 and 35 days (Fig. 1). At the end of the experimental period, the chickens were sacrificed, and the liver tissues were extracted, frozen with liquid nitrogen, and stored at − 80℃ until analysis.

Protein Extraction And Digestion

An equal amount of liver tissue from each chicken was lysed in 1 mL of 8 M urea and protein inhibitor with vortex mixing for 30 s. Then, the mixture was sonicated for 3 min in an ice bath to homogenize the tissue. The

homogenized tissue was then centrifuged at 14,000 rpm for 10 min, following which the supernatant was obtained for protein concentration measurement with the bicinchoninic acid assay. Then, 100 µg of protein was subjected to in-solution digestion. Thereafter, 100 µg of depleted protein in 100 mM Tris buffer (pH 3.0) in a total volume of 30 µL was first incubated with 6 M urea and 20 mM dithiothreitol at 56℃ for 30 min and then alkylated with fresh 50 mM iodoacetamide in 100 mM Tris buffer (pH 8.0) for 30 min in the dark at ambient temperature (24℃). The reaction was quenched with 100 mM Tris buffer (pH 8.0) and the protein was enzymatically digested overnight at 37℃ in a trypsin/LysC mix (1:50, enzyme:substrate). The reaction was quenched with formic acid and the peptides were desalted on an Oasis HLB column (Waters Corporation, Milford, MA, USA). Finally, the peptides obtained were dried using a speed vac.

Liquid Chromatography-tandem Mass Spectrometry Analysis And Data Analysis

The peptides were separated by liquid chromatography (LC) on an EASY-nLC 1000 system (Thermo Fisher Scientific, Rockford, IL, USA) equipped with a C18 column (2 µm particle size, 50 µm ID × 15 cm length; Thermo Fisher Scientific), using mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in 100% acetonitrile) at a flow rate of 300 nL/min. The gradient profile was set as follows: 5–40% B in 45 min and 40–80% B in 2 min. Mass spectrometry (MS) analysis was performed using a Q Exactive mass spectrometer (Thermo Fisher Scientific) with the spray voltage set at 2.3 kV. MS spectra were collected at a resolution of 70,000 at *m/z* 200 (350–2000 *m/z* mass range), followed by data-dependent higher-energy collisional dissociation (HCD) MS/MS spectra (at a resolution of 17,500 and collision energy of 25%) of the 20 most abundant ions. A dynamic exclusion time of 30 s was used.

For the identification of the chicken liver protein, the raw files were searched against the UniProt Chicken database using the Percolator node in Proteome Discoverer (v1.4). Oxidation was chosen as the dynamic modification and carbamidomethyl as the static modification. The parent ion mass error was set to ± 10 ppm and the fragment ion mass error to ± 0.02 Da. Peptides with full tryptic cleavage specificity were searched, with two missed cleavages allowed. The search parameters used were a precursor tolerance of 10 ppm and a fragment ion tolerance of 0.02 Da. Scaffold Q + S was used for the label-free quantitation of the proteins. All identified proteins were normalized by protein size and total spectrum count number, and the normalized data were filtered with a confidence level (CI) of > 95%. For further filtering, the false discovery rate was set to 0.05 and the count number to > 0 for 2 out of 2 replicates.

Gene Ontology Enrichment Analysis

The differentially expressed proteins were clustered using the Database for Annotation, Visualization and Integrated Discovery bioinformatics resources (DAVID v6.8, https://david.ncifcrf.gov/) with the Gene Ontology (GO) database, and the probability value was corrected with the Bonferroni method. The classifications from the *Gallus gallus* database were applied.

Pathway enrichment analysis was carried out using the WEB-based Gene SeT AnaLysis Toolkit (WebGestalt, http://www.webgestalt.org/) with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The classifications were again from the *Gallus gallus* database. The probability value was corrected with the Benjamini–Hochberg method.

Validation Of Proteins By Their Gene Expression

Total RNA was extracted from the liver tissue using an RNA extraction kit (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. The RNA concentration and purity were measured using the μDrop Plate (NanoDrop, Thermo Fisher Scientific). cDNA was synthesized from 1 μg of total RNA using the AccuPower RocketScript Cycle RT PreMix (dT20) (Bioneer). The randomly selected gene primers were designed with Primer 3 software (v.0.4.0) and are shown in Supplemental Table S1. The reverse transcription quantitative polymerase chain reaction (RT-qPCR) was conducted using the SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) on a CFX96 real-time PCR detection system (Bio-Rad). The RT-qPCR thermal cycle conditions were as follows: 95℃ for 5 min, followed by 40 cycles of 95℃ for 5 and 60℃ for 30 s. The relative gene expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method {Livak, 2001 #131} and normalized against the level of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*).

Statistical analysis

All experimental data were analyzed with the SAS 9.4 program and are expressed as the mean ± SE. Differences were analyzed using analysis of variance, and statistical differences among groups were analyzed with Duncan's multiple-range test. Statistical significance was set at $P < 0.05$.

Results

Differentially expressed proteins in response to chronic heat stress and early heat exposure

In total, 277 proteins (132 downregulated and 145 upregulated) were differentially expressed between the control and CH groups (Table S2). However, the chronic heat affect was reduced by early heat exposure for 95 (42 downregulated and 53 upregulated by chronic heat stress) of the 277 proteins (Table 1). The heat map and graph of the expression patterns of the differentially expressed proteins in the various groups are shown in Fig. 2. Of the 95 proteins that were positively affected by the early heat exposure, the putative interferon-stimulated gene 12 (ISG12) protein was the most highly expressed in response to chronic heat stress. Induced by interferons, ISG12 enables a variety of biologically active states, such as protection against viral replication and increase of the host defenses through the activation of immune cells, major histocompatibility complex antigens, etc. Therefore, the role of this protein against viral infections and in regulating the immune system may be regulated by early heat exposure.

