Expression of equine glucose transporter type 4 in skeletal muscle after glycogen-depleting exercise

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Objectives—To clone and sequence cDNA for equine insulin-responsive glucose transporter (glucose transporter type 4 [GLUT-4]) and determine effects of glycogen-depleting exercise and meal type after exercise on GLUT-4 gene expression in skeletal muscle of horses.

Sample Population—Muscle biopsy specimens from 7 healthy adult horses.

Procedures—Total RNA was extracted from specimens, and GLUT-4 cDNA was synthesized and sequenced. Horses were exercised on 3 consecutive days. On the third day of exercise, for 8 hours after exercise, horses were either not fed, fed half of daily energy requirements as hay, or fed an isocaloric amount of corn. The GLUT-4 mRNA was determined by use of real-time reverse transcriptase-polymerase chain reaction in muscle biopsy specimens obtained before 3 consecutive days of exercise and within 10 minutes and 4, 8, and 24 hours after the third exercise bout.

Results—A 1,629-bp segment was sequenced, of which 1,530 bp corresponded to the coding region and encoded a protein of 509 amino acids. Expression of GLUT-4 gene increased by 2.3, 4.3, 3.3, and 2.6 times 10 minutes and 4, 8, and 24 hours after exercise, respectively, compared with that prior to exercise. No differences were observed in GLUT-4 gene expression among conditions of feed withholding, corn feeding, and hay feeding during the 8 hours postexercise.

Conclusions and Clinical Relevance—Lack of increase of GLUT-4 gene expression after grain feeding and exercise may explain the apparently slower rate of glycogen synthesis after exercise in horses relative to that of other species. (*Am J Vet Res* 2005;66:379–385)

During exercise, the 2 primary substrates available for energy production in working skeletal muscle of mammals are carbohydrates (muscle glycogen and blood glucose) and fats (fatty acids from adipose tissue and intramuscular triglycerides).[1] Restoration of skeletal muscle glycogen stores is critical for exercise performance in human athletes who undertake multiple exercise bouts on the same or successive days.[2] Similarly, low muscle glycogen concentrations prior to exercise in horses lead to decreased exercise performance.[3] Muscle glycogen synthesis is catalyzed by the enzyme glycogen synthase. Availability of the substrate (glucose) rather than glycogen synthase activity appears to be the rate-limiting factor for muscle glycogen synthesis.[4,5] Stimulation of glucose transport into cells by insulin, muscular contraction, or both is mediated by translocation of the insulin-regulated glucose transporter (**glucose transporter type 4 [GLUT-4]**) from intracellular sites into the plasma membrane.[5] This is of particular importance in glucose homeostasis because it mediates insulin-mediated glucose uptake in skeletal muscle, the main site of glucose disposal, and in adipose tissue.[6] The GLUT-4 is expressed in skeletal and cardiac muscle and white and brown adipose tissues.[7] Gene expression of GLUT-4 increases in skeletal muscle after exercise as an early adaptation that facilitates replenishment of the muscle glycogen stores.[8] In addition, gene transcription of carbohydrate metabolism-related genes (GLUT-4, glycogenin, and pyruvate dehydrogenase kinase 4) is upregulated and some lipid-related genes (fatty acid translocase, and uncoupling binding protein 3) are downregulated during glycogen-depleting exercise followed by a high-carbohydrate diet in humans, which indicates a rapid capacity for exercise and diet to modulate changes in gene transcription.[9] In summary, GLUT-4 gene expression, GLUT-4 protein content, and translocation of GLUT-4 protein to the cellular membrane are key events in glucose homeostasis at rest and during exercise.

The cDNA nucleotide sequence of GLUT-4 mRNA has been reported in humans[10]; mice[11,12]; cattle[13]; rabbits[14]; and partially in dogs,[15] pigs,[16] sheep and goats,[17] and water buffaloes.[18] Results of recent studies[19,20] performed in horses indicate that exercise and training result in increased GLUT-4 protein content in skeletal muscle; however, to date, the effect of exercise and diet on equine GLUT-4 mRNA expression has been difficult to quantify, in part because the nucleotide sequence was unknown.

The purpose of the study reported here was to determine the effect of exercise that substantially depletes muscle glycogen and of type of meal after exercise on GLUT-4 gene expression in equine skeletal muscle. As a necessary preliminary step for mRNA quantitation by use of real-time **reverse transcriptase-polymerase chain reaction (RT-PCR)**, the equine GLUT-4 gene was cloned and sequenced. We hypothesized that exercise would increase GLUT-4 gene expression in equine skeletal muscle and starch-rich meals would further enhance GLUT-4 gene expression,

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compared with isocaloric meals of roughage or feed withholding.

