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Biochemistry

Metabolomic analysis of plasma and intramuscular adipose tissue between Wagyu and Holstein cattle

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ABSTRACT. In this experiment, we studied the effects of breed differences in intramuscular adipogenic capacity on the metabolomic profiles of plasma and intramuscular adipose tissue between Wagyu (high intramuscular adipogenic capacity) and Holstein (low intramuscular adipogenic capacity) using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS). We showed that the intramuscular fat content, intramuscular adipocyte size and the expression of adipogenic transcription factors (C/EBPβ and C/EBPα) of Wagyu were significantly higher than those of Holstein. Metabolites detected at significantly higher levels in Wagyu plasma were related to the tricarboxylic acid cycle, lipid synthesis, fatty acid metabolism, diabetes, and glucose homeostasis. In contrast, metabolites detected at significantly higher levels in Holstein plasma were related to choline metabolism, the ethanolamine pathway, glutathione homeostasis, nucleic acid metabolism, and amino acid metabolism. Metabolites detected at significantly higher levels in Holstein intramuscular adipose tissue were related to nucleic acid metabolism, amino acid metabolism, amino sugar metabolism, beta oxidation, and the ethanolamine pathway. There were no metabolites significantly higher levels in Wagyu intramuscular adipose tissue. These results indicate candidate biomarkers of breed differences in intramuscular adipogenic capacity between Wagyu and Holstein.

KEY WORDS: intramuscular adipose tissue, metabolomic analysis, plasma

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Recently, metabolomic analysis has been widely used to identify the biomarkers of disease [27]. Metabolomic analysis also has been applied to identify obesity-related biomarkers by analyzing samples of plasma and/or adipose tissue from obese patients [14]. Intramuscular adipose tissue accumulation within the skeletal muscle, which is categorized as an ectopic fat deposition, has been recognized as a new risk factor of metabolic syndrome in humans [3, 31]. However, the metabolomic biomarkers for intramuscular adipogenic capacity, including in humans and rodents, have not been identified.

Intramuscular adipose tissue accumulation is important in determining the meat quality grades of beef cattle. In particular, Japanese black cattle (Wagyu) are characterized by the capacity to accumulate higher amounts of intramuscular adipose tissue than Holstein [1, 46]. We hypothesized that the intramuscular adipogenic capacity of cattle would affect the metabolomic profiles of plasma and intramuscular adipose tissue. The aim of the present study was to elucidate the effects of intramuscular adipogenic capacity on the metabolomic profiles of plasma and intramuscular adipose tissue between Wagyu (high intramuscular adipogenic capacity) and Holstein (low intramuscular adipogenic capacity).

MATERIALS AND METHODS

Animals

Wagyu fattening steers (n=4) and Holstein fattening steers (n=4) were used in this study [44]. Cattle were slaughtered in accordance with the commonly applied fattening periods in Japan (Wagyu: 29–30 months of age, Holstein: 21–22 months of age). They received concentrate (78% total digestible nutrients and 14% crude protein on a dry matter basis) and orchard grass hay (56% total digestible nutrients and 8% crude protein on a dry matter basis) *ad libitum*. There was no significant difference in final body weight between the breeds (Wagyu, 776 ± 27.9 kg, and Holsteins, 766 ± 19.9 kg). Three days before slaughter, blood samples were collected from the jugular vein, and plasma was separated by centrifugation 3,000 × *g* at 4°C for 20 min and stored at −80°C until

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metabolomic analysis. Intramuscular adipose tissue within the longissimus lumborum was collected between the third and fourth lumbar vertebrae from the core of large marbling area by micro scissors immediately after slaughter. Intramuscular adipose tissue samples for determining adipogenic gene expression were collected in RNAlater reagent (Ambion, Carlsbad, CA, USA) and stored at −80°C for later analysis. Intramuscular adipose tissue samples for metabolomic analysis were snap-frozen in liquid nitrogen and stored at −80°C for later analysis. Intramuscular adipose tissue samples for determining adipocyte cellularity were fixed with osmium tetroxide buffer. Longissimus lumborum samples for intramuscular fat content analysis were stored at −30°C for later analysis. All animals received humane care as outlined in the Guide for the Care and Use of Experimental Animals (No.1631B004, National Agriculture and Food Research Organization).