Table 1
List of differentially expressed proteins of positive effected by early heat exposure

No	UniProt	Description	Name	MW (kDa)	Score		
					CC	CH	HH
Low expressed by chronic heat stress							
1	F1NJG4	Cytochrome P450 CYP2D49	CYP2D6	58	1	0.40	0.80
2	F1NBI2	Uroporphyrinogen_deCOase domain-containing protein	UROD	36	1	0.34	0.70
3	F1NK40	Uncharacterized protein	A2ML4	163	1	0.09	1.18
4	P05094	Alpha-actinin-1 (Alpha-actinin cytoskeletal isoform) (F-actin cross-linking protein) (Non-muscle alpha-actinin-1)	ACTN1	102	1	0.37	0.88
5	F1P298	Amidohydro-rel domain-containing protein	AMDHD1	37	1	0.04	0.85
6	P28337	Aminomethyltransferase, mitochondrial, EC 2.1.2.10 (Glycine cleavage system T protein, GCVT)	AMT	42	1	0.29	0.94
7	-	Calpain-11	CAPN11	80	1	0.11	0.69
8	Q5ZHR7	Clathrin light chain	CLTA	24	1	0.21	0.83
9	A0A1L1RWF6	Calponin	CNN3	37	1	0.25	0.71
10	A0A1D5PLS2	Citrate synthase	CS	60	1	0.34	0.93
11	A0A1L1RWI4	Uncharacterized protein	CYP2AC2	57	1	0.08	0.68
12	A0A3Q2U335	ADF-H domain-containing protein	DBNL	45	1	0.41	0.98
13	P28675	Decorin (Bone proteoglycan II) (PG-S2)	DCN	40	1	0.26	1.06
14	A0A3Q3AA40	Eukaryotic translation initiation factor 3 subunit G, eIF3g (Eukaryotic translation initiation factor 3 RNA-binding subunit, eIF-3 RNA-binding subunit) (Eukaryotic translation initiation factor 3 subunit 4)	EIF3G	29	1	0.33	0.90
15	A0A1D5P5T1	Eukaryotic translation initiation factor 3 subunit K, eIF3k (Eukaryotic translation initiation factor 3 subunit 12) (eIF-3 p25)	EIF3K	27	1	0.04	0.73
16	A0A3Q2TU97	Eukaryotic translation initiation factor 4E	EIF4E	25	1	0.22	1.16
17	A0A1D5PU09	PH domain-containing protein	FERMT2	81	1	0.15	0.83
18	E1C6R4	Peptidylprolyl isomerase, EC 5.2.1.8	FKBP8	44	1	0.03	0.69

No	UniProt	Description	Name	MW (kDa)	Score		
					CC	CH	HH
19	P21872	Trifunctional purine biosynthetic protein adenosine-3 [Includes: Phosphoribosylamine–glycine ligase, EC 6.3.4.13 (Glycinamide ribonucleotide synthetase, GARS) (Phosphoribosylglycinamide synthetase)	GART	107	1	0.17	0.93
20	A0A1D5PZ32	**GB1/RHD3-type G domain-containing protein**	GBP4L	70	1	0.04	1.13
21	F1NLE4	**Glutathione synthetase, GSH-S, EC 6.3.2.3**	GSS	52	1	0.07	0.68
22	P20057	**Hemopexin**	HPX	43	1	0.31	0.83
23	A0A1D5P1W7	Importin N-terminal domain-containing protein	KPNB1	120	1	0.37	0.88
24	Q5ZK33	Mitochondrial proton/calcium exchanger protein (Leucine zipper-EF-hand-containing transmembrane protein 1)	LETM1	86	1	0.06	0.71
25	A0A3Q3B025	LIM zinc-binding domain-containing protein	LIMA1	85	1	0.20	0.56
26	A0A1D5PMD9	Carboxylic ester hydrolase, EC 3.1.1.-	LOC769704	59	1	0.38	1.02
27	P00698	Lysozyme C, EC 3.2.1.17 (1,4-beta-N-acetylmuramidase C) (Allergen Gal d IV) (allergen Gal d 4)	LYZ	16	1	0.21	0.86
28	P14105	Myosin-9	MYH9	227	1	0.21	0.46
29	F1NTJ5	Myosin IB	MYO1B	125	1	0.26	0.73
30	A0A1L1RKT0	Glycylpeptide N-tetradecanoyltransferase, EC 2.3.1.97	NMT1	57	1	0.04	0.34
31	O13154	Protein kinase C and casein kinase substrate in neurons protein 2 (Focal adhesion protein of 52 kDa, FAP52)	PACSIN2	56	1	0.44	0.99
32	A0A1D5PLK6	BRO1 domain-containing protein	PDCD6IP	97	1	0.26	0.72
33	Q5ZLX0	Cytochrome b5 heme-binding domain-containing protein	PGRMC2	22	1	0.02	0.48
34	O42265	Proteasome subunit alpha type-1, EC 3.4.25.1 (Macropain subunit C2) (Multicatalytic endopeptidase complex subunit C2) (Proteasome component C2)	PSMA1	29	1	0.16	0.64
35	E1BWG7	Phosphotriesterase related protein	PTER	39	1	0.17	0.94

No	UniProt	Description	Name	MW (kDa)	Score		
					CC	CH	HH
36	A0A1D5PNR0	RNA-binding protein 8A	RBM8A	19	1	0.02	0.94
37	A0A1D5PDV6	Ribosomal protein S19	RPS19	15	1	0.39	0.86
38	Q5ZM66	40S ribosomal protein S26	RPS26	13	1	0.34	0.93
39	A0A1L1S0C5	PX domain-containing protein	SNX1	58	1	0.04	0.73
40	P0DMQ6	Sorbitol dehydrogenase, SDH, EC 1.1.1.- (Polyol dehydrogenase)	SORD	38	1	0.34	1.17
41	A0A1L1RY95	UBIQUITIN_CONJUGAT_2 domain-containing protein	UBE2N	17	1	0.37	1.14
42	A0A1D5NZ55	UDP-glucose glycoprotein glucosyltransferase 1	UGGT1	180	1	0.06	0.18
High expressed by chronic heat stress							
43	F1NGM0	Uncharacterized protein	EHD3	61	1	2.09	0.84
44	F1NNH9	**TPR_REGION domain-containing protein**	TOMM70	67	1	3.34	0.90
45	Q5ZHT1	**Acyl-CoA dehydrogenase family member 11**	ACAD11	87	1	8.29	1.16
46	F1NEF6	Uncharacterized protein	ACAD9	37	1	9.75	1.16
47	Q5ZKG5	**Low molecular weight phosphotyrosine protein phosphatase**	ACP1	18	1	20.07	1.16
48	E1BZT9	**Acetyl-coenzyme A synthetase**	ACSS1L	58	1	5.39	1.43
49	-	Uncharacterized protein	AKR1B10L4	36	1	10.32	1.16
50	P09572	**Sodium/potassium-transporting ATPase subunit alpha-1, Na(+)/K(+) ATPase alpha-1 subunit**	ATP1A1	113	1	6.28	1.16
51	A0A1D5PBD2	Uncharacterized protein	ATPIF1	13	1	4.32	1.43
52	A0A3Q3AZM2	Uncharacterized protein	CALM1	16	1	23.16	1.16
53	A9CP13	D-serine dehydratase	CHDSD	40	1	34.27	1.16
54	Q9I882	Protein kinase C inhibitor	chPKCI	14	1	3.93	1.33
55	A0A1D5PCT4	**Coronin**	CORO1C	64	1	2.41	0.95
56	P55164	**Beta-crystallin A2**	CRYBA2	23	1	83.20	1.16
57	A0A1L1RKJ5	**Carboxypeptidase, EC 3.4.16.-**	CTSA	53	1	34.27	9.25
58	Q5F412	**Dynein light chain**	DYL1	10	1	2.73	1.15
59	F1N9U8	**ETF domain-containing protein**	ETFA	34	1	19.93	1.16

