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Original Research Article

Glucose supplementation improves intestinal amino acid transport and muscle amino acid pool in pigs during chronic cold exposure

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ABSTRACT

Mammals in northern regions chronically suffer from low temperatures during autumn-winter seasons. The aim of this study was to investigate the response of intestinal amino acid transport and the amino acid pool in muscle to chronic cold exposure via Min pig models (cold adaptation) and Yorkshire pig models (non-cold adaptation). Furthermore, this study explored the beneficial effects of glucose supplementation on small intestinal amino acid transport and amino acid pool in muscle of cold-exposed Yorkshire pigs. Min pigs (Exp. 1) and Yorkshire pigs (Exp. 2) were divided into a control group (17 °C, $n = 6$) and chronic cold exposure group (7 °C, $n = 6$), respectively. Twelve Yorkshire pigs (Exp. 3) were divided into a cold control group and cold glucose supplementation group (8 °C). The results showed that chronic cold exposure inhibited peptide transporter protein 1 (PepT1) and excitatory amino acid transporter 3 (EAAT3) expression in ileal mucosa and cationic amino acid transporter-1 (CAT-1) in the jejunal mucosa of Yorkshire pigs ($P < 0.05$). In contrast, CAT-1, PepT1 and EAAT3 expression was enhanced in the duodenal mucosa of Min pigs ($P < 0.05$). Branched amino acids (BCAA) in the muscle of Yorkshire pigs were consumed by chronic cold exposure, accompanied by increased muscle RING-finger protein-1 (MuRF1) and muscle atrophy F-box (atrogin-1) expression ($P < 0.05$). More importantly, reduced concentrations of dystrophin were detected in the muscle of Yorkshire pigs ($P < 0.05$). However, glycine concentration in the muscle of Min pigs was raised ($P < 0.05$). In the absence of interaction between chronic cold exposure and glucose supplementation, glucose supplementation improved CAT-1 expression in the jejunal mucosa and PepT1 expression in the ileal mucosa of cold-exposed Yorkshire pigs ($P < 0.05$). It also improved BCAA and inhibited MuRF1 and atrogin-1 expression in muscle ($P < 0.05$). Moreover, dystrophin concentration was improved by glucose supplementation ($P < 0.05$). In summary, chronic cold exposure inhibits amino acid absorption in the small intestine, depletes BCAA and promotes protein degradation in muscle. Glucose supplementation ameliorates the negative effects of chronic cold exposure on amino acid transport and the amino acid pool in muscle.

1. Introduction

Mammals frequently experience sustained energy turnover, especially in chronic cold exposure. Cold exposure is a common problem and a stressor for animals that live in extreme environments. Usually, mammals experience active resistance, adaptation and exhaustion during chronic cold exposure and prolonged exposure to low temperatures may lead to irreversible negative effects (Selye, 1979). Extensive data have confirmed that cold limits animal growth and increases feed intake (Bayril et al., 2020; Toghiani et al., 2020). More worryingly, long-term cold exposure induces oxidative stress and inflammation in organs, even causing

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death in animals (Liu et al., 2019, 2022; Luo et al., 2019). Adaptation to low ambient temperature is one of the mechanisms vital to the survival of mammals. Undoubtedly, mammals need to expend more energy to cope with low temperature (Yu et al., 2015). To date, since fatty acids and glucose are the main energy sources, most studies have focused on heat production of brown adipose tissue and insulin sensitivity in mammals during cold exposure (Chevalier et al., 2015; Sanchez-Gurmaches et al., 2018; Leiria et al., 2019). However, neglected amino acids (AA) can also contribute to physiology as energy sources. So far, there are few reports on the effects of chronic cold exposure on the digestion and absorption of amino acids in mammals.

AA are well known nutrient substrates for protein synthesis. They also participate as bioactive molecules in nutrient metabolism (Nie et al., 2018). Indeed, when dietary proteins are hydrolyzed by digestive enzymes, AA and small peptides are released into the intestinal lumen. Subsequently, they are absorbed with the assistance of AA transporters in the epithelial cells of the intestinal mucosa. AA transporters specifically mediate the uptake and transport of AA present in living organisms (Broer and Palacin, 2011). Free AA circulate in the bloodstream and are then absorbed and used by various organs and tissues via AA transporters. Skeletal muscle represents the largest protein and AA reservoir (Qi and Lu, 2007) and enable movement of the body. AA transporters in muscle tissue adjust AA flux according to demand to maintain AA homeostasis. Muscle mass depends on the growth and development of muscles, which involves the proliferation and differentiation of muscle cells. Myogenic factor 5 (Myf5) and myoblast differentiation antigen (MyoD) are the determinants of myogenesis, which controls the fate of muscle cells. MyoD is regarded as an important marker of muscle cell proliferation and differentiation (Zammit, 2017). Myf5 is a myogenic regulatory factor involved in myoblast specification and maintenance (Panda et al., 2016). The expression of these 2 factors determines the fate of muscle cells. Skeletal muscle protein is constantly built up when mammals undergo rapid growth (Aisbett et al., 2017). In this physiological state, the rate of protein synthesis is greater than protein breakdown (Rennie et al., 2004). Muscle growth rate is inhibited when the balance between protein synthesis and breakdown is disrupted. An increased rate of protein breakdown results in muscle growth retardation or loss of muscle mass (Bodine and Baehr, 2014), even triggering some diseases (Kerksick and Leutholtz, 2005). Dystrophin is a macromolecular cytoskeletal protein widely used to evaluate muscle diseases or dysplasia (Mendell et al., 2010; Carter et al., 2018). At present, the regulation of dystrophin by chronic cold exposure in pigs remains to be explored. Furthermore, the effects of chronic cold exposure on small intestinal AA transport and AA pool of muscle in pigs are unknown.

The balance of mitochondrial dynamics (fission and fusion) is crucial for muscle contraction and metabolism. In general, the imbalance of division and fusion leads to abnormal mitochondrial function (Wei and Ruvkun, 2020). Mitofusin 1 (Mfn1), mitofusin 2 (Mfn2) and mitochondrial dynamin like GTPase (OPA1) contribute to mitochondrial docking and fusion, and stabilize associations between mitochondria (Kato and Nishitoh, 2015). Mitochondrial fission protein 1 (Fis1) and mitochondrial fission factor (MFF) in the mitochondrial outer membrane participate in fission (Gandre-Babbe and van der Bliek, 2008). Previous evidence suggests that muscle atrophy is associated with disruption of mitochondrial dynamic balance (Romanello et al., 2010; Lee et al., 2020). Thus, mitochondrial function in muscle tissue is closely related to muscle development. However, the effect of chronic cold exposure on mitochondrial balance in muscle tissue is incompletely characterized.

As an essential source of organic carbon, carbohydrate in the diet is critically important to the health of mammals. Carbohydrates such as starch and maltose are hydrolyzed into monosaccharides (such as glucose) by digestive enzymes in the intestinal lumen (Holst et al., 2016). Dietary glucose supplements are readily absorbed by glucose transporters (Chan and Leung, 2015). Therefore, it is worth exploring the effects of dietary glucose supplementation on muscle AA levels and muscle development under chronic cold exposure.

Min pigs are a local pig species in northern China with strong resistance to stress. Studies have shown that they have a stronger immune system than Yorkshire pigs (Teng et al., 2020). In addition, Min pigs have been demonstrated to have better cold adaptability (Li et al., 2017; Peng et al., 2019). Here, we explored the effects of chronic cold exposure on AA digestion, absorption and muscle development in a Min pig model (cold adaptation) and Yorkshire pig model (non-cold adaptation). In addition, we reveal the mechanism of cold adaptation based on AA metabolism under chronic cold exposure via these 2 animal models. Furthermore, we attempt to ameliorate the negative impacts of chronic cold exposure on AA transport and muscle AA pool in Yorkshire pigs by adding glucose to the diet. This study provides new evidence for cold and non-cold adaptations based on AA transport and the AA pool in muscle.

2. Materials and methods

2.1. Animal ethical statement

The protocols used during this study were approved by the Ethical and Animal Welfare Committee of Heilongjiang Province, China. The experimental proposals and procedures for the care and treatment of animals were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (NEAU - [2011] - 9). The procedure number is NEAUEC20200208.

2.2. Two chronic cold exposure experiments

Two chronic cold exposure experiments were carried out simultaneously (Fig. 1A). Twelve Min pigs (Exp. 1) were randomly divided into a control group (CM, 35.52 ± 1.18 kg, 17 ± 3 °C, $n = 6$) and cold stress group (CSM, 35.48 ± 1.29 kg, 7 ± 3 °C, $n = 6$). Twelve Yorkshire pigs (Exp. 2) were divided into a control group (CY, 24.83 ± 0.64 kg, 17 ± 3 °C, $n = 6$) and cold exposure group (CSY, 24.80 ± 0.61 kg, 7 ± 3 °C, $n = 6$). The environmental temperature of the CSM and CSY group was maintained by electronic heaters (GSM501, Guangzhou Rongce Electronics, Guangdong, China), and the environmental temperature of the CM and CY group was derived from natural conditions. All the pigs were fed separately from a single metabolic cage containing a water dispenser and feeding tank for free drinking and feeding. These 2 experiments lasted for 21 days.

2.3. Glucose supplementation in the diet of cold-exposed pigs

Next, a trial was carried out to supplement the diet of cold-exposed pigs with glucose. Twelve Yorkshire pigs (Exp. 3) were randomly divided into a control group (Con, 23.54 ± 0.84 kg, $n = 6$) and glucose supplement group (GS, 23.76 ± 0.78 kg, $n = 6$). The environmental temperature of the cold exposure group (under natural conditions) was 8 ± 3 °C. As in the previous experiment, all the pigs were fed separately from a single metabolic cage containing a water dispenser and feeding tank for free drinking and feeding. This trial lasted for 22 days.