Adipocyte cellularity and intramuscular fat content

Adipocyte cellularity was measured as described previously [43]. The adipocyte size was measured using WinROOF software (Mitani Corp., Fukui, Japan). More than 300 adipocytes for each sample were measured. The intramuscular fat content of the longissimus lumborum was obtained via Soxhlet extraction method [43].

Adipogenic gene expressions

Adipogenic gene expression was analyzed by real-time PCR as described previously [43]. The primer sequences were as follows: C/EBPβ, 5′- ACA GCG ACG AGT ACA AGA TCC -3′(forward) and 5′- GAC AGT TGC TCC ACC TTC TTC T -3′ (reverse); C/EBPα, 5′-ATC TGC GAA CAC GAG ACG -3′(forward) and 5′-CCA GGA ACT CGT CGT TGA A -3′ (reverse); leptin, 5′- GGA GAA GGT CCC GGA GGT T-3′ (forward) and 5′- GGA CCA GAC ATT GGC GAT CT-3′ (reverse); adiponectin, 5′-CAC AAT GGG GTC TAT GCA GAT -3′ (forward) and 5′- GTT ATG GTA GAG AAG GAA GCC TGT -3′ (reverse); ribosomal protein large P0 (RPLP0), 5′-CAA CCC TGA AGT GCT TGA CAT -3′ (forward) and 5′-AGG CAG ATG GAT CAG CCA -3′ (reverse). Reaction conditions were designed as follows: initial denaturation at 95°C for 60 sec followed by 40 cycles at 95°C for 15 sec, 55°C for 15 sec, and 70°C for 30 sec.

Metabolomic analysis of plasma and intramuscular adipose tissue

Fifty µl of plasma was added to 200 µl of methanol containing internal standards (H3304-1002, Human Metabolome Technologies (HMT), Tsuruoka, Japan) at 0°C to suppress enzymatic activity. The extract solution was thoroughly mixed with 150 µl of Milli-Q water, after which 300 µl of the mixture was centrifugally filtered through a 5 kDa cutoff filter (ULTRAFREE MC PLHCC, HMT) at 9,100 × *g*, 4°C for 120 min to remove macromolecules. Two hundred mg of frozen intramuscular adipose tissue was placed in a homogenization tube, along with zirconia beads (5mmφ and 3mmφ). Next, 730 µl of 50% acetonitrile/ Milli-Q water containing internal standards (H3304-1002, HMT) was added to the tube, after which the tissue was completely homogenized at 1,100 rpm, 4°C for 120 sec using a bead shaker (Shake Master NEO, Bio Medical Science, Tokyo, Japan). The homogenate was then centrifuged at 2,300 × *g*, 4°C for 5 min. Subsequently, 400 µl of upper aqueous layer was centrifugally filtered through a 5 kDa cutoff filter (HMT) at 9,100 × *g*, 4°C for 120 min to remove macromolecules. The filtrates of plasma and intramuscular adipose tissue were evaporated to dryness under vacuum and reconstituted in 50 µl of Milli-Q water for the next metabolomic analysis.

Metabolomic analysis was conducted using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) of Agilent 6210 (Agilent Technologies, Inc., Santa Clara, CA, USA) at HMT in accordance with previous studies [22]. CE-TOFMS was controlled by Agilent G2201AA ChemStation software (Agilent Technologies) and connected by a fused silica capillary (50 µm i.d. × 80 cm total length) with commercial electrophoresis buffer (H3301-1001 and I3302-1023 for cation and anion analyses, respectively, HMT) as the electrolyte. Detected peaks were processed by MasterHands software (Keio University, Yamagata, Japan) and annotated according to the HMT metabolite database library. Areas of the annotated peaks were normalized by internal standard levels and sample amounts to obtain the relative levels of each metabolite.