No	UniProt	Description	Name	MW (kDa)	Score		
					CC	CH	HH
60	F1NHG6	Uncharacterized protein	FAM162A	17	1	85.75	1.16
61	A0A1D5P3I6	**Peptidylprolyl isomerase, EC 5.2.1.8**	FKBP3	26	1	26.39	1.16
62	A0A1D5NYG3	Uncharacterized protein	FLNB	284	1	2.03	0.96
63	P21872	**Trifunctional purine biosynthetic protein adenosine-3** [Includes: **Phosphoribosylamine–glycine ligase**	GART	107	1	9.67	1.16
64	P00504	**Aspartate aminotransferase, cytoplasmic, cAspAT**	GOT1	46	1	2.94	1.33
65	F1NIJ6	**Glucose-6-phosphate isomerase**	GPI	26	1	14.09	1.16
66	-	Uncharacterized protein	GSPT1	68	1	5.13	1.16
67	A0A1D5P338	**Glutathione reductase**	GSR	50	1	21.09	9.25
68	F1NQS2	Uncharacterized protein	GSTAL3	26	1	13.86	1.16
69	E1BUB6	Uncharacterized protein	GSTT1L	28	1	4.29	1.45
70	E1BRU7	Uncharacterized protein	HKDC1	102	1	2.08	0.94
71	P35915	**Hydroxymethylglutaryl-CoA lyase, mitochondrial**	HMGCL	34	1	10.26	1.16
72	Q5ZM98	**Stress-70 protein, mitochondrial**	HSPA9	73	1	4.82	1.16
73	Q5ZKA2	**Isoleucine–tRNA ligase, mitochondrial**	IARS	147	1	2.92	1.36
74	E1BWB9	Uncharacterized protein	INF2	144	1	4.96	1.16
75	Q6IEC5	Putative ISG12(2) protein	ISG12(2)	10	1	89.96	40.86
76	A0A1D5PAF0	Uncharacterized protein	LOC107080643	27	1	4.67	1.05
77	E1BRI5	**Abhydrolase_2 domain-containing protein**	LYPLA2	25	1	3.16	1.46
78	E1BTL0	**Transket_pyr domain-containing protein**	OGDHL	115	1	3.25	1.16
79	F1P0M2	Uncharacterized protein	PCCA	79	1	3.00	0.95
80	A0A1L1RW22	**CTP_transf_like domain-containing protein**	PCYT2	40	1	18.41	1.16
81	Q5ZLT2	**Protein-serine/threonine kinase**	PDK3	44	1	2.92	1.30
82	A0A1L1RXJ1	Uncharacterized protein	QARS	96	1	7.57	1.16
83	E1C0F3	Uncharacterized protein	RAB7A	24	1	2.10	0.96
84	Q5ZM11	**Arginine–tRNA ligase, cytoplasmic**	RARS	75	1	2.08	0.94

No	UniProt	Description	Name	MW (kDa)	Score		
					CC	CH	HH
85	P41263	**Retinol-binding protein 4**	RBP4	23	1	16.20	1.16
86	E1BSA7	Uncharacterized protein	SEC24A	120	1	5.85	1.16
87	A0A1D5NVD4	Uncharacterized protein	SRSF7	28	1	13.35	1.16
88	A0A1D6UPQ3	**AA_TRNA_LIGASE_II domain-containing protein**	TARS	91	1	6.43	1.43
89	A0A1L1RMM0	Uncharacterized protein	tcp-1	58	1	11.99	1.16
90	F1NU71	**Mitochondrial import inner membrane translocase subunit TIM44**	TIMM44	51	1	4.23	1.42
91	P08629	**Thioredoxin, Trx**	TXN	12	1	3.43	1.70
92	A0A1D5PWT4	**Thioredoxin domain-containing protein**	TXN2	16	1	64.63	1.16
93	-	Uncharacterized protein	UGT2A1	61	1	24.35	8.10
94	A0A1D5PEU7	**CN hydrolase domain-containing protein**	VNN1	57	1	6.66	1.16
95	O93277	**WD repeat-containing protein 1**	WDR1	67	1	10.56	1.16

To further compare the differences among the CC, CH, and HH groups, the functions of the differentially expressed proteins were categorized using the WebGestalt toolkit and the GO resource, in which the three main functional categories are biological process, cellular component, and molecular function. Using the 95 proteins that were positively affected by early heat treatment, we obtained the top 10 GO terms for the three main categories (Table S3 and Fig. 3). In the biological process category, genes were associated with the small molecule metabolic process (13 genes), actin filament-based process (7 genes), actin cytoskeleton organization (7 genes), and cofactor metabolic process (7 genes). Additionally, the most related term was glutathione metabolic process. In the cellular component category, actomyosin had the highest significance among the GO terms, with the 4 genes actinin alpha 1 (*ACTN1*), fermitin family homolog 2 (*FERMT2*), LIM domain and actin binding 1 (*LIMA1*), and filamin B (*FLNB*) being associated with the actomyosin, stress fiber, contractile actin filament bundle, actin filament bundle, focal adhesion, cell-substrate adherens junction, adherens junction, and actin cytoskeleton terms. In the molecular function category, the genes were strongly associated with actin filament binding, with the 5 genes *ACTN1*, *FERMT2*, *LIMA1*, myosin heavy chain 9 (*MYH9*), and myosin 1B (*MYO1B*) being enriched in the actin filament binding, actin binding, cytoskeletal protein binding, and protein-containing complex binding terms.