Materials and Methods

Horses—In the first portion of the study, specimens of semitendinosus or semimembranosus muscles were obtained via cut-down incisions from 4 horses and spleen samples from one of them. Horses had been donated to The Ohio State University and were euthanatized because of chronic musculoskeletal disease. Specimens were collected within 20 minutes after euthanasia, immediately frozen in liquid nitrogen, and stored at –80°C until subsequent analysis. Skeletal muscle from hind limbs of mice also was harvested similarly within 10 minutes of euthanasia and used as a positive control during the PCR assays intended to clone and sequence equine GLUT-4. In the second portion of the study, 7 horses were used to determine the effects of exercise and feed withholding or isocaloric corn or hay meals on GLUT-4 mRNA quantitation in skeletal muscle. All animal experiments were conducted after approval by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University and were performed in compliance with their guidelines and recommendations.

Extraction of total RNA—Total RNA was isolated from tissues by use of the acid guanidium thiocyanate-phenol-chloroform extraction method[21,a] and reconstituted in nuclease-free water. Any possible contaminating DNA was removed by treating the specimens with DNase I.[b] The RNA was quantitated by use of UV spectrophotometry and evaluated via formaldehyde gel electrophoresis to confirm RNA integrity. The intra- and interassay coefficients of variation for RNA quantitation were < 6%.

Cloning and sequencing of equine GLUT-4—Cloning and sequencing were performed via standard RT-PCR techniques, initially with primers designed by comparing published sequences of GLUT-4 cDNA in human, rat, mouse, and cow and choosing oligonucleotides in areas with the highest homology among species (**Table 1**). Primers were designed with a software package[c] to obtain, in separate reactions, DNA fragments of 700 to 800 bp that overlapped a minimum of 100 bp and spanned the entire coding region of the equine GLUT-4 gene. Two-step RT-PCR reactions involved cDNA synthesis[d] with oligo(dT) primer and incubation at 50°C for 1 hour and PCR amplification of a 2-µL aliquot of the cDNA synthesis reaction. The PCR conditions involved denaturation at 94°C for 2 minutes and 40 cycles of denaturation (30 seconds at 92°C), annealing (45 seconds; Table 1), and extension (1 minute at 72°C), with a final extension step at 72°C for 5 minutes by use of a commercial PCR kit.[e] All RT-PCR reactions for the cloning experiments were performed with total RNA from mouse skeletal muscle as positive control, with nuclease-free water and total RNA from equine spleen as negative controls (because in other species, GLUT-4 is not expressed in spleen tissues) in addition to total RNA from equine skeletal muscle. Ten microliters of PCR product was resolved on a 1% agarose gel. The DNA fragments of predicted size were ligated into terminal transferase activity cloning vectors.[f,g] Candidate clones were screened by restriction enzyme digestion with *Eco*RI,[h] and positive clones were sequenced at the institutional sequenc-

Table 1—Primers used to determine the cDNA sequence of equine glucose transporter type 4 (GLUT-4; insulin-responsive glucose transporter), real-time reverse transcriptase-polymerase chain reaction (RT-PCR) of equine GLUT-4 and β-actin, and length of the product of amplification and annealing temperature used with each primer pair.

Target	Amplicon size (bp)	Annealing temperature (°C)	Primer sequence
GLUT-4 5' coding region (1–713)*	713	50	Forward 5′-AAA CAA GAT GCC GTC GGG-3′ Reverse 5′-GGT TCC GGA TGA TGT AGA GGT A-3′
GLUT-4 mid coding region (248–905)*	658	66	Forward 5′-TGG GCT CTC TCC GTG GCC ATC TT3′ Reverse 5′-GCT GCT GGC TCA GCT GCA GCA-3′
GLUT-4 3′ coding region (725–1,527)*	803	64	Forward 5′-GCC AGA AAG AGT CTR AAG CGC CTG A-3′ Reverse 5′-CCT CAG TCR TKC TCA TCT GGC CCT A-3′
GLUT-4 3′ coding region and UTR (725–1,623)*	899	64	Forward 5′-GCC AGA AAG AGT CTR AAG CGC CTG A-3′ Reverse 5′-TGG GGA AGA GAG GGT TAA AGT GCT GC-3′
GLUT-4 Real-time RT-PCR	63	55	Forward 5′-TTT GTG GCA TTC TTT GAG ATT GG-3′ Reverse 5′-CTG AAG AGC TCA GCC ACG A-3′
β-actin Real-time RT-PCR	142	58	Forward 5′-GAC AGG ATG CAG AAG GAG ATC ACA-3′ Reverse 5′-TGA TCC ACA TCT GCT GGA AGG T-3′

*The GLUT-4 primers used during sequencing and cloning experiments were designed on the basis of the consensus sequence among human, cattle, rat, and mouse GLUT-4 and may not exactly match the final equine GLUT-4 sequence reported in Figure 1.
Mixed-base site code: R = A or G, K = G or T.

ing facility with universal M13 forward and M13 reverse primers and an automated sequencing analyzer.[i] Sequence results were compared, by use of a software package,[j] with those of other species within the GenBank database at the National Center for Biotechnology Information.

