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Effects of protein phosphorylation on glycolysis through the regulation of enzyme activity in ovine muscle

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ABSTRACT

To verify the effect of protein phosphorylation on glycolysis and elucidate the regulatory mechanism from the perspective of enzyme activity, ovine muscle was treated with a kinase inhibitor, dimethyl sulfoxide, or a phosphatase inhibitor and the activities of glycogen phosphorylase, pyruvate kinase and phosphofructokinase were determined. The protein phosphorylation level was significantly different after incubation of muscle with kinase or phosphatase inhibitors. The pH value and lactate content revealed that a high phosphorylation level was the reason for the fast glycolysis. The glycogen phosphorylase, pyruvate kinase and phosphofructokinase activities were significantly higher in the phosphatase inhibitor group than in the other two groups ($p < 0.05$). Therefore, protein phosphorylation is involved in activating these three enzymes. In summary, protein phosphorylation plays a role in post-mortem glycolysis through the regulation of enzyme activity in ovine muscle.

1. Introduction

The biochemical changes referred to as post-mortem metabolism occurring in muscle after slaughter are very important parts of the conversion of muscle to meat (Paredi, Raboni, Bendixen, de Almeida, & Mozzarelli, 2012). Generally, they involve glycogenolysis converting glycogen into glucose and glycolysis converting glucose into lactate and H^+, with a drop in pH. Rapid and excessive glycolysis leads to pale, soft and exudative (PSE) meat, while insufficient glycolysis results in dark, firm and dry (DFD) meat (Immonen & Puolanne, 2000; Shen et al., 2006). Very minute differences in the ultimate pH and rates of pH decline cause great differences in meat properties. The underlying mechanisms are still not fully understood, but pH, and therefore carbohydrate metabolism, is always of key importance (Pösö & Puolanne, 2005).

Post-translational modifications play a regulatory role in protein structure, function, cell signalling and enzyme activity. Protein phosphorylation, one of the most frequent post translational modifications, is a key modulator in glycolysis metabolism. Most research on protein phosphorylation has mainly focused on the fields of medicine, physical chemistry and biochemistry, but in recent years it has intrigued scientists due to its regulatory role in meat quality during the post-mortem period. It was confirmed that protein phosphorylation of glycolytic enzymes explains some of the differences in meat tenderness (Li et al., 2017). Consistently, protein phosphorylation has been found to be involved in meat colour development (Li et al., 2018). However, the regulatory pathways are not clear, and it was speculated that protein phosphorylation may influence the meat rigor mortis and quality development by regulating glycolysis (Chen et al., 2018; Huang et al., 2011).

Glycolysis is a sequence of ten enzyme-catalysed reactions. Most glycolytic enzymes are phosphoproteins, including the three rate-limiting enzymes, hexokinase, phosphofructokinase and pyruvate kinase. Phosphofructokinase is activated after phosphorylation by AMP-activated protein kinase (AMPK), to upregulate glycolysis and maintain intracellular ATP homeostasis in the ischaemic heart (Marsin et al., 2000). Pak1-mediated phosphorylation of phosphoglycerate mutase at threonine 466 significantly increases its enzyme activity (Gururaj, Barnes, Vadlamudi, & Kumar, 2004). However, these studies have not been carried out in post-mortem muscle, as differences may exist in the machinery regulating glycolysis ante-mortem and post-mortem. During the post-mortem time under anaerobic conditions, Shen et al. (2006) also found that AMPK regulated glycolysis in post-mortem muscle at least partially through phosphorylation. Lametsch et al. (2011)

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indicated that the pH decline of the RN genotype could be a consequence of phosphorylation of glycolytic enzymes during the post-mortem metabolism. However, the activities of related enzymes were not detected and the changes in enzyme activity after phosphorylation were of less concern.

To verify the effect of protein phosphorylation on glycolysis and elucidate the regulatory mechanisms from the perspective of enzyme activity, we profiled protein phosphorylation in post-mortem muscle with kinase/phosphatase inhibitors and the activities of glycogen phosphorylase, pyruvate kinase and phosphofructokinase were determined. We hypothesise that protein phosphorylation may alter the activities of glycolytic enzymes, thus regulating the enzymatic reaction of glycolysis. This research broadens our knowledge about the regulation of protein phosphorylation in post-mortem muscle.