On the basis of the KEGG pathway analysis with the WebGestalt toolkit, we identified 13 significant pathways among the CC, CH, and HH groups (Table 2). Many pathways were involved in carbohydrate metabolism, including those of glycolysis/gluconeogenesis, citrate cycle, pentose and glucoronate interconversions, fructose and mannose metabolism, starch and sucrose metabolism, glyoxylate and dicarboxylate metabolism, and propanoate metabolism.

Table 2
List of KEGG pathways

Term ID	Term	No.	Genes	p-value
Carbohydrate metabolism				
00010	Glycolysis / Gluconeogenesis	3	ACSS1L, GPI, HKDC1	0.02
00020	Citrate cycle (TCA cycle)	2	CS, OGDHL	0.04
00040	Pentose and glucoronate interconversions	2	SORD, UGT2A1	0.02
00051	Fructose and mannose metabolism	2	HKDC1, SORD	0.04
00500	Starch and sucrose metabolism	3	GPI, HKDC1, UGT2A1	0.01
00630	Glyoxylate and dicarboxylate metabolism	4	AMT, CS, PCCA, ACSS1L	0.004
00640	Propanoate metabolism	2	ACSS1L, PCCA	0.04
Metabolism of cofactors and vitamins				
00670	One carbon pool by folate	2	AMT, GART	0.01
00860	Porphyrin and chlorophyll metabolism	2	UGT2A1, UROD	0.03
Xenobiotics biodegradation and metabolism				
00982	Drug metabolism (cytochrome P450)	2	GSTAL3, GSTT1L	0.04
00983	Drug metabolism (other enzymes)	3	LOC769704, GSTAL3, GSTT1L	0.02
00980	Metabolism of xenobiotics by cytochrome P450	2	GSTT1L, UGT2A1	0.05
Translation				
00970	Aminoacyl-tRNA biosynthesis	4	IARS, QARS, RARS, TARS	0.001
Amino acid metabolism				
00270	Cysteine and methionine metabolism	2	GOT1, GSS	0.06
Metabolism of other amino acids				
00480	Glutathione metabolism	3	GSR, GSS, GSTT1L	0.01
Lipid metabolism				
00140	Steroid hormone biosynthesis	2	CYP2D6, UGT2A1	0.06

Gene Expression Analysis For Validation Of The Abundant Protein

We analyzed the mRNA expression levels of glutamic-oxaloacetic transaminase 1 (*GOT1*), phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase (*GART*), glutathione *S*-transferase theta 1-like (*GSTT1L*), 2-oxoglutarate dehydrogenase-like, mitochondrial (*OGDHL*), UDP glucuronosyltransferase family 2 member A1 (*UGT2A1*), arginyl-tRNA synthetase, cytoplasmic (*RARS*), cytochrome P450 2D6 (*CYP2D6*), isoleucyl-tRNA synthetase (*IARS*), and glutathione synthetase (*GSS*) (Fig. 4). The *OGDHL* and *UGT2A1* expression levels were significantly higher in the CH

and HH groups than in the CC group ($P < 0.05$), where the pattern was similar to that of their protein expression levels; that is, the highest in the CH group, lowest in the CC group, and moderate in the HH group. The *GOT1*, *GART*, *GSTT1L*, *RARS*, *CYP2D6*, and *GSS* expression levels were significantly higher in the CH group than in the CC and HH groups ($P < 0.05$), whereas *IARS* expression was significantly lower in the CH group ($P < 0.05$). GOT1, GART, and GSTT1L showed the same gene and protein expression patterns, whereas CYP2D6, IARS, and GSS showed the opposite expression patterns.

Discussion

Heat stress reduces the health and activity of animals as well as agricultural productivity. Although many research studies on the physiological reactions of animals to heat have been conducted, there are no direct countermeasures to offset the negative effects and any such measure is limited to general environmental temperature reduction and feed additives. The use of early heat exposure for reducing the thermal stress of broilers has emerged as an economically viable method compared with other countermeasures. Although strategies for reducing heat stress have increased the heat resistance of animals, the productivity of the animals in the hot summer season has yet to be fully normalized. Therefore, a detailed understanding of the biological responses to heat stress is necessary to aid the development of techniques for normalizing the heat-related physiological and metabolic responses of animals. Among the livestock affected by high temperatures, poultry was selected as it is the most susceptible to heat and therefore the most suitable for studying the metabolic response during heat exposure.

Given that protein metabolism in animals can be altered by heat, researchers have studied the heat-induced proteomic responses in various animals, such as cattle, pigs, and birds. For example, {Victoria Sanz Fernandez, 2015 #241} reported that heat stress affects carbohydrate metabolism in pigs. Heat stress decreases ATP production through oxidative phosphorylation and increases energy production through aerobic sugar degradation, thereby significantly altering intracellular energy (11). This is similar to the Warburg effect that is used in tumor cells to gain energy (12). Our differentially expressed proteins were consistent with the results of these reports, in that proteins in pathways of carbohydrate metabolism—such as those in glycolysis/gluconeogenesis (acetyl-CoA synthetase 2-like, mitochondrial isoform X1 (ACSS1L), glucose 6-phosphate isomerase (GPI), hexokinase domain-containing 1 (HKDC1)), the citrate cycle (OGDHL), starch and sucrose metabolism (GPI, HKDC1, UGT2A1), and propanoate metabolism (ACSS1L, propionyl-CoA carboxylase subunit alpha (PCCA))—were upregulated in the liver tissue of the CH chickens compared with that of the CC and HH chickens. The overall metabolic process is shown in Fig. 5.