Statistical analysis

All results are presented as the means ± S.D. The differences in mean values between the groups were analyzed by Student's *t*-test. Values of $P<0.05$ were considered significant.

RESULTS

Adipocyte cellularity and adipogenic gene expression

The intramuscular fat content of longissimus muscle in Wagyu was significantly higher than that of Holstein (Fig. 1). The photomicrographs of osmium-fixed intramuscular adipocytes showed that larger sizes of adipocytes were more abundant in Wagyu than in Holstein (Fig. 2A). As compared to the Holstein, the intramuscular adipocyte size distribution in the Wagyu shifted toward larger diameters (Fig. 2B). The mean size of intramuscular adipocyte in Wagyu was significantly larger than that of Holstein (Fig. 2C). The expressions of C/EBPβ and C/EBPα genes in intramuscular adipose tissue of Wagyu were significantly higher than in Holstein. Expression of leptin gene in Wagyu was tended to be higher than in Holsten. In contrast, the expression of the adiponectin gene in Wagyu was significantly lower than in Holstein (Fig. 3).

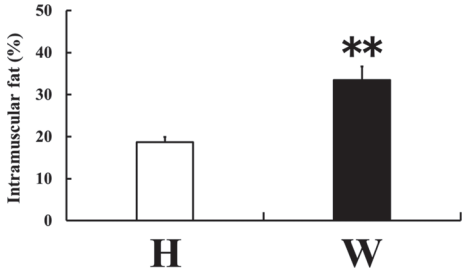

Fig. 1. Intramuscular fat content of longissimus muscle in Holstein (H) and Wagyu (W) cattle. The data represent the means ± standard deviation. ***P*<0.01.

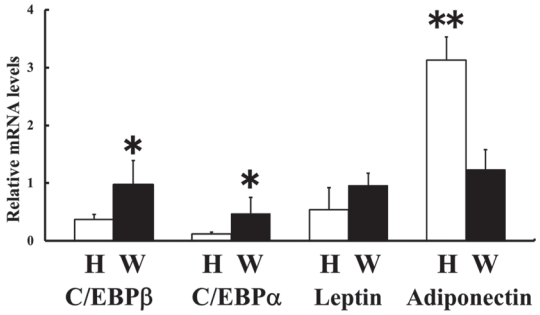

Fig. 3. Expression of C/EBPβ, C/EBPα, leptin and adiponectin gene in intramuscular adipose tissue of Holstein (H) and Wagyu (W) cattle. Ribosomal protein large P0 mRNA was used as an internal control. The data represent the means ± standard deviation. ***P*<0.01, **P*<0.05.