2. Materials and methods

2.1. Sample treatment

Six male sheep (Mongolian sheep × Small Tail Han Sheep, carcass weight 23.4 ± 1.09 kg) were fed with the same diet and conventionally Halal slaughtered by severing the trachea, carotid arteries, and oesophagus (with no stunning) in a local commercial abattoir slaughter plant in Inner Mongolia, China. Longissimus thoracis muscle from both sides of the carcasses were removed immediately (more than 500 g), trimming off visible fat and connective tissue, and then cut into pieces of approximately 1 cm × 1 cm. The samples at 0.5 h were collected directly and the remaining muscle pieces were divided into three sub-groups (120 g per sub-group). Three different reagents were added into each sub-group, mixed and crushed with a bowl chopper (QSJ-B02X5 0.6L; Bear Electrical Appliance Co., Ltd.). The three treatment sub-groups were as follows: (1) the dimethyl sulfoxide (DMSO) control group, where 4 mL of 0.6675% DMSO was added to the muscle samples (Li et al., 2017); (2) the kinase inhibitor group, where a kinase inhibitor (P0300, Sigma, 111.12 μg/mL) was added to the muscle samples to inhibit protein phosphorylation; and (3) the phosphatase inhibitor group, where a phosphatase inhibitor (PhosStop Roche, Mannheim, Germany, one tablet per 5 g muscle, dissolved in DMSO) was added to the muscle samples to inhibit protein dephosphorylation. The chopped meat of the three sub-groups was stored in disposable Petri dishes at 4 °C for collection at 2, 6, 12, 24 and 48 h post-mortem (PM). The pH values were measured with a Testo205 pH meter (Lenzkirch, Germany) and the samples were then snap-frozen in liquid nitrogen.

2.2. Glycogen measurement

The glycogen content was measured using a glycogen kit (A043; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Generally, 70 mg muscle were added to 210 μL NaOH and boiled for 20 min covered with a perforated membrane. After cooling with flowing water, 1.12 mL of ultrapure water was added to the samples. The glycogen content was determined at 620 nm by spectrophotometry (UV-1800; Suzhou Shimadzu Instrument Co., Ltd., China).

2.3. Lactate measurement

The lactate was determined using a lactate kit (A019-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). One gram of muscle was homogenised in 9 mL ice-cold saline for 3 × 15 s on ice (with intermediate cooling for 30 s), and then centrifuged at 3000*g*, 4 °C for 10 min. The supernatant was used for lactate measurements according to the manufacturer's instructions at a wavelength of 530 nm.

2.4. Protein extraction

Sarcoplasmic proteins were extracted as previously described (Chen et al., 2018). Generally, 1.2 g of muscle (0.2 g per sample) were added to 7.2 mL of extraction buffer (0.6057 g Tris, 0.0771 g DTT, one tablet complete protease inhibitor, 5 tablets phosphatase inhibitor PhosStop, pH 8.3; volume brought to 50 mL with ultrapure water) and homogenised for 3 × 10 s on ice with 15 s cooling between bursts using an IKA Werke GmbH & Co. KG (Staufen, Germany). After centrifuging (10,000*g*, 4 °C, 30 min), the supernatant containing the sarcoplasmic proteins was collected.

2.5. Gel electrophoresis and protein phosphorylation analysis

Proteins were separated by electrophoresis as described by Huang (Huang et al., 2011). The sample collected at 0.5 h PM was loaded onto each gel as a reference for the densitometric analysis. After electrophoresis, the gel was stained with Pro-Q Diamond dye (Invitrogen, Eugene, OR) and SYPRO Ruby dye (Invitrogen, Eugene, OR), to visualise the phosphoproteins (P) and total proteins (T). The P/T ratio was used to calculate the phosphorylation levels. The intensity of the protein bands was analysed using Quantity One software (version 4.6.2; Bio-Rad, Hercules, CA).

2.6. Protein identification

Protein bands on gels stained with colloidal Coomassie Brilliant Blue R-250 were excised (in duplicate) and washed twice in ultrapure water for 10 min. The following preparation steps were as described previously (Li et al., 2018). Peptides were identified by LC–MS/MS (Thermo Q-Exactive; Thermo Fisher Scientific, Waltham, MA) as described in the literature (Li, Li, Gao, Du, & Zhang, 2017). The raw spectral data from the analyser were extracted as peak lists using Thermo Proteome Discoverer 1.3.0.339 (Thermo Scientific). The peak lists were matched against the UniProt database by MASCOT (version 2.3.01).

2.7. Enzyme activity analysis

2.7.1. Glycogen phosphorylase (GP)

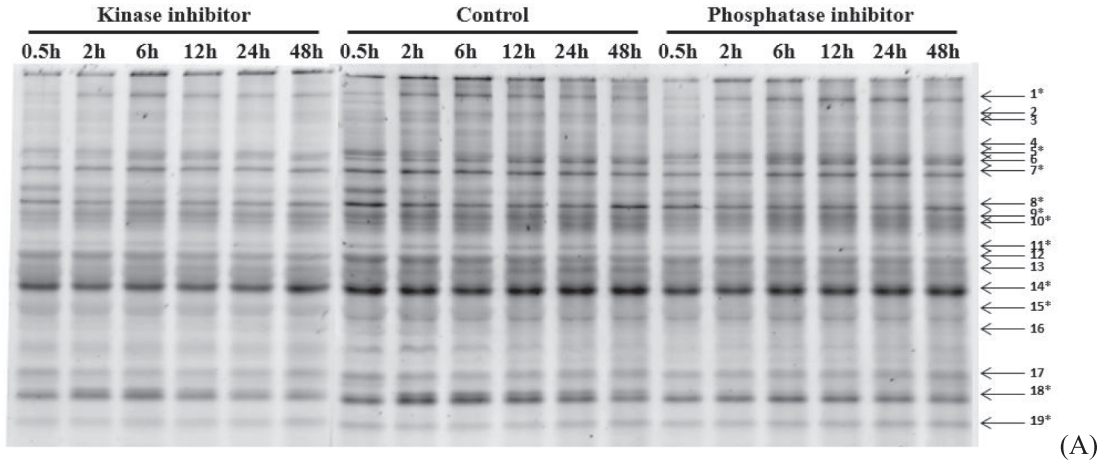
The activities of GP were measured using a commercial kit (GPA-2-Y; Suzhou Keming Biotechnology Co., Ltd., China) according to the manufacturer's instructions. Generally, 0.1 g of ovine muscle in 1 mL of extracting solution were homogenised (IKA Werke GmbH & Co. KG, Staufen, Germany) on ice for 3 × 10 s, with intermediate cooling on ice for 30 s. The homogenate was centrifuged at 8000*g*, 4 °C for 10 min and the supernatant was aliquoted for analysis. The activities of the GP were calculated according to the protein concentration (BCA assay; Pierce Chemical Company, Rockford, IL) of the sample.