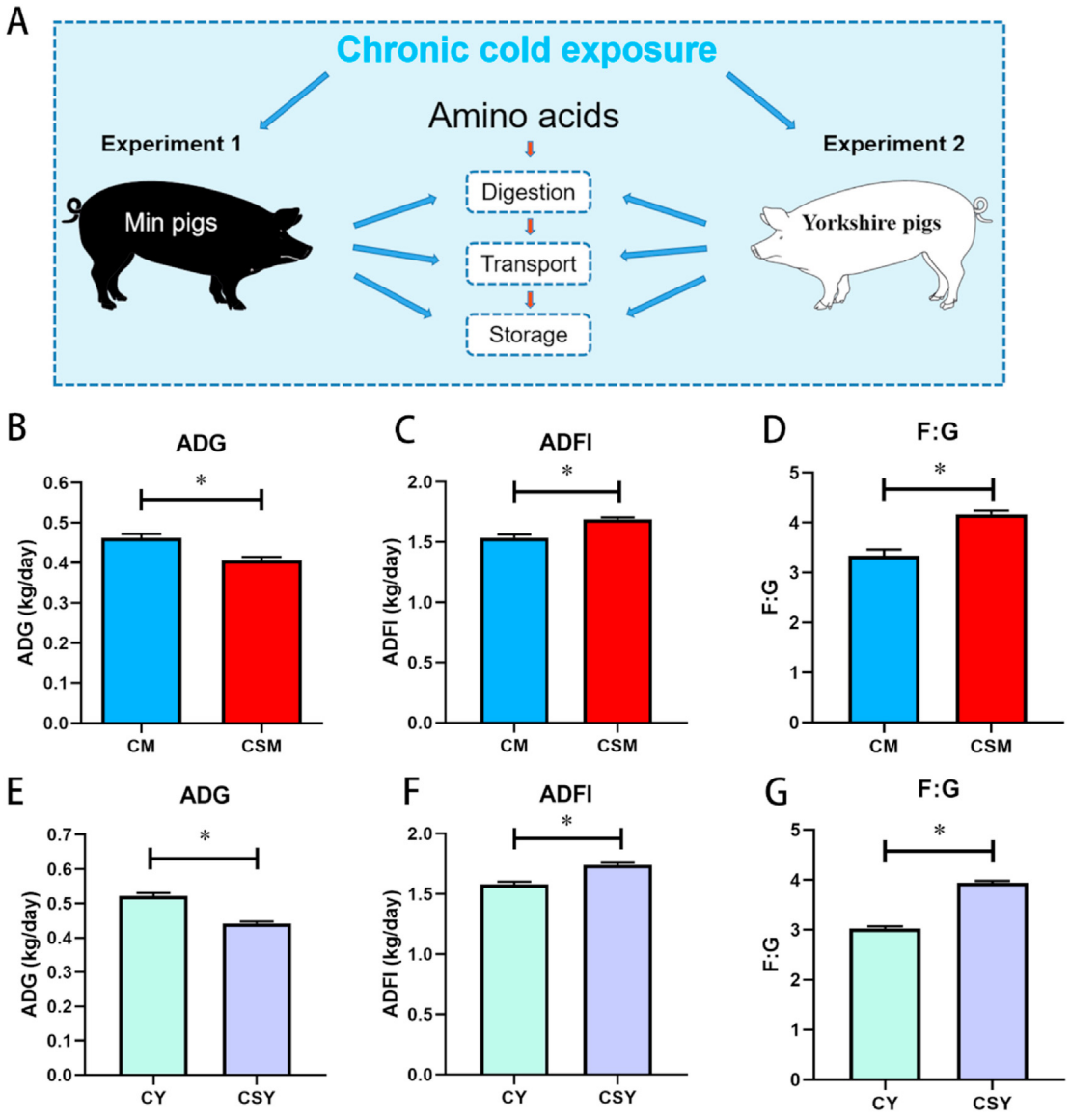

Fig. 1. Chronic cold exposure inhibits growth and increases feed consumption in pigs. (A) Design of Exp. 1 and 2. (B) The average daily gain (ADG) of Min pigs. (C) The average daily feed intake (ADFI) of Min pigs. (D) The feed to gain ratio (F:G) of Min pigs. (E) The ADG of Yorkshire pigs. (F) The ADFI of Yorkshire pigs. (G) The F:G of Yorkshire pigs. Data are expressed as the mean ± SEM, $n = 6$, $^*P < 0.05$.

The diets involved in this study were formulated (Tables 1 and 2) to reference the Ministry of Agriculture of the People's Republic of China (MOA, 2020) and National Research Council (NRC, 2012) recommended requirements. The experiment was carried out at Acheng Experimental Base of Northeast Agricultural University.

2.4. Sample collection

All pigs were electrocuted and slaughtered after fasting for 12 h. Venous blood was collected and centrifuged for 15 min at 300×g. Plasma samples were stored in −20 °C. Two grams of longissimus dorsi muscle was quickly collected in cryo-storage tubes and then frozen in liquid nitrogen. In addition, 1 cm^2 of longissimus dorsi muscle was cut and preserved in 4% paraformaldehyde for morphological analysis. Mucosal scrapings were collected from the duodenum, jejunum and ileum using a glass slide then transferred to liquid nitrogen for snap freezing. Finally, these samples were transferred to a −80 °C refrigerator for storage.

2.5. Digestibility of crude protein

The apparent total tract digestibility (ATTD) of crude protein (CP) was assessed. Fecal samples were collected during day 17 to 19 of the experiment according to published procedures (Nair et al., 2019). Twice a day at 07:00 and 20:00, individual feces were collected and weighed. To avoid ammonia loss, 10 mL of H_2SO_4 (10% vol/wt) was added and the feces were then stored at −20 °C. Before analysis, the fecal samples of each pig were thawed and homogenized. Then, 200 g of feces were removed and left to dry in a hot air oven at 60 ± 2 °C for 72 h and ground through a 1-mm screen. The feed offered to each pig was weighed daily to calculate the ATTD. CP in the feces and diet was assessed according to the previous method (Nair et al., 2019).

2.6. Detection of trypsin activity

Samples of 0.5 g of duodenal mucosa and jejunal mucosa were placed in ice-cold 0.9% sodium chloride solution (1:9, wt:vol). They

Table 1
Composition of experimental diets.[1]

Item	Content, %
Ingredients	
Corn	73.00
Soybean meal, de-hulled	15.30
Full-fat soybean meal, puffed	5.00
Fish meal	2.00
Soybean oil	1.00
L-Lysine	0.39
DL-Methionine	0.04
L-Threonine	0.12
L-Tryptophan	0.02
Calcium hydrogen phosphate	1.19
Limestone	0.66
Salt	0.28
Premix [2]	1.00
Nutrient levels [3]	
NE [4], Mcal/kg	2.50
Crude protein	16.03
Lysine	0.98
Methionine	0.29
Threonine	0.60
Leucine	0.17
Calcium	0.66
Total phosphorus	0.56
Available phosphorus	0.33
Sodium	0.14
Chlorine	0.19

[1] This diet was formulated to reference the Ministry of Agriculture of the People's Republic of China (MOA, 2020) and National Research Council (NRC, 2012) recommended requirements.
[2] Provided the following per kilogram of diet: Fe, 160 mg; Cu, 150 mg; Mn, 40 mg; Zn, 140 mg; Se, 0.4 mg; I, 0.5 mg; vitamin A, 8,000 IU; vitamin D_3, 2,000 IU; vitamin E, 30 mg; vitamin B_1, 1.60 mg; vitamin B_2, 5.00 mg; vitamin B_6, 5.00 mg; vitamin B_{12}, 0.01 mg; pantothenic acid, 20 mg; niacin, 15 mg; biotin, 0.05 mg.
[3] Nutrient levels were calculated values.
[4] NE: net energy.

Table 2
Composition of glucose diets.[1]

Item	Content, %
Ingredients	
Corn	60.68
Soybean meal, de-hulled	17.53
Full-fat soybean meal, puffed	5.00
Fish meal	2.00
Soybean oil	1.00
Glucose	10.00
L-Lysine	0.35
DL-Methionine	0.05
L-Threonine	0.11
L-Tryptophan	0.01
Calcium hydrogen phosphate	1.25
Limestone	0.62
Salt	0.40
Premix [2]	1.00
Nutrient levels [3]	
NE [4], Mcal/kg	2.63
Crude protein	16.03
Lysine	0.98
Methionine	0.29
Threonine	0.60
Leucine	0.17
Calcium	0.66
Total phosphorus	0.56
Available phosphorus	0.34
Sodium	0.19
Chlorine	0.26

[1] This diet was formulated to reference the Ministry of Agriculture of the People's Republic of China (MOA, 2020) and National Research Council (NRC, 2012) recommended requirements.
[2] Provided the following per kilogram of diet: Fe, 160 mg; Cu, 150 mg; Mn, 40 mg; Zn, 140 mg; Se, 0.4 mg; I, 0.5 mg; vitamin A, 8,000 IU; vitamin D_3, 2,000 IU; vitamin E, 30 mg; vitamin B_1, 1.60 mg; vitamin B_2, 5.00 mg; vitamin B_6, 5.00 mg; vitamin B_{12}, 0.01 mg; pantothenic acid, 20 mg; niacin, 15 mg; biotin, 0.05 mg.
[3] Nutrient levels were calculated values.
[4] NE: net energy.

were then thawed and homogenized for 60 s and centrifuged at 13,000 × *g* for 20 min at 4 °C. After that, the supernatant was collected for assaying. Trypsin activity was analyzed using a previous method (Chen et al., 2018). Commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used to assess the protein concentration and trypsin activity, according to the manufacturer's guidelines.