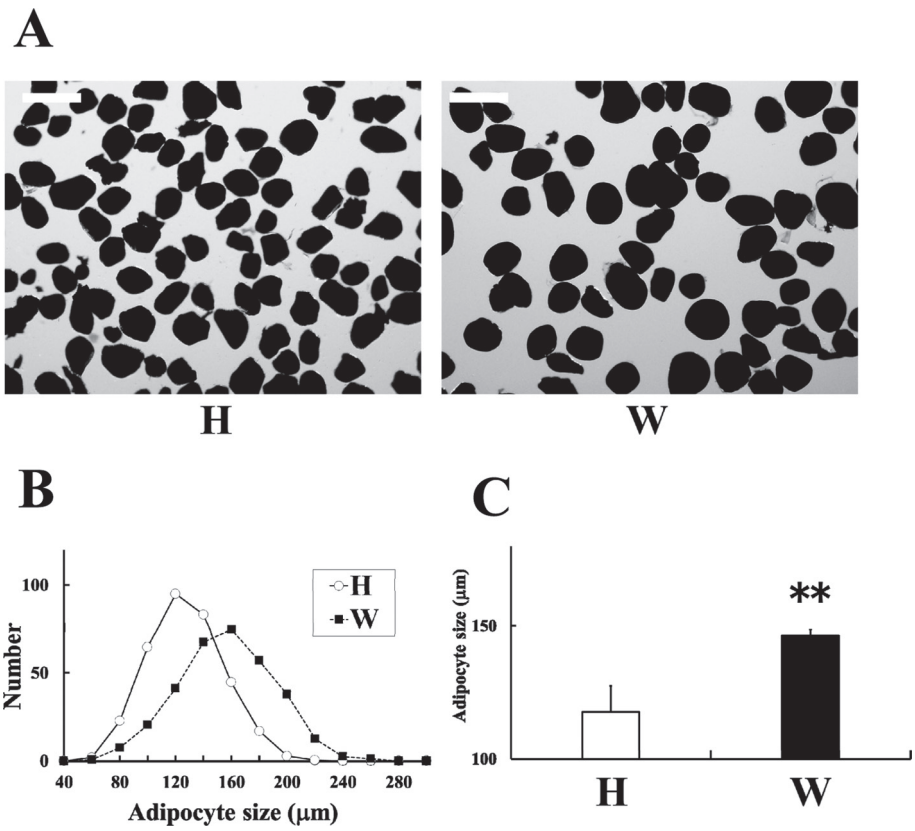

Fig. 2. Intramuscular adipocyte cellularity between Holstein and Wagyu cattle. (**A**) Osmium tetroxide-fixed intramuscular adipocytes in Holstein (H) and Wagyu (W) cattle. The white scale bar indicates 300 μm. (**B**) Distributions of the diameters of intramuscular adipocytes in Holstein (H) and Wagyu (W) cattle. (**C**) Mean diameter of intramuscular adipocytes in Holstein (H) and Wagyu (W) cattle. The data represent the means ± standard deviation. ***P*<0.01.

Metabolomic analysis of plasma and intramuscular adipose tissue

CE-TOFMS analysis detected 196 metabolites in the plasma samples of Wagyu and Holstein cattle (Supplementary Table 1). A total of 30 metabolites were significantly different between Wagyu and Holstein (Table 1). Twenty-two metabolites in Wagyu plasma were significantly higher than Holstein. Eight metabolites in Holstein plasma were significantly higher than Wagyu.

Two hundred and ninety-four metabolites were detected in the intramuscular adipose tissue samples of Wagyu and Holstein cattle (Supplementary Table 2). Twelve metabolites in Holstein intramuscular adipose tissue were significantly higher than Wagyu. There were no metabolites significantly higher level in Wagyu intramuscular adipose tissue (Table 2).