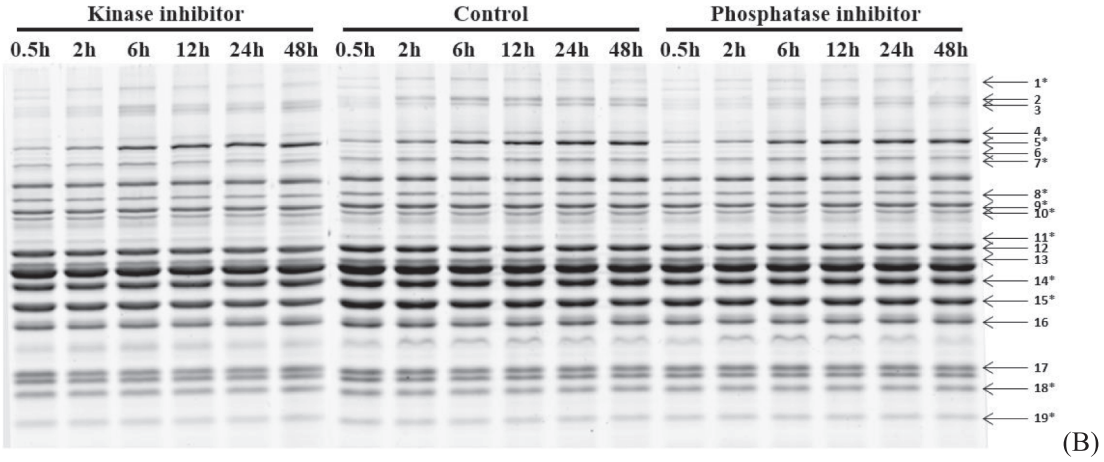
2.7.2. Pyruvate kinase (PK)

The activities of PK were determined using a pyruvate kinase activity assay kit (A076-1; Nanjing Jiancheng Bioengineering Institute, China). Generally, 0.1 g of ovine muscle were rapidly homogenised in 0.9 mL of saline and then centrifuged at 2500 rpm for 10 min to remove insoluble materials. The supernatant (50 μL) was used for the test. The detection wavelength was 340 nm.

2.7.3. Phosphofructokinase (PFK)

The activities of PFK were measured using a phosphofructokinase test kit (A129; Nanjing Jiancheng Bioengineering Institute, China). Generally, 0.1 g of ovine muscle were homogenised on ice in 0.9 mL of ice-cold phosphofructokinase assay buffer and then centrifuged at 4 °C, 8000*g* for 10 min to remove insoluble materials. The activities of the phosphofructokinase were determined spectrophotometrically at 340 nm.