Real-time RT-PCR for measurement of equine GLUT-4 gene expression in skeletal muscle—Species-specific primers for real-time RT-PCR of the equine GLUT-4 gene were designed by adapting those used in rats,[22] and primers for the equine β-actin gene[k] were adapted from those used in humans[23] (Table 1). To compensate for variations in input RNA amounts and efficiency of reverse transcription, β-actin mRNA was quantified and the GLUT-4 mRNA results were normalized to these values. β-Actin mRNA concentrations have been reported not to change during training in rat skeletal muscle[24]; therefore, β-actin was considered an adequate housekeeping gene. In contrast, another housekeeping gene, **glyceraldehyde-3-phosphate dehydrogenase (GAPDH)**, has been commonly used, but insulin induces GAPDH gene expression in cell cultures.[25] Therefore, we considered GAPDH not to be an adequate housekeeping gene because feeding after exercise was anticipated to increase plasma insulin concentrations and possibly induce GAPDH gene expression.

Real-time RT-PCR with SYBR green was used to quantify mRNA expression, the principles of which have been described in detail elsewhere.[26] In this method, amplification is monitored as it occurs by collection of fluorescence data during each cycle of amplification, which allows for quantification in the log-linear range of amplification. Fluorescence occurs because of intercalation of SYBR green into the double-stranded DNA generated in the PCR assay. Concentrations of mRNA were quantitated by use of the fit-points method of a software program.[l] Real-time RT-PCR reactions were performed in duplicate by use of a quantitative, real-time, 1-step RT-PCR with SYBR green kit[m] and a detection system.[n] Each 20-µL reaction contained 1X SYBR green RT-PCR master mix, forward and reverse primers (1µM each; Table 1), and total RNA (100 ng). Real-time RT-PCR conditions involved reverse transcription at 50°C for 20 minutes; denaturation at 95°C for 15 minutes, and 45 cycles of denaturing (95°C for 15 seconds), annealing (55° and 58°C for GLUT-4 and β-actin, respectively, for 20 seconds), extension (72°C for 10 seconds), and fluorescence data acquisition. Specific amplification of equine GLUT-4 and equine β-actin was confirmed in all specimens by melting temperature analysis of the product of amplification and by resolving on a 1% agarose gel. During the preliminary studies, specific amplification of equine GLUT-4 and equine β-actin also was confirmed via sequencing. To quantify mRNA copy number, a standard curve was generated by use of equine GLUT-4 and equine β-actin RNA previously synthesized via in vitro transcription. The RNA standards were transcribed from the linearized cDNA cloned into pCR2.1 plasmid[f] by use of T7 RNA polymerase.[o] Purified RNA transcripts were verified by use of formaldehyde gel electrophoresis for size and integrity, quantified by use of UV spectrophotometry, and tested for amplification with the gene-specific primers with and without reverse transcriptase (to rule out DNA contamination). Aliquots of 10^9 copies/µL were stored at –80°C, and a fresh aliquot was diluted in nuclease-free water prior to use to obtain standard curves ranging from 10^7 to 10^2 transcripts/µL.

Effect of exercise and meal type after exercise on GLUT-4 gene expression—The effects of feeding isocaloric meals of varying starch and fiber content (or food withholding) in combination with exercise were examined in a semibalanced, randomized, 3-way crossover study. Seven horses underwent 3 consecutive days of strenuous treadmill exercise intended to lower muscle glycogen concentrations by at least 55% of the initial values.[3,27] Exercise intensity and duration of the glycogen-depleting exercise protocol used in this study are described in greater detail elsewhere.[27] In each of 3 trials during the 8 hours after the third consecutive day of exercise, horses were either offered meals of cracked corn (mean ± SD, 2.2 ± 0.2 kg/meal [approx 7.4 Mcal **digestible energy** {**DE**}/meal] grain trial), offered isocaloric meals of grass and alfalfa hay (3.4 ± 0.4 kg/meal [7.4 Mcal DE/meal] hay trial), or had food withheld. Trials were separated by 2 weeks, and the order of trials was randomized for individual horses. Because of musculoskeletal injuries, 3 of 7 horses did not complete all 3 trials. Horses 1 through 6 completed the glycogen-depleting trials followed by