In glycolysis, HKDC1 (a member of the hexokinase family) plays an important role in regulating glucose metabolism by catalyzing the ATP-dependent phosphorylation of glucose-6-phosphate at the first step of the glycolytic pathway (13). Although the specific biological functions of HKDC1 are still unclear, it has been proposed to play an especially greater role in glucose metabolism when the fetus needs to be supplied with sufficient nutrients during pregnancy (14). In a study of the sugar degradation pathways of various hyperthermophilic archaea, the extremophiles were found to degrade glucose, maltose, cellobiose, and starch through modified Embden-Meyerhof pathways (15). Additionally, it has been found that enzyme activity increases exponentially at high temperatures (above 55℃) (16). GPI, which catalyzes the reversible isomerization of glucose-6-phosphate and fructose-6-phosphate, plays a role in glycolysis and gluconeogenesis in the cytoplasm and is also involved in the pentose phosphate pathway (17). This protein has been studied as a neurotrophic factor for promoting the survival of skeletal and sensory neurons and inducing immunoglobulin secretion, and as a tumor-secreting cytokine that plays a role in tumor angiogenesis and metastasis and cell migration, proliferation, and apoptosis (18, 19). GPI deficiency induces hemolytic anemia,

whereas its overexpression is related to carcinogenesis and its elevated serum level is used as a prognostic biomarker of colon, rectal, breast, lung, and kidney cancers (20). Therefore, the upregulation of both HKDC1 and GPI by chronic heat stress would promote glycolysis and activate pathways to obtain energy from glucose in the body. However, such overexpression may induce cell damage as well. Sorbitol dehydrogenase (SORD), which converts sorbitol to fructose in the polyol pathway, is closely related to various diabetic complications (viz., neuropathy, retinopathy, cataracts, and nephropathy) (21). The decreased expression of SORD causes an excessive accumulation of sorbitol, leading to osmotic damage to the retinal endothelial cells and pericytes through oxidative-nitridation stress and activation of the protein kinase C pathways, with resultant inflammation and growth factor imbalance (22). Our results showed that the expression of SORD was reduced by chronic heat stress, which is coincident with the finding that SORD is inactivated with increasing temperature (23). However, the SORD reduction in the CH group was recovered in the HH group, indicating that early heat exposure plays a protective role against heat stress in the liver cells.

In the tricarboxylic acid (TCA) cycle, the main precursor acetyl-CoA is essential for energy generation toward the mitochondria (24). During a lack of carbohydrate intake or utilization, acetate is used as an essential source for producing acetyl-CoA under ketogenic conditions and is involved in heat generation (25). In broilers, ACSS1L catalyzes the synthesis of acetyl-CoA for use in glycolysis. The feed intake of the CH broilers was significantly decreased compared with that of the CC broilers, and thus the expression of ACSS1L was increased in that group owing to the insufficient nutrients for metabolism. Citrate synthase (CS) catalyzes the condensation reaction to form citrate from oxaloacetate and acetyl-CoA, which are the first steps in the TCA cycle. It is also used as an enzymatic marker of intact mitochondria (26). CS activity has been found to be decreased by mitochondrial dysfunction and inhibited by oxidative stress (27). Another study confirmed the protective function of CS in thermally stressed yeast cells (28), where deletion of the CS-coding gene *cit1* resulted in temperature-sensitive ROS accumulation, nuclear and DNA fragmentation, and phosphatidylserine translocation, all of which are hallmarks of cytological apoptosis. From the results of our study, we can postulate that heat stress causes damage to the mitochondria as a result of the downregulation of CS, but early heat exposure can regulate the CS expression level. OGDHL, which is located in the mitochondria, is also a TCA cycle-related enzyme and indirectly responsible for the induction of apoptosis. The increased expression of OGDHL contributes to the acceleration of the TCA cycle (29). It was found in a previous study that OGDHL overexpression significantly induced ROS production and lipid peroxidation in the mitochondria of cervical cancer cells and resulted in apoptosis by inducing caspase-3 activity and PARP protein expression, whereas OGDHL suppression significantly inhibited these processes and promoted cell proliferation (30). These results suggest that overexpressed OGDHL plays an important role in inducing ROS-mediated apoptosis. In our study, chronic heat stress induced OGDHL expression, which suggests that it may induce ROS production followed by cell damage. Thus, the increase in carbohydrate metabolism observed in chronically heat-stressed broilers may be the result of a lack of energy in the body.

In general, heat stress increases energy consumption for panting to dissipate heat, maintaining homeostasis, and protecting the cells (31). In a study on the fatty acid levels in broilers under heat stress, the plasma concentration of non-esterified fatty acids was reduced in the heat-stressed animals, and better absorption and storage of triglycerides in the intestine or liver were observed (32). This is because the heat emitted from fat metabolism is higher than that generated through carbohydrate metabolism. Similarly, in heat-stressed dairy cows, energy was preferentially supplied by the carbohydrates to reduce the body's own heat generation (33). However, in our study, chronic heat stress, which increases the requirement of a considerable amount of energy, increased the expression of enzymes of the acyl-CoA dehydrogenase (ACAD) family (i.e., ACAD11 and ACAD9) to obtain energy through fat. This confirms that a considerable amount of energy is required, along with a reduction in feed intake, in response to

chronic heat stress. Although heat stress significantly reduced the nutrient (and thus potential energy) intake by the broilers, a large amount of energy was needed from metabolism to cope with the stress. Thus, the expression of carbohydrate metabolism-related proteins changed.

Conclusions

The feasibility of the early heat exposure method for reducing heat stress in broilers was studied, as this method has been reported to have a protective effect against heat stress. Although there are published studies on the effects of heat stress on cell growth, heat-shock protein expression, hormones, and blood parameters, to our best knowledge, no research has been conducted on the mechanisms related to the thermal reactions. Thus, in this study, we carried out a proteomic analysis to confirm which proteins/processes are regulated by early heat exposure under chronic heat stress. The results showed that chronic heat stress affected the expression of proteins involved in carbohydrate metabolism and carbon metabolism, which generally result in less heat production than lipid metabolism for energy production. If the expression of these proteins increases, cell damage may increase as well. However, the early heat treatment recovered the proteins that were induced (or reduced) by chronic heat stress, indicating that early heat exposure increases the heat resistance of the animal to inhibit biological damage. The knowledge about these physiological changes would be useful for the development of programs for breeding animals with high heat resistance. Moreover, this early heat exposure method and the results of our study may be helpful in reducing heat stress in animals, thereby improving animal welfare in general.