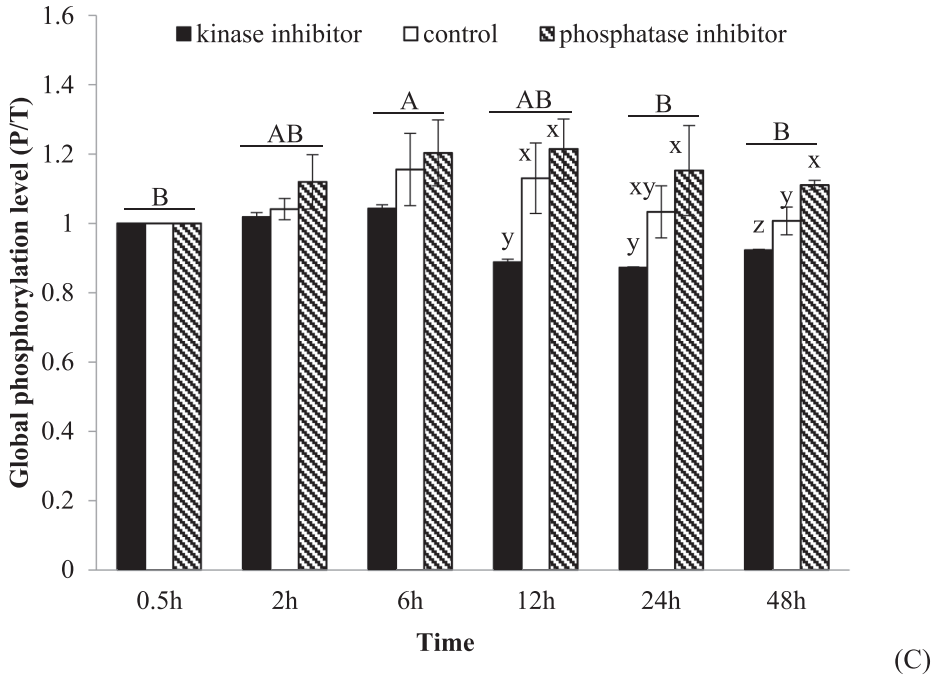


Fig. 1. Gel-base analysis and phosphorylation level of sarcoplasmic protein extracted from ovine Longissimus thoracis. (A) Images of gels stained with Pro-Q Diamond to visualize phosphoproteins (P). (B) Images of gels stained with SYPRO Ruby to visualize total proteins (T). P/T abbreviation means the ratio of phosphoproteins to total proteins, representing the phosphorylation level. Bands marked with * were selected for protein identification by LC–MS/MS. (C) Global phosphorylation level of sarcoplasmic protein. Different letters (x, y, z) at the same time points represent significant difference among the three groups ($p < 0.05$). Different letters (A, B) in the same group represent significant differences at different PM times ($p < 0.05$).

2.8. Statistical analysis

The data were analysed using SAS statistical software (version 9.2). The effect of the treatment and time and their interactions with pH, lactic acid and phosphorylation level were analysed with general linear models (GLM). Tukey's test ($p < 0.05$) was conducted if the means were statistically different. The correlation of all measurements was analysed *via* Pearson's correlation with PM time. All data are expressed as the mean ± SD (standard deviation).

3. Results and discussion

3.1. Global phosphorylation of sarcoplasmic protein

The global phosphorylation level of the sarcoplasmic protein was evaluated by densitometry analysis of phosphoproteins and total proteins on gels after staining (Fig. 1). The global phosphorylation level of the sarcoplasmic protein increased early PM and then decreased ($p < 0.05$). These results are consistent with Huang's data about protein phosphorylation in muscle with intermediate pH decline rates (Huang et al., 2011). After incubation for 12 h, the phosphorylation level of the kinase inhibitor group was significantly lower than in the other two groups, and the phosphorylation level of the phosphatase inhibitor group was significantly higher at 48 h PM compared to the control group ($p < 0.05$).

Protein phosphorylation and dephosphorylation play diverse roles in cellular regulation and signalling, which are regulated by the competing activities of protein kinases and phosphatases (Manning, Whyte, Martinez, Hunter, & Sudarsanam, 2002). Protein kinases catalyse the transfer of phosphate groups from ATP to molecules, whereas phosphatases remove phosphate groups from molecules. P0300 as a kinase inhibitor is a synthetic peptide used for studying the cAMP-dependent protein kinase, inhibiting this protein kinase competitively. PhosStop Roche used as a phosphatase inhibitor has a broad spectrum of action against phosphatases, inhibiting protein phosphatases competitively. The samples were "intact" meat, which involves a series of physico-biochemical changes. The inhibitors only show significant difference after 12 h of incubation, which may be because they need time to enter the muscle cell to function. The significant difference among the three groups revealed that the inhibitors were effective in modulating the level of protein phosphorylation.

3.2. Glycolytic rate

The pH value and lactic acid content of the three groups are shown in Fig. 2. Generally, the ovine muscle pH decreased dramatically within the first 12 h, but remained stable afterwards. Meanwhile, the lactic acid corresponds well with the pH value, which increased within the first 12 h but remained stable afterwards. As to the three groups, the pH values in the phosphatase inhibitor group were significantly lower at 2 h and 6 h than those in the control group, and the lactic acid in the phosphatase inhibitor group was significantly higher than that in the kinase inhibitor group during the PM time except at 6 h ($p < 0.05$). There was no significant difference in pH values and lactic acid between the control and kinase inhibitor groups throughout the whole PM period.

Pearson correlation coefficients of all indicators detected in this study are shown in Table 1, from which we can see that the pH value was negatively correlated with lactate during the PM time. Lactate is the ultimate product of anaerobic metabolism and with the formation of lactate, one hydrogen from NADH and one hydrogen from solution are removed from the cytoplasm (Ferguson & Gerrard, 2014). Ferguson claimed there was utility in lactate formation for extending anaerobic muscle metabolism and suggested that lactate accumulation is a good indicator of the extent and rate of glycolysis.

Ovine muscle in the phosphatase inhibitor group with a high phosphorylation level had higher lactate and a lower pH value compared to the control group. Li et al. (2017) reported that the muscle of the kinase inhibitor group with a low phosphorylation level had lower lactate and a higher pH value compared to the control group. It is logical to conclude that a high protein phosphorylation is one of the reasons for the fast decline in the pH value.