2.7. Determination of free AA

The free AA profile was determined in the longissimus dorsi muscle by using a previous method (Sun et al., 2020). A 100-mg sample was hydrolyzed with 8 mL of 6 mol/L HCl (reflux for 24 h at 110 °C) to convert protein-bound AA to free AA. Then, 1 mL of the hydrolysate was obtained and lyophilized. The freeze-dried sample was homogenized with 1 mL of 0.02 mol/L HCl, and then centrifuged at 14,000 × *g* for 15 min. The final supernatants were used for AA analysis via a High-speed Amino Acid Analyzer (Hitachi L-8900, Tokyo, Japan).

2.8. Total RNA extraction, reverse transcription and relative quantitative real-time PCR

The tissue samples were homogenized in 1 mL TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and the total RNA was isolated according to the manufacturer's instructions. The quality of the total RNA was determined by confirming that the ratio of OD_{260} and OD_{280} was between 1.8 and 2.0. The total RNA was reverse-transcribed using a PrimeScript TM RT reagent kit (TaKaRa, Biotechnology, Dalian, China). The total RNA was reverse-transcribed using an Integrated First-strand cDNA Synthesis Kit (Dining, Beijing, China). Next, the 2 × Fast qPCR Master Mixture (Dining, Beijing, China) was used to perform real-time PCR in an ABI 7500 Fast Real-Time PCR System (Foster City, CA, USA). Every reaction was performed at least twice. The relative amount of each target mRNA was normalized to the β-actin mRNA level. Information on all the primers is shown in Table S1. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ normalized to β-actin expression.

2.9. Western blot analysis

Samples were homogenized in RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with PMSF (Beyotime Biotechnology, Shanghai, China). The protein concentrations were determined by using a BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. After SDS-PAGE, protein was transferred to a polyvinylidene fluoride (PVDF) membrane through electrophoretic transfer. The membrane was blocked in TBST which contained 5% nonfat dry milk at room temperature for 2 h. The blots were incubated with primary antibodies overnight at 4 °C. Subsequently, primary antibodies against cationic amino acid transporter-1 (CAT-1), excitatory amino acid transporter 3 (EAAT3), peptide transporter protein 1 (PepT1), mitochondrial fission factor (MFF), muscle atrophy F-box (atrogin-1), muscle RING-finger protein-1 (MuRF1) and β-actin were added. The blots were incubated with primary antibodies overnight at 4 °C. After thoroughly washing 3 times by TBST, the membranes were incubated with (HRP Goat Anti-Rabbit

IgG [H + L]) for 2 h at 25 °C. After that, the membranes were washed 3 times and antibody reactivity was detected by chemiluminescence through the BeyoECL Star fluorescence detection kit (Beyotime Biotechnology, Shanghai, China). These bands were imaged by a gel imaging and analysis system (UVItec, Cambridge, Britain), and band intensity was assessed using the Image J system, with correction for background and loading controls. β-Actin was used to normalize the intensity of the bands. The antibody information mentioned above is shown in Table S2.

2.10. Determination of dystrophin in longissimus dorsi muscle

A 10% longissimus dorsi muscle homogenate (samples were added to physiological saline at a 1:9 ratio) was first prepared. Total protein concentration in the homogenate was detected by a BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, China). Then, an ELISA kit was used to detect dystrophin in longissimus dorsi muscle according to the instructions (Enzyme Biosystems, Shanghai, China).

2.11. Histopathological examination

Muscle tissue fixed in 4% paraformaldehyde was embedded in paraffin (5 μm thickness sections). The sections were stained with hematoxylin and eosin (H&E). Then, they were captured by light microscopy to observe the development of the longissimus dorsi muscle at 200× magnification. Images were captured (scale bar = 100 μm) by a Nikon DS-F12 digital camera (Nikon, Tokyo, Japan).

2.12. Statistical analysis

In this study, each pig was considered to be a statistical unit. Firstly, the normality and homogeneity of variances of the data were evaluated. Then, all data were analyzed by *t*-Test (SPSS 22.0; IBM-SPSS Inc., Chicago, IL, USA) and visualized via GraphPad Prism (Graph Pad Software Inc., San Diego, CA, USA). The data are expressed as the mean ± SEM. $P < 0.05$ was considered statistically significant (* means $P < 0.05$ and ** means $P < 0.01$). A trend was defined as $0.05 < P < 0.1$.

3. Results

3.1. Feeding and growth of Min pig models and Yorkshire pig models

In Exp. 1, chronic cold exposure significantly decreased average daily gain (ADG), increased average daily feed intake (ADFI) and feed to gain ratio (F:G) of Min pigs (Fig. 1B–D, $P < 0.05$). Similarly, in Exp. 2, the ADG of Yorkshire pigs was decreased, the ADFI and F:G were increased by chronic cold exposure (Fig. 1E–G, $P < 0.05$).

3.2. The ATTD of crude protein (CP) of Min pig models and Yorkshire pig models

The ATTD of CP for Min pigs during chronic cold stress is shown in Fig. 2A. Compared with the CM group, the ATTD of CP was significantly increased in the CSM group ($P < 0.05$). The ATTD of CP for Yorkshire pigs during chronic cold stress is shown in Fig. 2B. The ATTD of CP in Yorkshire pigs was not altered ($P > 0.05$).

3.3. Trypsin activity of Min pig models and Yorkshire pig models

Trypsin activity in the duodenal mucosa and jejunal mucosa was measured in Exp. 1 and Exp. 2. For Min pigs, trypsin activity in the duodenal mucosa and jejunal mucosa of the CSM group was not changed by chronic cold stress (Fig. 2C and D and , $P > 0.05$). However, for Yorkshire pigs, the trypsin activity in the jejunal mucosa of the CSY group tended to be attenuated (Fig. 2F, $P = 0.064$), although the trypsin activity in the duodenal mucosa was not altered (Fig. 2E, $P > 0.05$).

3.4. Amino acid transport in the small intestine of Min pig models and Yorkshire pig models

Next, we examined the effects of chronic cold exposure on AA transporters in the small intestine. In Exp. 1, the mRNA and protein expression of CAT-1, PepT1 and EAAT3 in the duodenal mucosa was upregulated in the CSM group (Figs. 3A, and 4A–4C, $P < 0.05$). The expression of AA transporters in the jejunal mucosa was not affected by chronic cold exposure in the CSM group (Fig. 3B, $P > 0.05$). For the ileal mucosa, the mRNA expression of PepT1 in the CSM group was up-regulated (Fig. 3C, $P < 0.05$).

In Exp. 2, compared with the CY group, the expression of CAT-1, PepT1, EAAT3 and 4F2hc in the duodenal mucosa of the CSY group was not changed (Fig. 3D, $P > 0.05$). However, the expression of CAT-1 in the jejunal mucosa of the CSY group was inhibited (Figs. 3E and 4D, $P < 0.05$). The mRNA and protein expression of PepT1 and EAAT3 in the ileal mucosa of the CSY group was also down-regulated (Figs. 3F, 4E and 4F, $P < 0.05$).

3.5. Free amino acids and amino acid transporters in the longissimus dorsi muscle of Min pig models and Yorkshire pig models

The development of muscle tissue depends largely on AA. We characterized the mRNA expression of AA transporters in the longissimus dorsi muscle, and the levels of free AA in the longissimus dorsi muscle of Min pigs (Fig. 5C) and Yorkshire pigs (Fig. 5E) under chronic cold exposure. In Exp. 1, the expression of LAT-2 in longissimus dorsi muscle was enhanced in the CSM group (Fig. 5A, $P < 0.05$). Interestingly, glycine increased in the muscle of the CSM group compared with the CM group (Fig. 5D, $P < 0.05$).

In Exp. 2, for the Yorkshire pig models, the expression of LAT-2 in longissimus dorsi muscle was enhanced by chronic cold exposure (Fig. 5B, $P < 0.05$). Surprisingly, the concentration of leucine also had a tendency to be decreased (Fig. 5F, $P = 0.096$), and the concentration of isoleucine and valine in the CSY group was decreased (Fig. 5G and H, $P < 0.05$). In particular, all 3 AA were BCAA.

3.6. The dystrophin concentration in muscle of Min pig models and Yorkshire pig models

We focused on the dystrophin concentration in both models during chronic cold exposure. In Exp. 1, the dystrophin concentration in Min pig models was not affected by chronic cold exposure (Fig. 6A, $P > 0.05$). However, in Exp. 2, the dystrophin concentration in the longissimus dorsi muscle was decreased in the CSY group (Fig. 6B, $P < 0.05$).

3.7. The mitochondrial dynamic balance in the muscle of Min pig models and Yorkshire pig models

In addition, we investigated the mitochondrial dynamic balance in the longissimus dorsi muscle. In Exp. 1, the expression of PPARG coactivator 1 alpha (PGC-1α), Mfn1, Mfn2, MFF, Fis1 and OPA1 was not changed in the CSM group (Fig. 6C, $P > 0.05$). In Exp. 2, for the Yorkshire pig models, MFF mRNA and protein expression was inhibited in the longissimus dorsi muscle of the CSY group compared with the CY group (Fig. 6D and G, $P < 0.05$).