Table 1. Main metabolites detected in plasma

Compound name	Relative area				Comparative analysis	
	Wagyu		Holstein		Wagyu vs. Holstein	
	Mean	S.D.	Mean	S.D.	Ratio	*P*-value
Anserine	2.6×10^{-4}	3.6×10^{-5}	9.5×10^{-5}	2.9×10^{-5}	2.8	0.001
Isethionic acid	2.9×10^{-4}	3.1×10^{-5}	1.3×10^{-4}	2.6×10^{-5}	2.2	0.0002
1-Methylhistidine 3-Methylhistidine	5.6×10^{-3}	3.4×10^{-4}	2.8×10^{-3}	5.3×10^{-4}	2.0	0.0002
N^6-Acetyllysine	5.4×10^{-4}	9.2×10^{-5}	3.2×10^{-4}	9.7×10^{-5}	1.7	0.017
Homovanillic acid Hydroxyphenyllactic acid	1.6×10^{-4}	1.8×10^{-5}	9.5×10^{-5}	4.0×10^{-5}	1.7	0.032
2-Hydroxyvaleric acid	4.2×10^{-4}	1.0×10^{-4}	2.4×10^{-4}	3.6×10^{-5}	1.7	0.032
3-Hydroxybutyric acid	2.3×10^{-2}	5.9×10^{-3}	1.4×10^{-2}	3.8×10^{-3}	1.7	0.045
O-Acetylcarnitine	1.8×10^{-3}	9.6×10^{-5}	1.1×10^{-3}	2.5×10^{-4}	1.6	0.007
N^6, N^6, N^6-Trimethyllysine	1.1×10^{-3}	1.3×10^{-4}	6.9×10^{-4}	2.4×10^{-4}	1.6	0.026
Creatinine	2.7×10^{-2}	3.8×10^{-3}	1.8×10^{-2}	3.7×10^{-3}	1.5	0.021
cis-Aconitic acid	1.9×10^{-3}	6.7×10^{-5}	1.3×10^{-3}	1.4×10^{-4}	1.4	0.002
Glycerol 3-phosphate	1.6×10^{-4}	2.7×10^{-5}	1.2×10^{-4}	8.0×10^{-6}	1.4	0.045
Hippuric acid	4.4×10^{-3}	7.3×10^{-4}	3.1×10^{-3}	6.2×10^{-4}	1.4	0.034
Isocitric acid	1.6×10^{-3}	2.4×10^{-4}	1.1×10^{-3}	4.9×10^{-5}	1.4	0.027
Citric acid	3.5×10^{-2}	4.0×10^{-3}	2.7×10^{-2}	2.9×10^{-3}	1.3	0.023
1-Methyl-4-imidazoleacetic acid	1.2×10^{-4}	9.9×10^{-6}	9.1×10^{-5}	1.9×10^{-5}	1.3	0.042
Asymmetric dimethylarginine	4.7×10^{-4}	1.6×10^{-5}	3.9×10^{-4}	2.7×10^{-5}	1.2	0.005
Glycerophosphocholine	7.4×10^{-4}	4.8×10^{-5}	6.1×10^{-4}	4.8×10^{-5}	1.2	0.011
Threonic acid	1.3×10^{-3}	1.3×10^{-4}	1.1×10^{-3}	1.0×10^{-4}	1.2	0.047
Thymidine	1.8×10^{-4}	1.2×10^{-5}	1.6×10^{-4}	5.7×10^{-6}	1.1	0.028
Cytidine	4.3×10^{-4}	3.0×10^{-5}	5.6×10^{-4}	8.4×10^{-5}	0.8	0.050
Serine	1.3×10^{-2}	1.8×10^{-3}	1.7×10^{-2}	1.2×10^{-3}	0.8	0.014
Ornithine	1.5×10^{-2}	1.6×10^{-3}	2.0×10^{-2}	3.5×10^{-3}	0.7	0.039
Sarcosine	6.6×10^{-4}	1.2×10^{-4}	9.0×10^{-4}	1.3×10^{-4}	0.7	0.037
Phosphoethanolamine	1.5×10^{-4}	3.3×10^{-5}	2.5×10^{-4}	5.2×10^{-5}	0.6	0.021
2-Aminoisobutyric acid 2-Aminobutyric acid	2.8×10^{-3}	9.8×10^{-4}	6.0×10^{-3}	1.2×10^{-3}	0.5	0.007
Piperidine	1.7×10^{-4}	6.3×10^{-5}	3.1×10^{-4}	6.5×10^{-5}	0.5	0.023

Values are expressed as mean ± standard deviation.