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   1  AAACAAGATGCCGTCGGGTTTTCAACAGATCGGCTCAGAAGATGGGGAACCGCCTCAGCA
            M  P  S  G  F  Q  Q  I  G  S  E  D  G  E  P  P  Q    17
  61  GCGAGTAACTGGGACCCTGGTCCTCGCAGTATTTTCTGCTGTGCTTGGCTCCCTGCAGTTT
      Q  R  V  T  G  T  L  V  L  A  V  F  S  A  V  L  G  S  L  Q    37
 121  TGGCTACAACATTGGGGTCATCAATGCCCCACAGAAGGTGATTGAACAGAGCTACAATGA
      F  G  Y  N  I  G  V  I  N  A  P  Q  K  V  I  E  Q  S  Y  N    57
 181  GACATGGCTGGGGAGGCAGGGGCCTGAGGGGCCCAGCTCCATCCCACCAGGCACCCTCAC
      E  T  W  L  G  R  Q  G  P  E  G  P  S  S  I  P  P  G  T  L    77
 241  CACCCTCTGGGCTCTCTCCGTGGCCATCTTTTCTGTGGGCGGCATGATCTCCTCCTTCCT
      T  T  L  W  A  L  S  V  A  I  F  S  V  G  G  M  I  S  S  F    97
 301  CATTGGCATCATCTCTCAGTGGCTGGGAAGGAAAAGGGCAATGCTGGTCAACAATGCCCT
      L  I  G  I  I  S  Q  W  L  G  R  K  R  A  M  L  V  N  N  A   117
 361  GGCAGTGCTGGGGGGCAGCCTCATGGGCCTGGCTGACACTGCTGCCTCCTATGAGATGCT
      L  A  V  L  G  G  S  L  M  G  L  A  D  T  A  A  S  Y  E  M   137
 421  CATTCTTGGACGGTTCCTCATTGGCGCCTACTCAGGGCTGACGTCAGGGCTGGTGCCCAT
      L  I  L  G  R  F  L  I  G  A  Y  S  G  L  T  S  G  L  V  P   157
 481  GTATGTGGGGGAGATCGCCCCCACTCACCTGCGGGGTGCCTTGGGGACACTCAACCAACT
      M  Y  V  G  E  I  A  P  T  H  L  R  G  A  L  G  T  L  N  Q   177
 541  GGCCATCGTCATTGGCATTCTGATCGCCCAGGTGCTGGGCTTGGAGTCTATGCTGGGCAC
      L  A  I  V  I  G  I  L  I  A  Q  V  L  G  L  E  S  M  L  G   197
 601  TGCCACCCTATGGCCACTGCTCCTGGGCATCACAGTGCTGCCTGCCCTTCTGCAGTTGGT
      T  A  T  L  W  P  L  L  L  G  I  T  V  L  P  A  L  L  Q  L   217
 661  CCTACTGCCCTTCTGCCCAGAAAGCCCTCGCTACCTCTACATCAGCCGGAACCTGGAGGG
      V  L  L  P  F  C  P  E  S  P  R  Y  L  Y  I  S  R  N  L  E   237
 721  GCCCGCCAGAAAGAGTCTGAAGCGCCTGACAGGCTGGGCTGACGTGTCTGGAGTGCTGGC
      G  P  A  R  K  S  L  K  R  L  T  G  W  A  D  V  S  G  V  L   257
 781  TGAGCTAAAGGAAGAGAAGCGGAAGCTGGAGCGTGAGCGGCCACTGTCCCTGCTCCAGCT
      A  E  L  K  E  E  K  R  K  L  E  R  E  R  P  L  S  L  L  Q   277
 841  CCTGGGCAGCCGTGTCCACCGGCAGCCCCTAGTCATTGCAGTTGTGCTGCAGCTCAGCCA
      L  L  G  S  R  V  H  R  Q  P  L  V  I  A  V  V  L  Q  L  S   297
 901  GCAGCTATCGGGCATCAACGCTGTTTTCTATTATTCAACCAGCATCTTCGAGAAGGCAGG
      Q  Q  L  S  G  I  N  A  V  F  Y  Y  S  T  S  I  F  E  K  A   317
 961  AGTAGGGCAGCCAGCCTATGCCACCATAGGAGCTGGTGTGGTCAACACAGTCTTCACCTT
      G  V  G  Q  P  A  Y  A  T  I  G  A  G  V  V  N  T  V  F  T   337
1021  GGTCTCGGTGTTTTTGGTAGAACGAGCTGGGCGCCGGACACTCCATCTCCTGGGCCTGGC
      L  V  S  V  F  L  V  E  R  A  G  R  R  T  L  H  L  L  G  L   357
1081  GGGAATGTGTGGCTGTGCCATCTTGATGACTGTGGCCCTGCTTCTGCTGGAGCGAGTTCC
      A  G  M  C  G  C  A  I  L  M  T  V  A  L  L  L  L  E  R  V   377
1141  AGCCATGAGCTATGTCTCCATCGTGGCCATCTTTGGCTTTGTGGCATTCTTTGAGATTGG
      P  A  M  S  Y  V  S  I  V  A  I  F  G  F  V  A  F  F  E  I   397
1201  CCCTGGCCCCATCCCCTGGTTCATCGTGGCTGAGCTCTTCAGCCAGGGACCCCGCCCGGC
      G  P  G  P  I  P  W  F  I  V  A  E  L  F  S  Q  G  P  R  P   417
1261  AGCCATGGCTGTGGCTGGCTTCTCCAACTGGACGTGCAACTTCATCATTGGCATGGGCTT
      A  A  M  A  V  A  G  F  S  N  W  T  C  N  F  I  I  G  M  G   437
1321  CCAGTATGTCGCGGATGCTATGGGTCCCTACGTCTTCCTTCTATTTGCGGTCCTCCTGCT
      F  Q  Y  V  A  D  A  M  G  P  Y  V  F  L  L  F  A  V  L  L   457
1381  TGGCTTCTTCATCTTCACCTTCTTAAGAGTGCCTGAAACCCGAGGCCGGACGTTTGACCA
      L  G  F  F  I  F  T  F  L  R  V  P  E  T  R  G  R  T  F  D   477
1441  GATCTCAGCCGCTTTCCACCGGACACCCTCTCTTTTAGAGCAGGAGGTGAAGCCCAGCAC
      Q  I  S  A  A  F  H  R  T  P  S  L  L  E  Q  E  V  K  P  S   497
1501  GGAACTTGAGTATTTAGGGCCAGATGAGAATGACTGAGGGCAGGCAGGGGTGGGAGAGCC
      T  E  L  E  Y  L  G  P  D  E  N  D  *                       509
1561  GGTGCTCTCTACCCCCCTCAGAGCCCCCTCCTTCTCTGCAGCACTTTAACCCTCTCTTCC
1621  CCA
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Figure 1—Complementary DNA and deduced amino acid sequences of equine glucose transporter type 4 (GLUT-4; insulin-responsive glucose transporter). Numbers on the left indicate nucleotides, and numbers on the right indicate amino acids beginning with methionine. The start (ATG) and stop (TGA) codons specifying the 1,530-bp open reading frame are in bold.