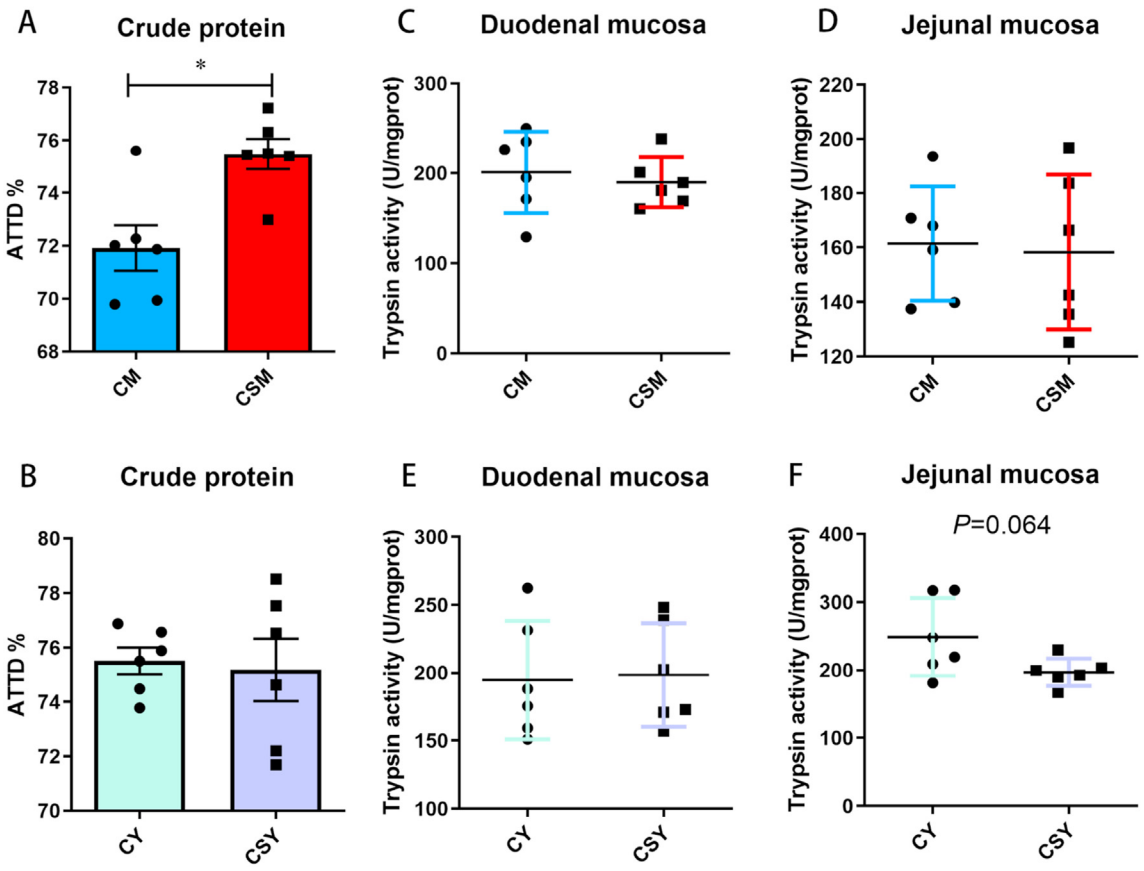

Fig. 2. Effects of chronic cold exposure on the apparent total tract digestibility (ATTD) of crude protein and trypsin activity in small Intestine. (A) The ATTD of crude protein of Min pig models. (B) The ATTD of crude protein of Yorkshire pig models. (C) Trypsin activity in duodenal mucosa of Min pig models. (D) Trypsin activity in jejunal mucosa of Min pig models. (E) Trypsin activity in duodenal mucosa of Yorkshire pig models. (F) Trypsin activity in jejunal mucosa of Yorkshire pig models. Data are expressed as the mean ± SEM, $n = 6$, *$P < 0.05$. CM and CY indicate the control groups; CSM indicates the cold stress group; and CSY indicates the cold exposure group.

3.8. Chronic cold exposure induces muscle cell atrophy and promotes protein degradation in Yorkshire pigs

Aiming at some negative effects of Yorkshire pig models during chronic cold exposure, we focused on muscle morphology of Yorkshire pig models in Exp. 2. As shown in Fig. 6E and F, notably, muscle cell atrophy was observed in the longissimus dorsi muscle of the CSY group compared with the CY group. Subsequently, expression of genes related to muscle development was detected. The expression of MyoD1 and Myf5 was not affected in the CSM group ($P > 0.05$). The expression of MyoD1 and Myf5 in the longissimus dorsi muscle was inhibited in the CSY group (Fig. S1, $P < 0.05$). Next, we investigated the expression of key proteins in the protein degradation pathway of Yorkshire pig models with muscular dysplasia induced by chronic cold exposure. MuRF1 and atrogin-1 protein expression was up-regulated in the CSY group (Fig. 6H, $P < 0.05$).

3.9. Glucose supplementation improves the ATTD of crude protein of cold-exposed pig models

Based on the above results, we attempted to use glucose as an energy supplement to improve AA transport and AA pools in the muscle of pigs exposed to chronic cold exposure (Exp. 3, Fig. 7A). In the absence of interaction between chronic cold exposure and glucose supplementation, the ATTD of crude protein, the expression of key AA transporters in the intestinal mucosa and longissimus dorsi muscle of cold-exposed pigs supplemented with dietary glucose is characterized in Fig. 7. Dietary glucose supplementation increased the ATTD of crude protein in cold-exposed pigs (Fig. 7B, $P < 0.05$).

3.10. Glucose supplementation improves the expression of amino acid transporters in the intestinal mucosa of cold-exposed pig models

Then, in Exp. 3, for the expression of key AA transporters in the intestinal mucosa, CAT-1 mRNA and protein expression in the jejunal mucosa, the expression of PepT1 mRNA and protein in the ileal mucosa of the GS group was promoted (Fig. 7D–F, $P < 0.05$). The expression of EAAT3 mRNA in the ileal mucosa was promoted (Fig. 7E, $P < 0.05$), but protein levels were not altered (Fig. 7F, $P > 0.05$). AA transporters in the duodenal mucosa were not modulated (Fig. 7C, $P > 0.05$).

In addition, 4F2 heavy chain (4F2hc) and L-amino acid transporter-2 (LAT-2) mRNA expression in the longissimus dorsi muscle was not changed (Fig. 7G, $P > 0.05$).

3.11. Glucose supplementation improves AA pools and protein degradation in longissimus dorsi muscles of cold-exposed pigs

Subsequently, in Exp. 3, we focused on the effects of dietary glucose supplementation on AA pools and protein degradation in longissimus dorsi muscles of cold-exposed Yorkshire pigs. We characterized the AA pools in the longissimus dorsi muscle (Fig. 8A). Interestingly, leucine and isoleucine concentrations were

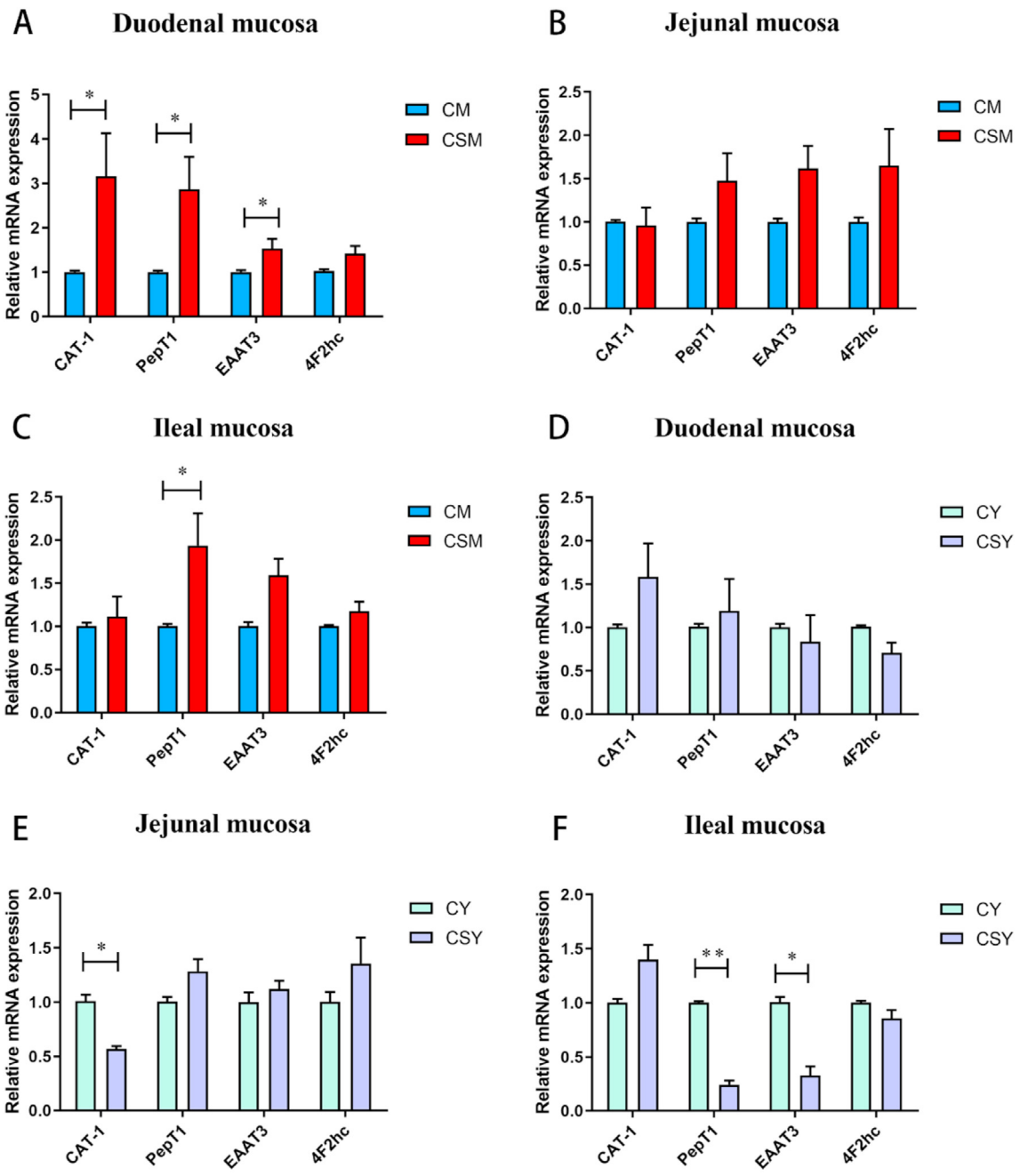

Fig. 3. Chronic cold exposure regulated the mRNA expression of amino acid transporters in small intestinal mucosa. (A) The duodenal mucosa of Min pig models. (B) The jejunal mucosa of Min pig models. (C) The ileal mucosa of Min pig models. (D) The duodenal mucosa of Yorkshire pig models. (E) The jejunal mucosa of Yorkshire pig models. (F) The ileal mucosa of Yorkshire pig models. Data are expressed as the mean ± SEM, $n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$. CM and CY indicate the control groups; CSM indicates the cold stress group; and CSY indicates the cold exposure group. CAT-1 indicates cationic amino acid transporter-1; PepT1 indicates peptide transporter protein 1; EAAT3 indicates excitatory amino acid transporter 3; 4F2hc indicates 4F2 heavy chain.