Table 2. Main metabolites detected in intramuscular adipose tissue

Compound name	Relative area				Comparative analysis	
	Wagyu		Holstein		Wagyu vs. Holstein	
	Mean	S.D.	Mean	S.D.	Ratio	*P*-value
Hexanoic acid	1.2×10^{-4}	2.0×10^{-5}	1.8×10^{-4}	1.4×10^{-5}	0.7	0.003
5-Hydroxylysine	1.3×10^{-5}	2.2×10^{-6}	1.9×10^{-5}	3.3×10^{-6}	0.7	0.025
4-Oxopyrrolidine-2-carboxylic acid	3.9×10^{-4}	1.1×10^{-4}	6.0×10^{-4}	5.8×10^{-5}	0.7	0.020
Cytidine	1.5×10^{-4}	1.5×10^{-5}	2.5×10^{-4}	5.7×10^{-5}	0.6	0.041
Phosphoethanolamine	1.1×10^{-3}	1.9×10^{-4}	1.8×10^{-3}	3.8×10^{-4}	0.6	0.022
Glutamic acid	2.9×10^{-2}	7.0×10^{-3}	4.6×10^{-2}	6.1×10^{-3}	0.6	0.012
Uric acid	6.0×10^{-4}	6.1×10^{-5}	9.4×10^{-4}	6.4×10^{-5}	0.6	0.0002
N-Acetylglycine	1.1×10^{-4}	3.1×10^{-5}	2.3×10^{-4}	4.9×10^{-5}	0.5	0.009
N-Glycolylneuraminic acid	7.1×10^{-5}	1.3×10^{-5}	1.3×10^{-4}	1.9×10^{-5}	0.5	0.003
myo-Inositol 2-phosphate	1.1×10^{-4}	2.1×10^{-5}	2.2×10^{-4}	4.8×10^{-5}	0.5	0.016
Putrescine	1.6×10^{-4}	3.4×10^{-5}	4.2×10^{-4}	1.5×10^{-4}	0.4	0.037
O-Acetylhomoserine 2-Aminoadipic acid	3.4×10^{-4}	1.3×10^{-4}	1.1×10^{-3}	4.8×10^{-4}	0.3	0.047

Values are expressed as mean ± standard deviation.

DISCUSSION

In the present study, we showed that the intramuscular fat content and intramuscular adipocyte size of Wagyu were significantly higher than those of Holstein. We also showed that the expression of the C/EBP family (C/EBPβ and C/EBPα) genes in intramuscular adipose tissue of Wagyu were significantly higher than those of Holstein. Adipogenic transcription factors of the C/EBP family promotes adipocyte differentiation, and the expression of these transcription factors are upregulated in obesity [5]. These results suggest that expression pattern of C/EBP family are affected by the differences of intramuscular adipogenic capacity between Wagyu and Holstein. We also showed that expression of leptin gene in intramuscular adipose tissue of Wagyu was tended to be higher than that of Holsten. In contrast to the C/EBP family and leptin, we showed that the expression of the adiponectin gene in intramuscular adipose tissue of Wagyu was significantly lower than that of Holstein. Adiponectin and leptin are adipocyte-derived secretory factor called adipocytokine [32, 45]. Leptin expression is upregulated in obese hypertrophied adipocytes [8]. In contrast, adiponectin expression is downregulated in obese hypertrophied adipocytes [2, 33]. These results suggest that the expression patterns of leptin and adiponectin genes are affected by the difference of intramuscular adipocyte size between Wagyu and Holstein.

The plasma metabolites detected at significantly higher levels in Wagyu cattle were related to the tricarboxylic acid (TCA) cycle (cis-aconitic acid, citric acid, isocitric acid) [21], lipid synthesis (3-hydroxybutiric acid, glycerol 3-phosphate) [47], fatty acid metabolism (o-acetylcarnitine) [40], diabetes (methylhistidine, asymmetric dimethylarginine) [34, 35], and glucose homeostasis (hippuric acid) [30]. Ho *et al.* showed that plasma metabolites of TCA cycle were positively associated with body mass index [10]. Metabolites of the TCA cycle in adipose tissue were also reported to be increased in human obesity [21]. In addition, Wagyu have higher body fat content than Holstein [20, 42]. Obese conditions closely related to diabetes, glucose homeostasis and fatty acid metabolism in humans [12, 28]. Our present results showed that metabolites associated with diabetes, glucose homeostasis and fatty acid metabolism in Wagyu were higher than those of Holstein. Therefore, plasma metabolites detected at higher levels in Wagyu might reflect the obesity and associated phenotypes. In contrast, the main metabolites detected at significantly higher levels in Holstein were related to the choline metabolism (sarcosine, serine) [36], the ethanolamine pathway (phosphoethanolamine) [6], glutathione homeostasis (2-aminoisobutyric acid, 2-aminobutyric acid) [11], nucleic acid metabolism (cytidine) [18], and amino acid metabolism (ornithine) [41]. Plasma metabolites related to glutathione, nucleic acid, and amino acid metabolism might reflect the higher carcass lean and lower carcass fat proportion of Holstein [20, 42]. In addition, the choline metabolism and ethanolamine pathway were reported to be associated with differences in ectopic fat accumulation [6, 24] as discussed below. These results indicate candidate biomarkers of breed differences in intramuscular adipogenic capacity between Wagyu and Holstein. Further studies are needed to identify plasma biomarkers relating to intramuscular adipogenic capacity.