3.3. Gel band identification

Nineteen protein bands were detected on the gels, among which 11 protein bands that significantly differed in phosphorylation levels were excised and identified (Table 2). In total, 12 unique proteins were identified and most of them were clustered into the pathways of glycogenolysis and glycolysis. They were PFK, filamin C, GP, phosphoglucomutase 1, PK, glucose-6-phosphate isomerase, fructose-bisphosphate aldolase, enolase 1, enolase 3, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase and adenylate kinase isoenzyme 1. Among these enzymes, PFK and PK are rate-limiting enzymes in glycolysis, while GP catalyses the rate-limiting step in glycogenolysis. With an attempt to explain the glycolysis, the properties of glycogen phosphorylase, phosphofructokinase, and pyruvate kinase have been studied (Scheffler & Gerrard, 2007). Therefore, these enzymes should be given more attention.

3.4. The activity of glycolytic enzymes

3.4.1. Glycogen phosphorylase

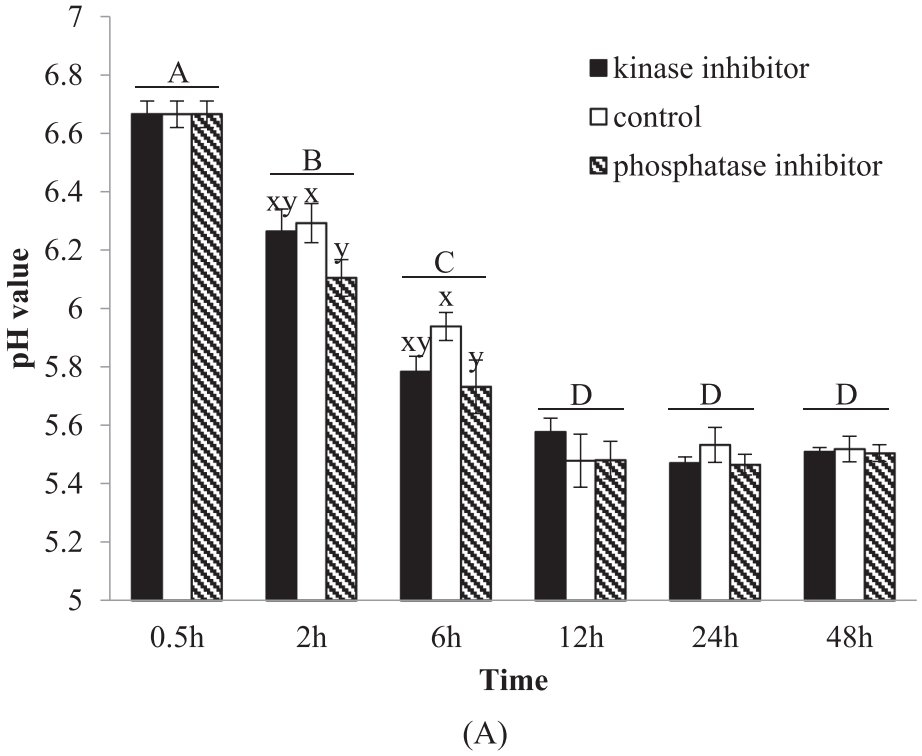
The phosphorylation level and activity of GP are shown in Table 3. The activity of GP was basically stable during the PM time. As for the three groups, the activity of GP in the phosphatase inhibitor group was significantly higher than in the other two groups ($p < 0.05$). The phosphorylation level of GP in the phosphatase inhibitor group at 2 h PM was significantly higher than that in the control group, which was not obtained at the other PM times ($p < 0.05$). The correlation analysis in Table 2 shows that the activity of GP showed a positive correlation with the phosphorylation level of GP during the PM time.

GP catalyses the breakdown of glycogen to glucose-1-phosphate for glycolysis and plays a key role in controlling glycogenolysis (Aizawa et al., 2017). GP exists in phosphorylated GP a and dephosphorylated GP b forms. When GP b is phosphorylated at serine 14, it changes its structure, transforming it into the active GP a form, which represents the first step in the activation of the enzyme (Johnson, 1992; Schwägele, Buesa, & Honikel, 1996; Sprang et al., 1988). The change in structure involves the amino- and carboxyl-terminal domains of GP rotating apart by 5°, which increases the access of substrates to the catalytic site (Sprang, 1991). The activity of GP was significantly higher in the phosphatase inhibitor group, theoretically, more GP a forms and a higher phosphorylation level should occur in the phosphatase inhibitor group. Although there is no statistical difference, there is numerical difference between the control and phosphatase inhibitor groups. The increased activity of GP further accelerates the rate of glycogenolysis, resulting in a low glycogen content and pH value. Therefore, phosphorylation of GP is important in the regulation of glycolysis.