increased in the GS group (Fig. 8B and D, $P < 0.05$), and valine also tended to be upregulated (Fig. 8C, $P = 0.093$). Interestingly, elevated levels of tyrosine were also detected in the GS group (Fig. 8E, $P < 0.05$). The concentration of dystrophin in the longissimus dorsi muscle of the GS group was increased compared with the Con group (Fig. 8F, $P < 0.05$). Compared with the control group, dietary glucose supplementation inhibited atrogin-1 expression in cold-exposed pigs (Fig. 8G, $P < 0.05$). MuRF1 also had a tendency to be suppressed (Fig. 8G, $P = 0.09$). Moreover, dietary glucose supplementation significantly enhanced the expression of Myf5 in longissimus dorsi muscle of cold-exposed pigs (Fig. S2, $P < 0.05$).

4. Discussion

Low temperature is one of the critical factors among various environmental stressors that mammals have to face, especially in northern latitudes during winter. Cold stress severely impedes the regular growth of mammals and challenges the immune system and metabolism. Early data have certainly established that cold stress in livestock is common in winter (Young, 1983). Recent studies have shown that long-term exposure of animals to low temperatures inhibits growth, along with adverse effects such as inflammation (Liu et al., 2019, 2022; Luo et al., 2019). As the

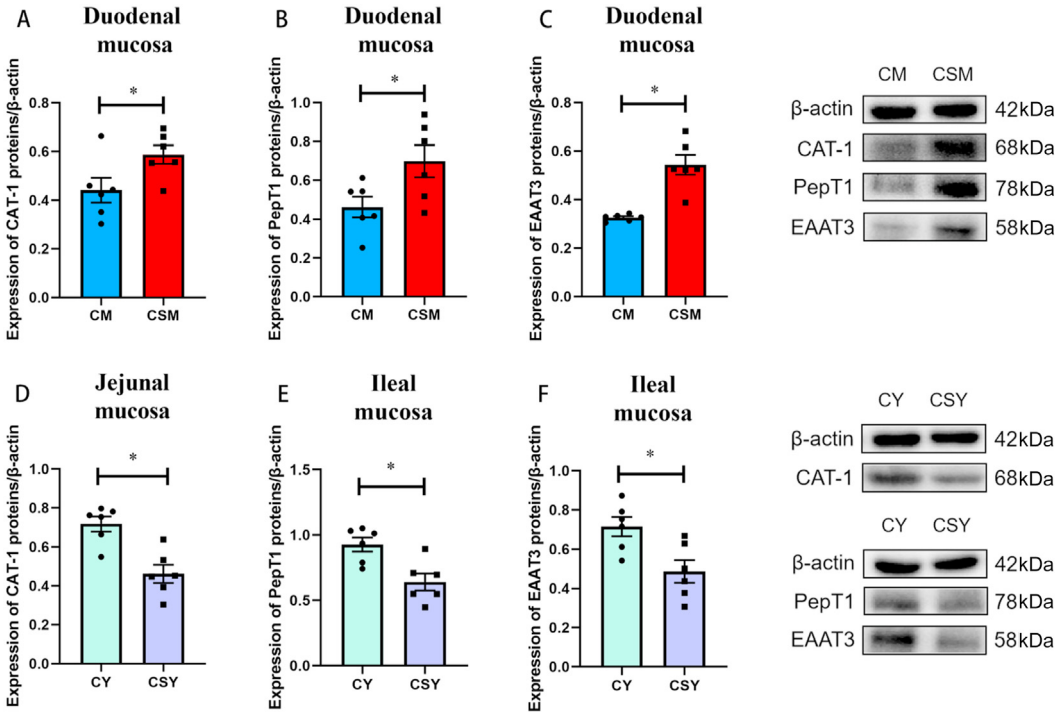

Fig. 4. Analysis of the relative expression of amino acid transporter proteins in small intestinal mucosa. (A-C) Cationic amino acid transporter-1 (CAT-1), peptide transporter protein 1 (PepT1) and excitatory amino acid transporter 3 (EAAT3) in duodenal mucosa of Min pig models. (D) CAT-1 in jejunal mucosa of Yorkshire pig models. (E and F) PepT1 and EAAT3 in ileum mucosa of Yorkshire pigs. Data are expressed as the mean ± SEM, $n = 6$, $^*P < 0.05$. CM and CY indicate the control groups; CSM indicates the cold stress group; and CSY indicates the cold exposure group.

building blocks of peptides and proteins, AA are necessary for all life-sustaining processes. Normal AA metabolism is an important process to sustain life (Keutgen and Pawelzik, 2008). AA transporters are responsible for the exchange and flow of free AA between tissues and blood. However, it is unclear how chronic cold exposure modulates small intestinal AA transporters and muscle AA pools, both during cold adaptation and during non-cold adaptation. In this research, firstly, we investigated the effects of long-term low temperature on the utilization of AA using Min pigs and Yorkshire pigs as models. In order to adapt to the cold, the initial body weight of Min pigs was different from that of the Yorkshire pigs, but they were both in the stage of rapid growth. The results showed that the ATTD of crude protein in the CSM group was enhanced, which was not observed in the CSY group. This suggested a possibility in the Min pig model that there exists a mechanism to improve the utilization of crude protein in the diet, although the growth rates of both Min pigs and Yorkshire pigs were limited by chronic cold exposure. Diet is the primary modality for mammals to obtain exogenous AA. Dietary protein is hydrolyzed by proteases and broken down into di- and tripeptides and free AA (Vranova et al., 2013; Gao et al., 2020). The small intestinal is the primary place for digestion and absorption of crude protein in animals (Liu et al., 2020), especially the duodenum and jejunum. This depends on trypsin in the intestinal lumen. Trypsin is a typical digestive enzyme that is secreted by the pancreas (Shen et al., 2013). Trypsin is produced by serine proteases and secreted in an inactive trypsinogen form by pancreatic acinar cells, and released into the intestinal lumen at the duodenum (Ferrari et al., 2021). We examined the effects of chronic cold stress on trypsin activity in duodenal and jejunal mucosa. The results showed that the trypsin activity in jejunal mucosa had a trend toward inhibition in the CSY group, suggesting that protein digestion ability was weakened. Studies have shown that chronic cold stress modulates intestinal inflammatory cell infiltration, intestinal development and mucosal barrier function (Liu et al., 2019; Sun et al., 2022). Thus, chronic cold exposure may dilute the ability for the small intestine to digest crude protein by inducing damage to the small intestine.

The absorption of exogenous AA in the intestine determines the metabolism or deposition of AA in extraintestinal tissues (Baker, 2009; Rezaei et al., 2013). This process relies on AA transporters and peptide transporters. AA transporters have distinctive substrate specificities (Prasad et al., 1999; Closs et al., 2006). Currently, 2 modes of AA transport are recognized. The first state is one AA is transported into the cell in exchange for the excretion of another AA (Verrey et al., 1999; Bode, 2001; Meier et al., 2002). The second pathway is to transport AA by coupling of Na^+, K^+, H^+ (Broer, 2002). The flow of AA in tissues and organs is regulated by AA transporters. They are actively involved in maintaining AA homeostasis. CAT-1 is a member of the sodium-independent cationic AA transporters that transport arginine specifically, and it also has a high affinity for lysine (Liao et al., 2008; Chafai et al., 2017; He et al., 2020). As an H^+ dependent peptide transport protein, PepT1 is responsible for the absorption of oligopeptides (mainly dipeptides and tripeptides) and peptide-derived substances (Hu et al., 2008; Xu et al., 2014). EAAT3 in the small intestine can transport free anionic AA, such as glutamate, and also mediates the uptake of cysteine (Chen and Swanson, 2003; Fan et al., 2004; Ye et al., 2016). We found that the mRNA and protein expression of CAT-1, PepT1 and EAAT3 in duodenal mucosa were upregulated in the CSM group. Moreover, PepT1 mRNA expression in ileal mucosa was also increased in the CSM group. These results indicated that the small intestine of Min pigs has a stronger ability to transport AA in the diet under chronic cold exposure. This was an important reason for the increase of ATTD of crude protein induced by low temperature. Previous studies have shown that the small intestine of animals exposed to low temperatures spontaneously increases absorption area to enhance the use of nutrients in diet (Chevalier et al., 2015). This may also be one of the reasons why Min pigs are better adapted to