The metabolites detected at significantly higher levels in Holstein intramuscular adipose tissue were related to nucleic acid metabolism (cytidine, uric acid, glutamic acid) [18, 39], amino acid metabolism (5-hydroxylysine, N-acetylglycine, putrescine) [9, 15, 26], amino sugar metabolism (N-glycolylneuraminic acid) [23], beta oxidation (hexanoic acid) [7], and the ethanolamine pathway (phosphoethanolamine) [6]. Interestingly, the metabolomic analysis of intramuscular adipose tissue showed that no metabolites were significantly higher level in Wagyu intramuscular adipose tissue. Butte *et al*. reported that plasma N-acetylglicine level in non-obese children was significantly higher than obese children [4]. Perng *et al*. showed that serum N-acetylglicine level was inversely associated with metabolic syndrome risk score [25]. In addition, adiponectin increases adipose tissue metabolism via enhances of fatty acid beta oxidation and energy consumption, and subsequently reduces triglycerides accumulation [13]. In the present study, we showed that the expression of the adiponectin gene and metabolites associated with amino acid metabolism and beta oxidation in intramuscular adipose tissue of Holstein were higher than those of Wagyu. The higher energy consumption of intramuscular adipocytes affected by adiponectin in Holstein might negatively affect the excess energy storage within adipocytes (hypertrophy). These results suggest that, in addition to the lower expression of the C/EBP family as shown in Fig. 3, higher energy consumption might affect the smaller sizes of intramuscular adipocytes in Holstein.

We showed that cytidine and phosphoethanolamine levels were significantly higher both in plasma and intramuscular adipose tissue of Holstein than that of Wagyu. The plasma cytidine concentration is related to the choline metabolism: the administration of choline increased plasma cytidine levels [17, 38]. In contrast, choline deficiency stimulates liver fat accumulation [29]. In addition, phosphatidylethanolamine is generated from phosphoethanolamine via the ethanolamine pathway [24], and the ethanolamine pathway abnormality causes a shift in triglyceride synthesis, resulting in a progression of hepatic steatosis [6]. Both intramuscular fat and liver fat are categorized as ectopic fat depositions [3, 31]. These results suggest that the higher cytidine and phosphoethanolamine levels in plasma and intramuscular adipose tissue might reflect the lower intramuscular adipogenic capacity in Holstein.

We showed that the N-glycolylneuraminic acid level in the intramuscular adipose tissue of Wagyu was significantly lower than that of Holstein. N-glycolylneuraminic acid deficiency increased sensitivity to muscular dystrophies and led to severe disease phenotypes [19, 23]. In addition, genetic and histological similarities between bovine intramuscular adipose tissue accumulation (marbling) and human muscular dystrophies have been suggested [16, 37]. These results suggest that the lower N-glycolylneuraminic acid level in the intramuscular adipose tissue might reflect the higher intramuscular adipogenic capacity in Wagyu.

In conclusion, we show that the metabolomic profiles of plasma and intramuscular adipose tissue are different between Wagyu and Holstein. These results indicate new biomarkers related to intramuscular adipogenic capacity.

CONFLICTS OF INTEREST. The authors declare no conflict of interest.

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