List Of Abbreviations

Nrf2: Nuclear factor erythroid 2-related factor 2; MAP2K1: Mitogen-activated protein kinase kinase 1; GSTM1: Glutathione S-transferase mu 1; STIP1: stress-induced phosphoprotein 1; GRP94: Glucose-regulated protein 94; SERPINA3: Serpin family A member 3; LC: liquid chromatography; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; ISG12: Interferon-stimulated gene 12; ACTN1: Actinin alpha 1; FERMT2: fermitin family homolog 2; LIMA1: LIM domain and actin binding 1; FLNB: filamin B; MYH9: myosin heavy chain 9; MYO1B: myosin 1B; GOT1: Glutamic-oxaloacetic transaminase 1; GART: Phosphoribosylgrlycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase; GSTT1L: Glutathione S-transferase theta 1-like; OGDHL: 2-oxoglutarate dehydrogenase-like, mitochondrial; UGT2A1: UDP glucuronosyltransferase family 2 member A1; RARS: Arginyl-tRNA synthetase, cytoplasmic; CYP2D6: Cytochrome P450 2D6; IARS: Isoleucyl-tRNA synthetase; GSS: Glutathione synthetase; ACSS1L: Acetyl-CoA synthetase 2-like, mitochondrial isoform X1; GPI: Glucose 6-phosphate isomerase; HKDC1: Hexokinase domain-containing 1; PCCA: Propionyl-CoA carboxylase subunit alpha; SORD: Sorbitol dehydrogenase; TCA: tricarboxylic acid; CS: Citrate synthase; ACAD: Acyl-CoA dehydrogenase;

Declarations

Ethics approval and consent to participate

Experimental procedures for animals were approved by the Animal Ethics Committee of the Jeonbuk National University (CBNU2018-097), Republic of Korea.

Consent for publication

Not applicable.

Availability of data and material

The datasets produced and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Writing-original draft preparation, DK; writing-review and editing, KS; conceiving and design, DK and KS; supervision, KS; analysis, DK; Both authors read and approved the final manuscript.

Acknowledgements

DK: Department of Animal Biotechnology, Jeonbuk National University, Korea, PhD.

KS: Department of Animal Biotehchnology, Jeonbuk National University, Korea, Professor.

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Figures

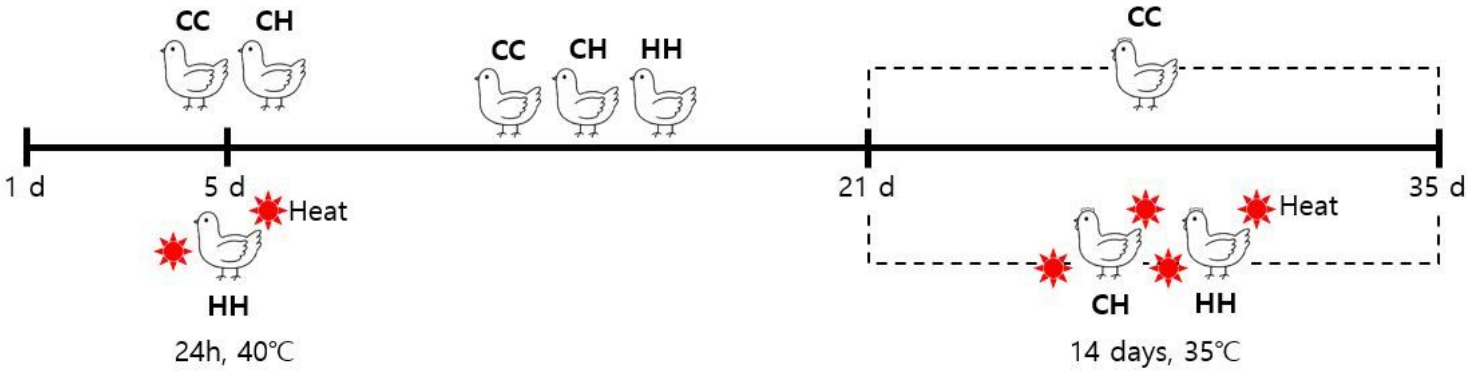

Figure 1

Schematized schedule of heat exposure condition. CC: raised at a suitable temperature without heat exposure; CH: chronic heat exposed group; HH: early and chronic heat exposed group.

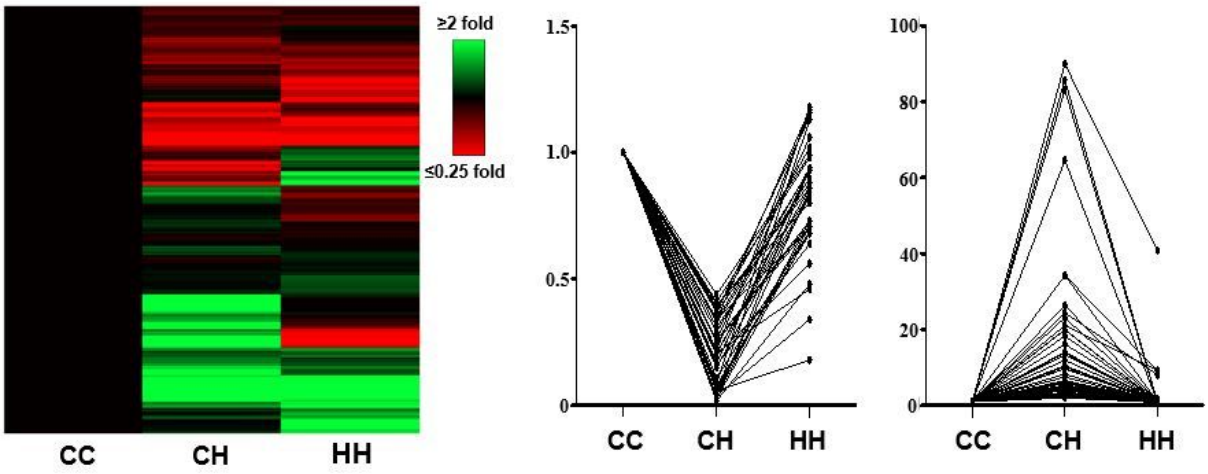

Figure 2

Heat map using differentially expressed proteins and expression patterns of positive effected proteins by early heat. CC: raised at a suitable temperature without heat exposure; CH: chronic heat exposed group; HH: early and chronic heat exposed group.