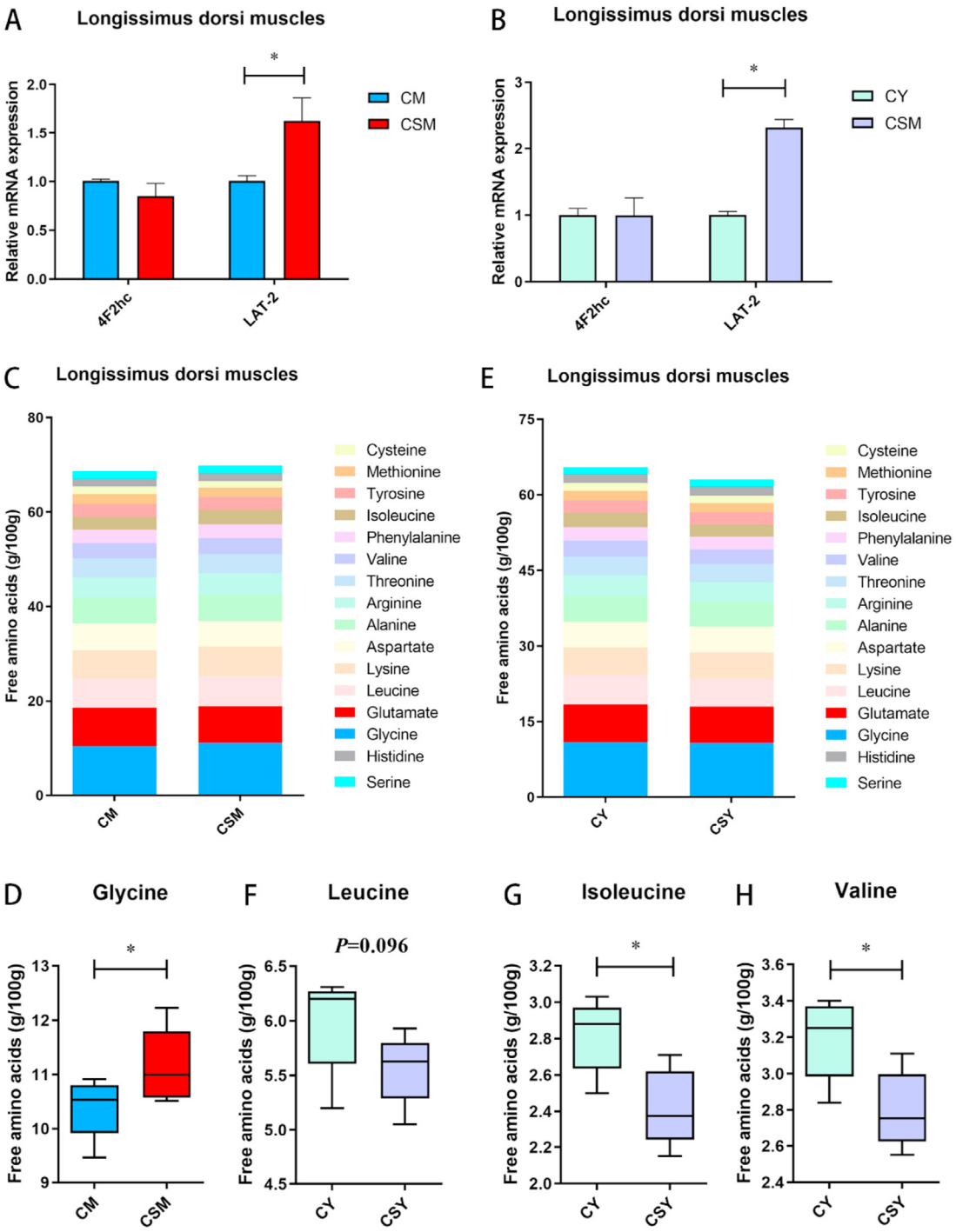

Fig. 5. Analysis of free amino acid composition and amino acid transporters in longissimus dorsi muscles. (A) Amino acid transporters in longissimus dorsi muscles of Min pig models. (B) Amino acid transporters in longissimus dorsi muscles of Yorkshire pig models. (C and D) Free amino acid composition of Min pig models. (E-H) Free amino acid composition of Yorkshire pig models. Data are expressed as the mean ± SEM, $n = 6$, $^*P < 0.05$. CM and CY indicate the control groups; CSM indicates the cold stress group; and CSY indicates the cold exposure group. LAT-2 indicates L-amino acid transporter-2; 4F2hc indicates 4F2 heavy chain.

cold and have higher intake of AA in the diet. However, of note, the results for AA transporters in the small intestine of Yorkshire pig were reversed. The mRNA and protein expression of CAT-1 in the jejunal mucosa of the CSY group were inhibited. Furthermore, the mRNA and protein expression of PepT1 and EAAT3 in the ileal mucosa in the CSY group were also inhibited. These data indicated that the absorption of exogenous free AA in Yorkshire pigs under chronic cold exposure was limited. Previous studies have shown that chronic cold stress induces intestinal damage (Su et al., 2018; Liu et al., 2019). AA transporters were restricted in Yorkshire pigs during chronic cold exposure, likely due to impaired intestinal development. However, additional evidence is required. There is plenty of evidence that energy deprivation can exacerbate intestinal and muscle injury (Steinberg and Kemp, 2009; Zhou et al., 2011). In this study, we attempted to increase the energy intake of pigs by adding glucose to the diet. We found that glucose

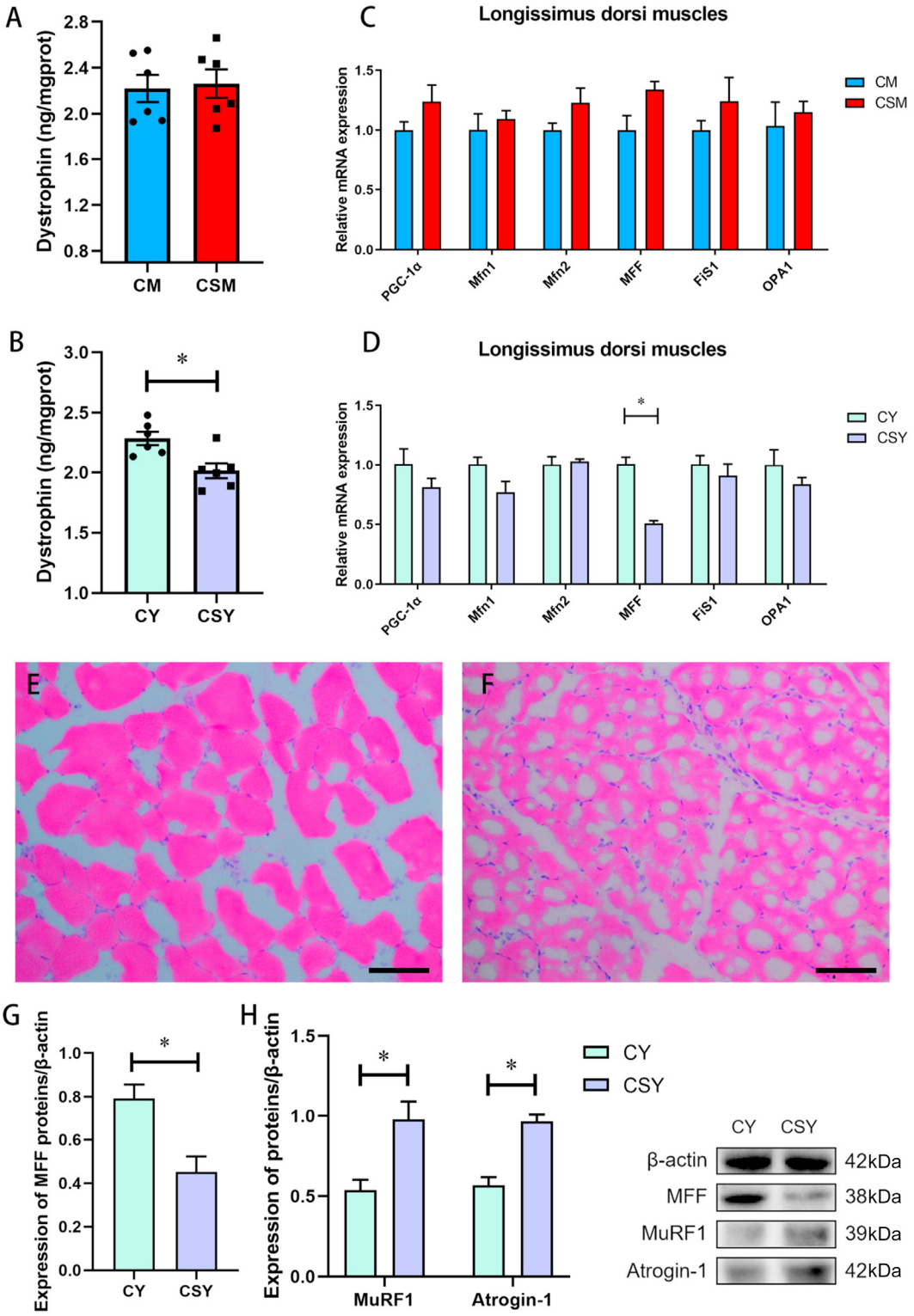

Fig. 6. Effects of chronic cold exposure on dystrophin, the expression of genes associated with mitochondrial dynamic balance and proteins that are key to protein degradation in longissimus dorsi muscles. (A) Dystrophin concentration in longissimus dorsi muscles of Min pig models ($n = 6$). (B) Dystrophin concentration in longissimus dorsi muscles of Yorkshire pig models ($n = 6$). (C) Expression of genes related to mitochondrial function in Min pig models ($n = 6$). (D) Expression of genes related to mitochondrial function in Yorkshire pig models ($n = 6$). (E and F) Muscle morphology of Yorkshire pig models (magnification, 200×; scale bar, 100 μm). (G) Protein expression level of MFF in longissimus dorsi muscles of Yorkshire pig models ($n = 4$). (H) Expression of protein degradation pathway in longissimus dorsi muscles of Yorkshire pig models ($n = 4$). Data are expressed as the mean ± SEM, $n = 6$, $^*P < 0.05$. CM and CY indicate the control groups; CSM indicates the cold stress group; and CSY indicates the cold exposure group. PGC-1α = PPARG coactivator 1 alpha; Mfn1 = mitofusin 1; Mfn2 = mitofusin 2; MFF = mitochondrial fission factor; OPA1 = mitochondrial dynamin like GTPase; Fis1 = mitochondrial fission protein 1.