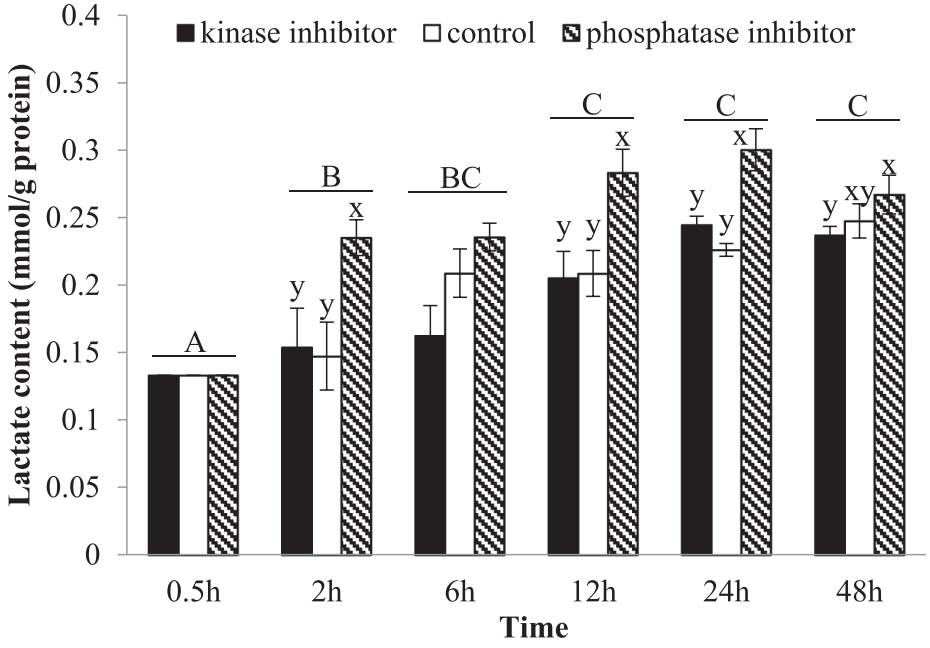
3.4.2. Pyruvate kinase

The phosphorylation level and the activity of PK are shown in Table 3. There was no significant difference in phosphorylation level among the three groups except at 12 h PM, where the phosphorylation level of PK in the phosphatase inhibitor group was significantly higher than that in the control group ($p < 0.05$). As for the enzymatic activity, there was a significant difference among the three groups from 6 h onwards, with the activity in the phosphatase inhibitor group being significantly higher than in the other two groups ($p < 0.05$).

PK is a critical rate-limiting enzyme that catalyses the irreversible conversion of phosphoenolpyruvate to pyruvate (Gupta & Bamezai,



(A)



(B)

Fig. 2. pH value of ovine Longissimus thoracis (A). Lactate content of ovine Longissimus thoracis (B). Different letters (x, y) at the same time point represent significant difference among the three groups ($p < 0.05$). Different letters (A, B, C, D) in the same group represent significant differences at different PM times ($p < 0.05$).

Table 1
Pearson correlation coefficients of glycolytic rate, glycolytic enzymes phosphorylation level and activities.

	Glycogen	GP activity	GP p-level	PK activity	PK p-level	PFK activity	PFK p-level	Global p-level	pH	lactate
Glycogen	1									
GP activity	**−0.5270**	1								
	0.0246									
GP p-level	0.1006	**0.6326**	1							
	0.6912	**0.0048**								
PK activity	−0.0577	**0.7325**	**0.6190**	1						
	0.8200	**0.0005**	**0.0062**							
PK p-level	−0.4174	**0.5842**	0.1543	0.2837	1					
	0.0848	**0.0109**	0.5409	0.2540						
PFK activity	−0.2105	**0.7592**	**0.5528**	**0.7711**	0.3872	1				
	0.4019	**0.0003**	**0.0174**	**0.0002**	0.1124					
PFK p-level	−0.3801	**0.7709**	**0.5605**	**0.5938**	**0.4918**	**0.5030**	1			
	0.1197	**0.0002**	**0.0155**	**0.0094**	**0.0382**	**0.0333**				
Global p-level	**−0.4960**	**0.5884**	0.2009	0.3455	**0.4919**	0.2336	**0.6220**	1		
	0.0363	**0.0102**	0.4241	0.1603	**0.0381**	0.3510	**0.0060**			
pH	**0.7199**	0.0352	**0.5842**	0.4599	−0.3325	0.1623	0.0353	−0.137	1	
	0.0008	0.8896	**0.0109**	0.0548	0.1776	0.5200	0.8895	0.5878		
lactate	**−0.9123**	0.3638	−0.1715	−0.1046	**0.4838**	0.0682	0.3472	0.3682	**−0.848**	1
	< 0.0001	0.1378	0.4962	0.6797	**0.0419**	0.7881	0.1580	0.1327	**< 0.0001**	

Abbreviations: GP, glycogen phosphorylase; PK, pyruvate kinase; PFK, phosphofructokinase; p-level, phosphorylation level.
The ovine Longissimus thoracis were crushed and incubated at 4 °C, and then snap frozen in liquid nitrogen after collection at different times.

Table 2
Protein identified from the 11 protein bands on gels.