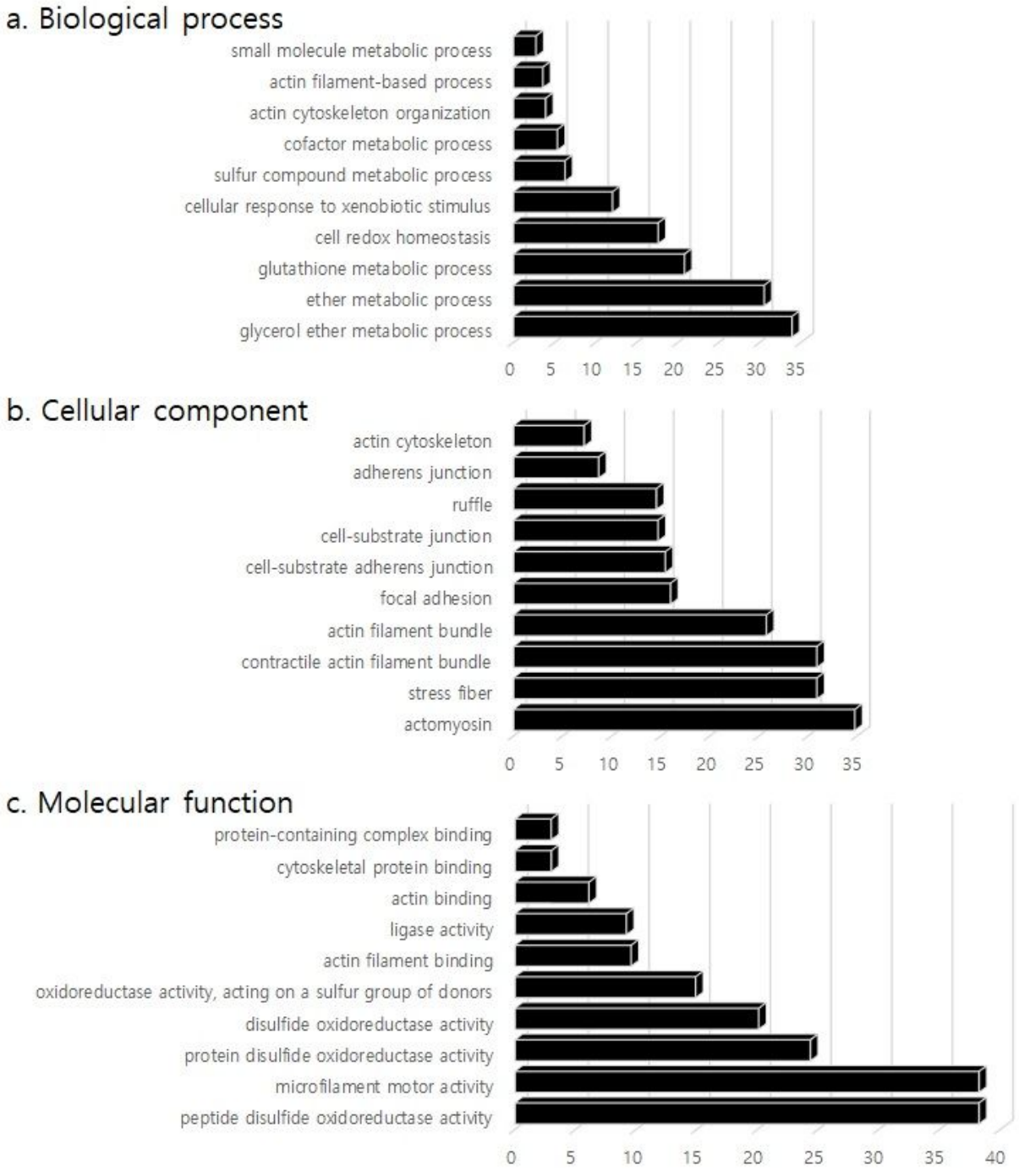

Figure 3

Gene Ontology terms of positive effected proteins of differentially expressed proteins by early heat exposure