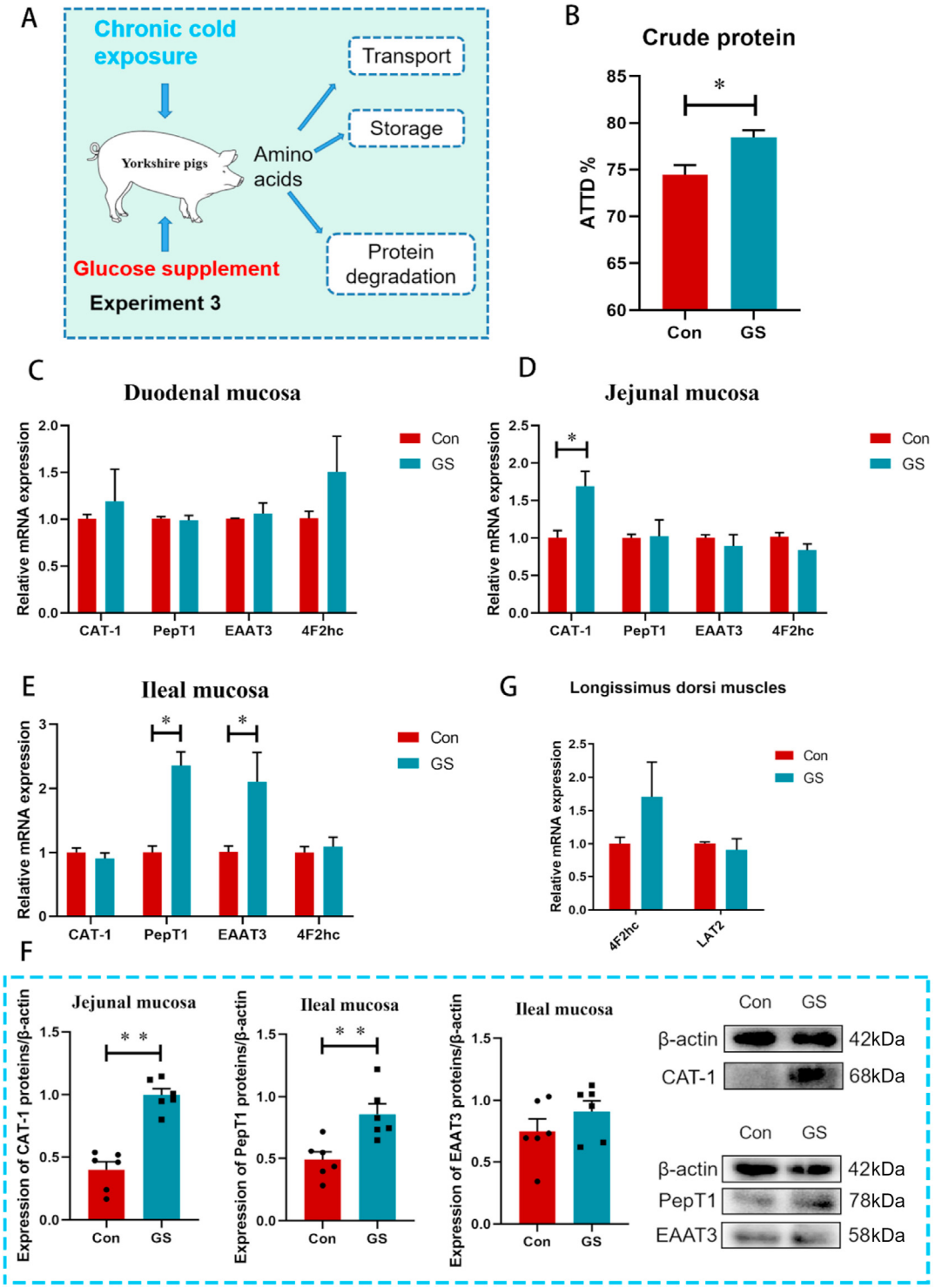

Fig. 7. Dietary glucose supplementation improved the apparent total tract digestibility (ATTD) of crude protein, the expression of amino acid transporters in small intestinal mucosa under chronic cold exposure. (A) Design of Exp. 3. (B) The ATTD of crude protein. (C) Amino acid transporters mRNA expression in duodenal mucosa. (D) Amino acid transporters mRNA expression in jejunal mucosa. (E) Amino acid transporters mRNA expression in ileal mucosa. (E) Protein expression in jejunal and ileal mucosa. (G) Amino acid transporters in the longissimus dorsal muscles. Data are expressed as the mean ± SEM, $n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$. Con = the control group, and GS = the glucose supplement group. CAT-1 = cationic amino acid transporter-1; PepT1 = peptide transporter protein 1; EAAT3 = excitatory amino acid transporter 3; 4F2hc = 4F2 heavy chain.

supplementation in the diet promoted the mRNA and protein expression of CAT-1 in jejunal mucosa and PepT1 in the ileal mucosa of a cold-exposure Yorkshire pig model. AA transport in the small intestine was improved. This may also be responsible for the increased ATTD of crude protein in the GS group.

The presence of proteinogenic AA in the diet is vital for muscle growth. Providing the AA needed to synthesize muscle protein requires an adequate intake of dietary protein. We characterized free AA in the longissimus dorsi muscle of Min pigs and Yorkshire pigs under chronic cold exposure. Interestingly, there were large

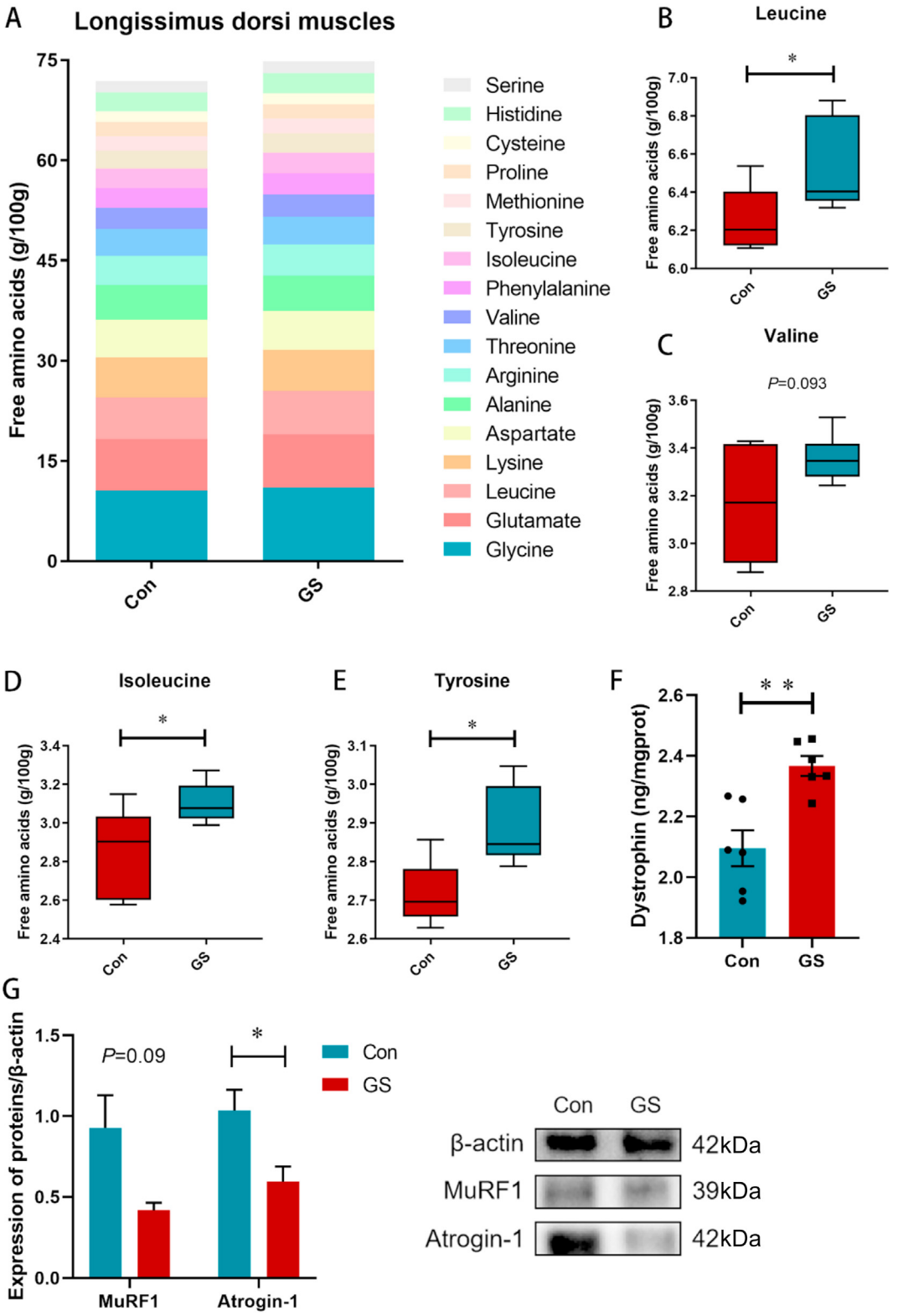

Fig. 8. Glucose supplementation improves AA pools and protein degradation in longissimus dorsi muscles of cold-exposed pigs. (A-E) Free amino acid composition ($n = 6$). (F) Dystrophin concentration in longissimus dorsi muscles ($n = 6$). (G) Expression of protein degradation pathway in longissimus dorsi muscles ($n = 4$). Data are expressed as the mean ± SEM. $^{*}P < 0.05$. $^{**}P < 0.01$. Con indicates the control group, and GS indicates the glucose supplement group. MuRF1 = muscle RING-finger protein-1; Atrogin-1 = atrophy F-box.