No.	Accession no[a]	Protein name	Mass[b]	Score[c]	Matches[d]	Sequence[e]
Band 1	W5NZK9	Filamin C	278,503	5152	282(1 6 8)	59(45)
Band 5	O18751	Glycogen phosphorylase	97,702	1994	188(86)	38(25)
Band 7	W5QDD4	ATP-dependent 6-phosphofructokinase	95,200	1874	123(73)	21(16)
Band 8	W5PJB6	Phosphoglucomutase 1	65,544	3401	188(1 2 5)	24(21)
Band 9	W5QC41	Pyruvate kinase	62,180	3084	164(1 1 4)	22(19)
Band 10	W5P323	Glucose-6-phosphate isomerase	63,079	3466	220(1 5 1)	16(13)
Band 11	W5PIG6	Enolase 1	49,727	1096	86(52)	15(11)
	W5P663	Enolase 3	47,382	1006	86(44)	14(11)
Band 14	W5P1X9	Fructose-bisphosphate aldolase	39,925	1855	122(69)	20(18)
Band 15	W5PDG3	Glyceraldehyde-3-phosphate dehydrogenase	36,241	1502	117(51)	14(9)
Band 18	W5P5W9	Triosephosphate isomerase	23,021	2637	116(89)	10(10)
Band 19	C5IJA8	Adenylate kinase isoenzyme 1	21,750	2140	142(99)	11(8)

The ovine Longissimus thoracis were crushed and incubated at 4 °C, and then snap frozen in liquid nitrogen after collection at different times. The 11 bands selected were significantly different in phosphorylation level among the three groups.
a Accession numbers were derived from the UniProt database.
b Theoretical molecular weight (recorded in UniProt database).
c For the proteins identified in more than one band, the highest score was presented.
d Number of matched peptides, total matched peptides (credible matched peptides).
e Number of matched amino acid sequence, total matched sequence (credible matched sequence).

2010). It has been reported that PK has two isoforms in muscle: isoform 2 arises from isoform 1 through phosphorylation (Schwägele et al., 1996). Phosphorylation causes pyruvate kinase to retain higher activity under acidic conditions (Schwägele et al., 1996). Heiden et al. (2010) reported that PK was found to be more active after phosphorylation of the enzyme. In the phosphatase inhibitor group, the phosphorylation level and the activity had the same trends with PM time. Of the three groups, phosphatase inhibitor group has relatively higher phosphorylation level and activity. Since protein phosphorylation alters the structure, activity and stability of the protein, it is likely that phosphorylation of PK has a positive effect on the activity of PK.

3.4.3. Phosphofructokinase

The phosphorylation level and activity of PFK are shown in Table 3. There was no significant difference in phosphorylation level between the kinase inhibitor and control groups PM. After 24 h incubation, the phosphorylation level of PFK in the phosphatase inhibitor group was significantly higher than that in the control group ($p < 0.05$). The activities of PFK were significantly higher in the phosphatase inhibitor group at 6 h and 12 h than in the other two groups ($p < 0.05$). The activity of PFK was positively correlated with the phosphorylation level (Table 2).

PFK catalyses the conversion of fructose-6-phosphate and ATP to fructose-1,6-bisphosphate and ADP, the first committed step in the glycolytic pathway. Phosphorylation of PFK alters the kinetic behaviour of the enzyme and regulates the compartmentalisation of the enzyme *in vitro* and *in vivo* (Luther & Lee, 1986). Some studies have demonstrated that PFK is activated after phosphorylation by AMPK, an AMP-activated protein kinase, promoting glycolysis (Marsin et al., 2000). The kinase inhibitor P0300 used in the present research is a specific kinase inhibitor, and inhibits the cAMP-dependent protein kinase, specially protein kinase A, contributing no significant difference in PFK phosphorylation level between the kinase inhibitor and control groups. AMPK can phosphorylate phosphofructokinase 2, which catalyses the formation of fructose-2,6-bisphosphate. This product is an allosteric activator of PFK-1, a key rate-limiting enzyme of glycolysis (Scheffler &

Table 3
Phosphorylation level and activity of glycogen phosphorylase, pyruvate kinase and phosphofructokinase in ovine Longissimus thoracis.

		Phosphorylation level (P/T)				
		0.5 h	2 h	6 h	12 h	24 h
Glycogen phosphorylase	kinase inhibitor	1.75 ± 0.38	1.16 ± 0.34^{xy}	0.45 ± 0.33	0.58 ± 0.13	0.75 ± 0.27
	control	1.73 ± 0.20	0.72 ± 0.17^{y}	0.55 ± 0.16	0.79 ± 0.45	0.52 ± 0.39
	phosphatase inhibitor	1.84 ± 0.26	2.53 ± 0.52^{x}	1.32 ± 0.55	1.21 ± 0.56	1.20 ± 0.63
Pyruvate kinase	kinase inhibitor	0.33 ± 0.03	0.39 ± 0.03	0.67 ± 0.03	0.85 ± 0.04^{xy}	0.58 ± 0.06
	control	0.43 ± 0.02	0.54 ± 0.02	0.54 ± 0.23	0.50 ± 0.34^{y}	0.36 ± 0.29
	phosphatase inhibitor	0.30 ± 0.03	0.94 ± 0.23	0.94 ± 0.35	1.19 ± 0.20^{x}	0.86 ± 0.43
Phosphofructokinase	kinase inhibitor	2.49 ± 0.60	2.90 ± 0.25	2.75 ± 0.04	2.36 ± 0.09	2.51 ± 0.07^{xy}
	control	3.11 ± 0.04	2.74 ± 0.17	2.62 ± 0.26	2.46 ± 0.45	2.27 ± 0.42^{y}
	phosphatase inhibitor	2.92 ± 0.45	3.35 ± 0.56	3.47 ± 0.79	3.29 ± 0.71	3.65 ± 0.75^{x}