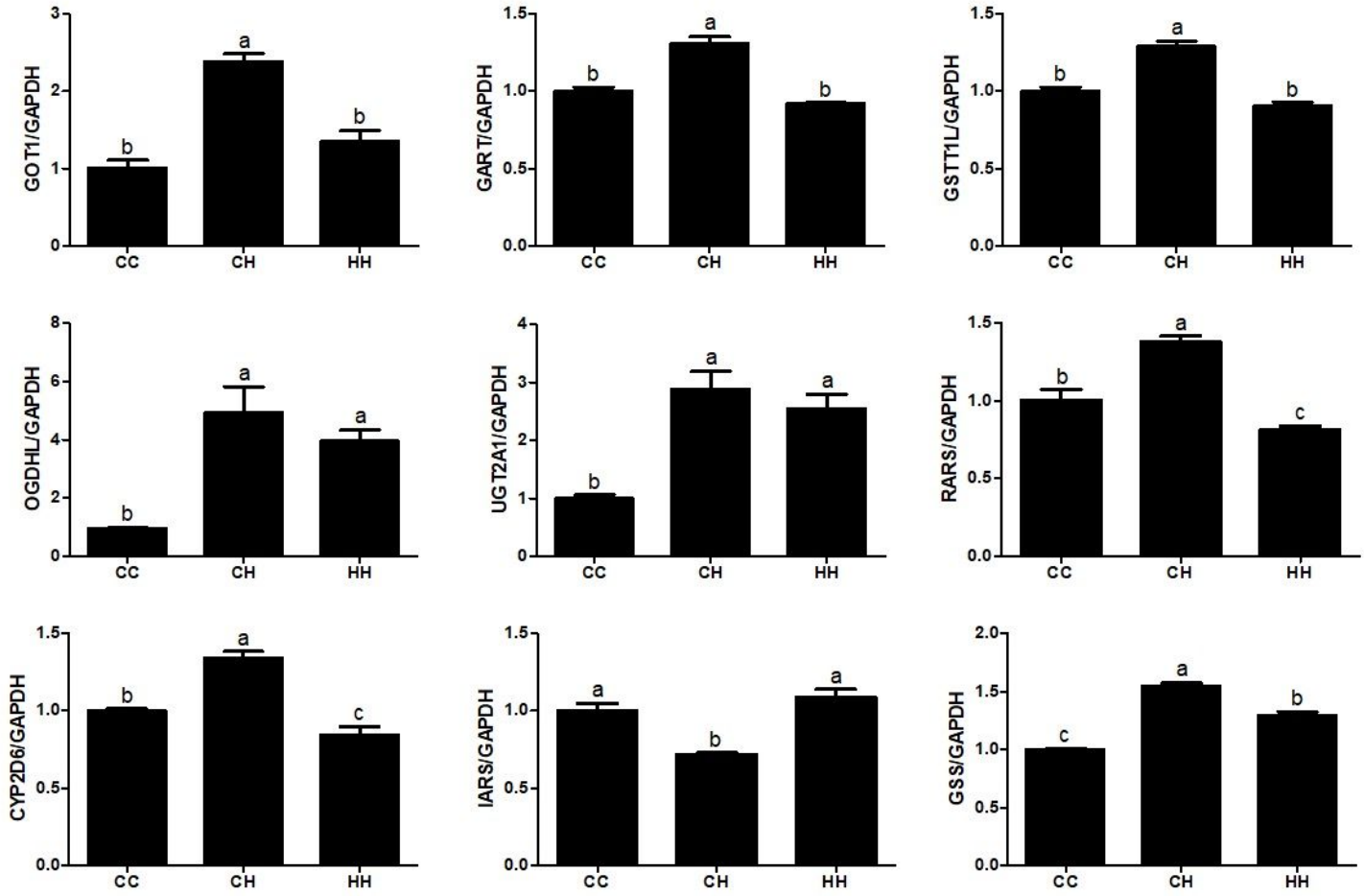

Figure 4

Gene expression in the liver tissue. CC: raised at a suitable temperature without heat exposure; CH: chronic heat exposed group; HH: early and chronic heat exposed group. The error bar represent the standard error

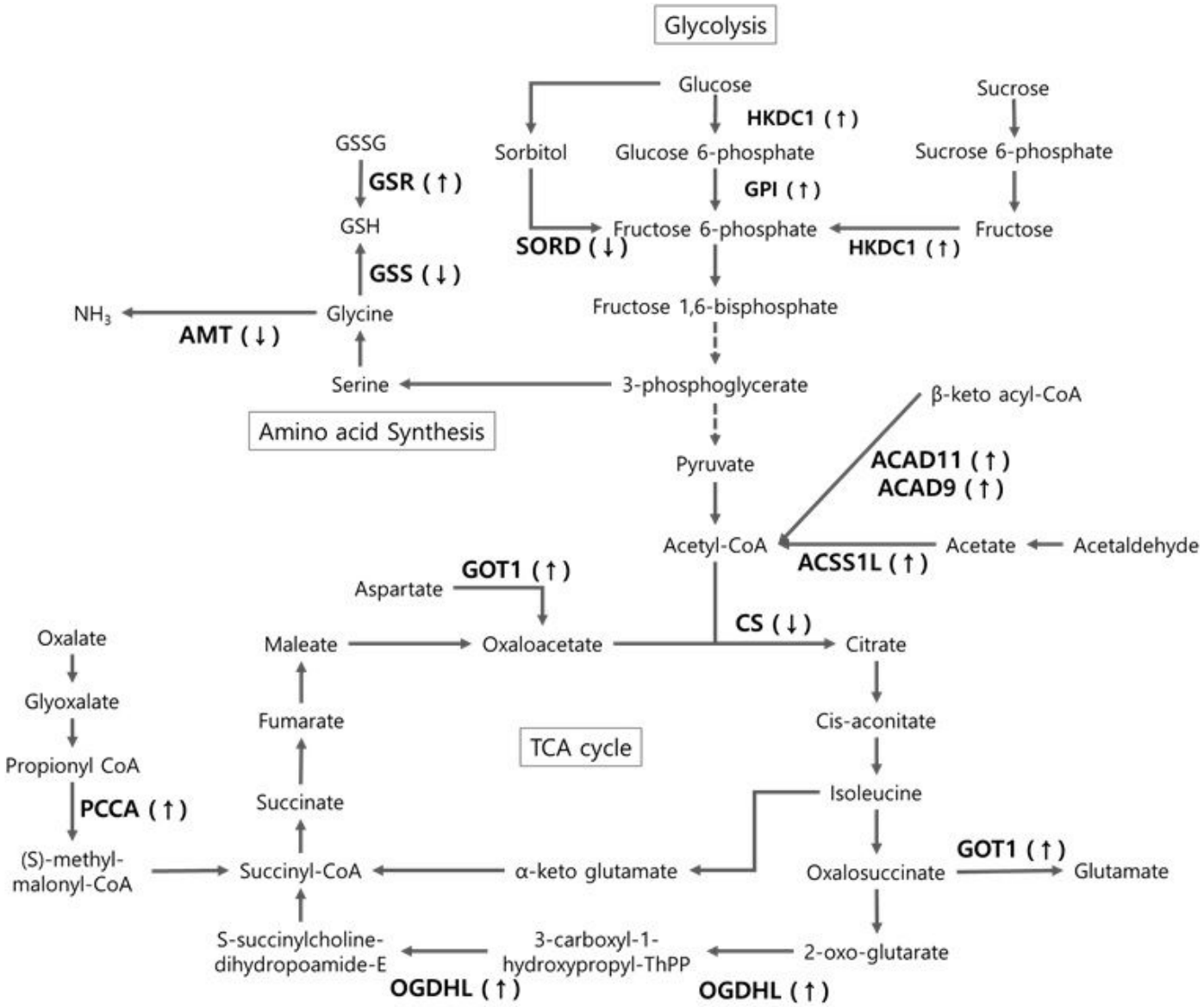

Figure 5

Metabolic schematic of differentially expressed proteins

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