differences between Min pigs and Yorkshire pigs. Our data showed that the concentration of glycine was increased in the longissimus dorsi muscle of the CSM group. Glycine, as non-essential AA, is the precursor of glutathione biosynthesis. Glycine and serine are interconvertible via serine hydroxymethyl transferase (He et al., 2018; Zhou et al., 2018). During chronic cold exposure, glycine levels in the longissimus dorsi muscle increased, which was beneficial for enabling the antioxidant system to resist oxidative damage induced by chronic cold exposure. Conversely, the concentration of valine, leucine and isoleucine in the longissimus dorsi muscle of the CSY group decreased. These 3 essential AA form the BCAA family. Skeletal muscle is a major site of BCAA utilization. BCAA can be converted into energy directly and improve muscle function. Dietary supplementation of BCAA suppresses muscle loss (Magne et al., 2013; Wall and van Loon, 2013), probably due to upregulation of mTOR (Jewell et al., 2013; Jackman et al., 2017). Chronic cold exposure depleted BCAA in the longissimus dorsi muscle of Yorkshire pigs. This was perhaps because cold stress forced the breakdown of BCAA in muscle tissue to provide energy. The difference in free AA in the longissimus dorsi muscle between Min pigs and Yorkshire pigs under chronic cold exposure was of enormous interest to us. Therefore, we evaluated the expression of AA transporters in the longissimus dorsi muscle. Our results showed that the mRNA expression of LAT-2 in the longissimus dorsi muscle of Min pigs and Yorkshire pigs was enhanced by chronic cold exposure. LAT-2 is member of the neutral AA transporters, which are involved in the transport of BCAA (Fuchs and Bode, 2005; Wang and Holst, 2015). More neutral AA were absorbed into the muscle in both models driven by chronic cold exposure. Even so, the level of BCAA in the muscle of cold-exposed Yorkshire pigs was still reduced. These results demonstrated that chronic cold exposure depleted large amounts of BCAA in the longissimus dorsi muscle in a non-cold adaptation model. Notably, glucose supplementation elevated the level of BCAA in the longissimus dorsi muscle. In addition, tyrosine levels were also elevated. Apparently, the AA pools in muscle of cold-exposed pigs were improved.

Skeletal muscle is the protein pool of the body, in which 60% of proteins are stored within it in various forms. The regulation of skeletal muscle protein synthesis is aligned closely with systemic and energy status. Skeletal muscle protein is widely mobilized into AA during stress. The BCAA fulfil a unique role in skeletal muscle protein metabolism. Studies have shown that enhanced catabolism of BCAA is related to energy consumption and mitochondrial function (Butte et al., 2015). Chronic cold exposure induced consumption of BCAA. This observation evoked our curiosity about mitochondrial function in the longissimus dorsi muscle. Subsequently, we assessed the expression of genes related to mitochondrial fusion and fission in the longissimus dorsi muscle under chronic cold exposure. The balance of mitochondrial dynamics is related to the production of ATP, especially in muscles that require a vast amount of continuous energy (Rossman et al., 2020). Fusion and fission of mitochondria are crucial remodeling processes involved in the cellular adaptation of mitochondria (Pernas and Scorrano, 2016; Wai and Langer, 2016). MFF promotes mitochondrial fission via recruiting DRP1 (Otera et al., 2010). In this research, the expression of MFF in the longissimus dorsi muscle in the CSY group was down-regulated by chronic cold exposure. This indicated that mitochondrial fission might be inhibited in Yorkshire pigs during chronic cold exposure, although PGC-1α expression was not altered. Mitochondrial dysfunction caused by the lack of fission can reduce ATP levels in cells and inhibit cell proliferation (Parone et al., 2008). Chronic cold exposure promoted the likelihood of inhibition of muscle cell proliferation in the Yorkshire pig model.

Skeletal muscle development depends on the balance between protein synthesis and degradation (Bodine and Baehr, 2014). During fasting, muscular dystrophy and other states, the ubiquitin-proteasome pathway is enhanced to accelerate protein degradation (Baptista et al., 2010; Hernandez-Garcia et al., 2016). It is the major machinery responsible for protein degradation in eukaryotic cells (Hochstrasser, 2009). Here, we assessed the expression of key proteins in the ubiquitin-proteasome pathway in the longissimus dorsi muscle of a Yorkshire pig model under chronic cold exposure. The results showed that chronic cold exposure enhanced the expression of atrogin-1 and MuRF-1 in the longissimus dorsi muscle of a Yorkshire pig model. Obviously, the activation of these 2 systems is not conducive to the deposition of muscle protein. We also investigated the effect of dietary glucose supplementation on

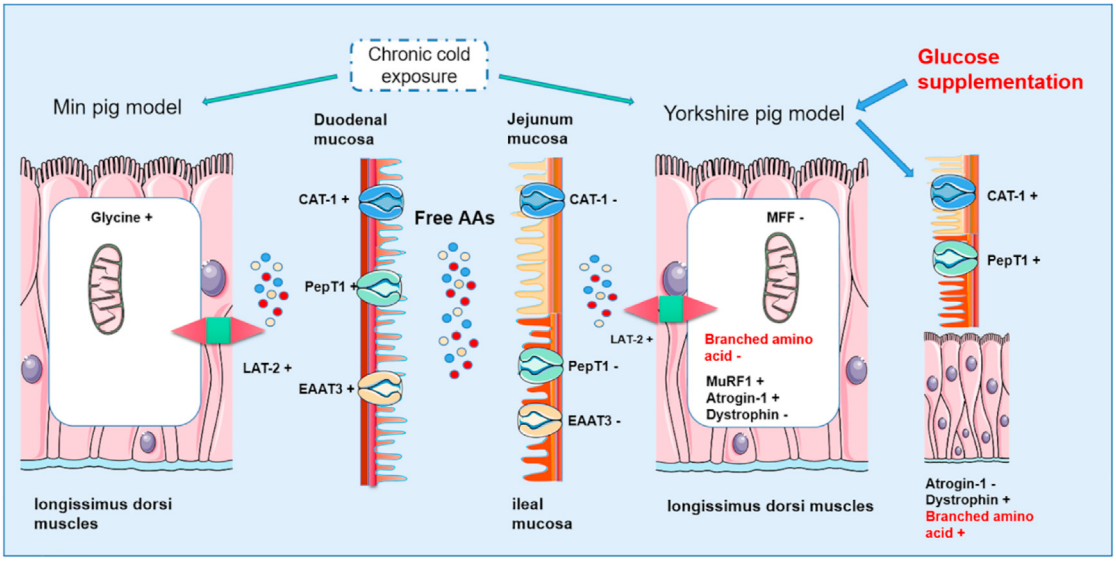

Fig. 9. Effects of chronic cold exposure on amino acid transport and muscle development in small intestine of Min and Yorkshire pigs, and the relieving effect of dietary glucose supplementation. AAs = amino acids; CAT-1 = cationic amino acid transporter-1; PepT1 = peptide transporter protein 1; EAAT3 = excitatory amino acid transporter 3; LAT-2 = L-amino acid transporter-2; MFF = mitochondrial fission factor; MuRF1 = muscle RING-finger protein-1; Atrogin-1 = atrophy F-box. (+) Increased. (−) Decreased.

protein degradation pathways in the longissimus dorsi muscle of cold-exposed pigs. The results showed that protein degradation was inhibited in the GS group, accompanied by down-regulation of protein expression of atrogin-1 and MuRF-1. This facilitated protein deposition in muscle under chronic cold exposure. Furthermore, with increased protein degradation, we found that the concentration of dystrophin in the longissimus dorsi muscle was decreased in the CSY group. Impaired structural integrity in the skeletal muscle leads to reduced cell size and contractile capacity and different myopathies in mammals (Bonaldo and Sandri, 2013). Weakness and loss of muscle function is induced by the lack of dystrophin in muscle (Godin et al., 2012). The decrease in dystrophin concentration induced by chronic cold exposure is detrimental to the muscles of Yorkshire pigs. Myoblast differentiation antigen (MyoD1) and myogenic factor 5 (Myf5) are members of the myogenic regulatory factor family and play central roles in development of muscle (Mohammadabadi et al., 2021) and they determine the fate of myoblasts. MyoD1 is also regarded as an important marker of muscle cell proliferation and differentiation, and its expression level regulates the growth rate muscle (Beilharz et al., 1992). Myf5 also regulates muscle differentiation and myocyte proliferation (Timmons et al., 2007). Studies have shown that inhibition of MyF5 results in reduced generation of skeletal muscle (Plank et al., 2014). In our study, we found that both MyoD1 and Myf5 expression were reduced in the longissimus dorsi muscle of Yorkshire pigs under chronic cold exposure, which might restrict muscle development. Combined with the above evidence, our study suggested that chronic cold exposure was detrimental to muscle development in Yorkshire pig models.

5. Conclusion

In summary, chronic cold exposure promotes AA transport in the small intestine and muscle to actively adapt to low temperature in Min pig models with better cold adaptation. Moreover, glucose supplementation ameliorated the disturbance of small intestinal AA transport and the destruction of muscle AA pools (mainly BCAAs) induced by chronic cold exposure in poorly cold-acclimated Yorkshire pigs (Fig. 9). Our findings provide new evidence for the adverse effects of chronic cold exposure in pigs and suggest a strategy for dietary glucose supplementation.

Author contributions

Teng Teng: Data curation, formal analysis, investigation, methodology, software and writing - original draft. **Xin Song:** Investigation, formal analysis, data curation, supervision and validation. **Guodong Sun**: Investigation, software and visualization. **Hongwei Ding**: Methodology and software. **Haoyang Sun**: Validation. **Guangdong Bai**: Visualization. **Baoming Shi**: Conceptualization, funding acquisition, project administration, resources, supervision, writing - review and editing.

Declaration of competing interest

No conflict of interest exits in the submission of this manuscript. I (Baoming Shi) would like to declare on behalf of my co-authors (Teng Teng, Guodong Sun, Hongwei Ding, Xin Song, Haoyang Sun and Guangdong Bai) that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.10.009.

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