	Phosphorylation level (P/T)	Activity					
	48 h	0.5 h	2 h	6 h	12 h	24 h	48 h
Glycogen phosphorylase	0.86 ± 0.26	23.48 ± 3.01	15.60 ± 5.75^{y}	18.63 ± 2.17^{y}	18.51 ± 0.86^{y}	18.08 ± 3.06^{y}	16.97 ± 2.51^{y}
	0.50 ± 0.26	23.48 ± 3.01	18.37 ± 1.65^{xy}	16.70 ± 4.13^{y}	17.31 ± 2.95^{y}	21.74 ± 1.20^{y}	17.82 ± 0.60^{y}
	1.15 ± 0.46	23.48 ± 3.01	28.24 ± 3.64^{x}	30.39 ± 0.77^{x}	28.89 ± 0.76^{x}	28.64 ± 1.48^{x}	24.22 ± 2.81^{x}
Pyruvate kinase	0.36 ± 0.09	38.32 ± 2.92	25.41 ± 2.17	23.57 ± 1.70^{y}	22.33 ± 1.24^{y}	24.43 ± 1.57^{xy}	18.73 ± 0.24^{y}
	0.33 ± 0.13	38.32 ± 2.92	23.45 ± 3.60	23.81 ± 2.07^{y}	18.04 ± 0.76^{y}	20.10 ± 3.61^{y}	22.14 ± 1.29^{y}
	0.37 ± 0.02	38.32 ± 2.92	26.01 ± 1.83	37.32 ± 2.59^{x}	41.39 ± 3.79^{x}	32.83 ± 7.16^{x}	29.30 ± 2.23^{x}
Phosphofructokinase	2.64 ± 0.09^{xy}	15.21 ± 1.10	12.55 ± 2.53	11.98 ± 1.45^{y}	12.11 ± 1.33^{y}	11.84 ± 0.67	12.02 ± 0.24
	2.38 ± 0.53^{y}	15.21 ± 1.10	11.45 ± 2.27	11.12 ± 1.79^{y}	11.49 ± 0.74^{y}	12.84 ± 0.59	11.82 ± 1.95
	3.49 ± 0.37^{x}	15.21 ± 1.10	13.57 ± 0.76	17.04 ± 1.77^{x}	18.48 ± 1.16^{x}	14.02 ± 4.25	12.79 ± 1.75

Data are expressed as mean ± standard deviation ($n = 6$). Means with different letters (x, y) in the same column are significantly different among the three groups ($p < 0.05$). P/T abbreviation means the ratio of phosphoproteins to total proteins, representing the phosphorylated level. The unit of enzyme activity for glycogen phosphorylase, pyruvate kinase and phosphofructokinase is nmol/min/mg protein, U/g protein and nmol/min/mg protein respectively.

Gerrard, 2007). In the present study, the activity of PFK was positively correlated with the phosphorylation level.

Glycolysis is a sequence of enzymatic reactions that are determined by the activities of glycolytic enzymes. GP, PK and PFK are rate-limiting enzymes that control the rate of PM metabolism. The activities of these enzymes were significantly higher in the phosphatase inhibitor group with high phosphorylation level, which revealed protein phosphorylation might play a positive role in the regulation of glycolysis. Shen and Du (2005) reported that the glycolysis and pH decline are indirectly affected by the phosphorylation status of AMP-activated protein kinase (AMPK) in PM muscle. Therefore, we can infer that glycolysis is indirectly affected by protein phosphorylation through the regulation of enzyme activity.

4. Conclusion

The kinase inhibitor and phosphatase inhibitor were effective at modulating the level of protein phosphorylation, with a higher phosphorylation level in the phosphatase inhibitor group and a lower level in the kinase inhibitor group after 12 h of incubation. The muscle in phosphatase group with high protein phosphorylation had a higher lactate content and lower pH values, which revealed that protein phosphorylation is one of the reasons for the decline in pH value. The activities and phosphorylation of GP, PK and PFK were analysed in the present study. Protein phosphorylation was positively correlated with the activity of these rate-limiting enzymes, which confirms the hypothesis that protein phosphorylation may influence the activities of glycolytic enzymes, thus regulating the enzymatic reaction of glycolysis.

Declaration of Competing Interest

The authors have declared no conflicts of interest.

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