corn feeding or feed withholding, and horses 1 through 4 and 7 completed the glycogen-depleting trials followed by hay feeding. Other results of this study are reported elsewhere.[28]

Collection of blood and muscle specimens and biochemical analyses—Blood samples were obtained before and hourly for 8 hours after exercise by use of catheters (14 gauge, 5.25 inches)[p] inserted into the left jugular vein. Plasma and serum samples were harvested by centrifugation and stored at –80°C until subsequent analysis. Plasma glucose and serum **immunoreactive insulin (IRI)** concentrations were measured in duplicate as described elsewhere.[29,30] Muscle biopsy specimens were obtained percutaneously at a depth of 6 cm from the middle gluteal muscle by use of needle biopsy before the 3 consecutive days of glycogen-depleting exercise and 10 minutes and 4, 8, and 24 hours after the last bout of exercise. Muscle specimens were placed immediately in liquid nitrogen and stored at –80°C until analysis. Muscle glycogen concentration was determined as previously described.[29]

Statistical analyses—Data are presented as mean ± SEM. Plasma glucose, serum IRI, and muscle glycogen concentrations as well as GLUT-4 mRNA, β-actin mRNA, and GLUT-4 mRNA normalized to β-actin expression were analyzed by use of an ANOVA mixed model with repeated measures, with treatment (feed withholding, hay, and grain), time, and the interaction of treatment and time as fixed effects. The null hypothesis was rejected at $P < 0.05$ for the main effects (treatment and time) and $P < 0.10$ for the interaction. Significant differences identified by use of ANOVA were isolated by use of the Dunn-Sidak post hoc test. Statistical computations were performed with a software package.[q]

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Equine       1 MPSGFQQIGSEDGEPPQQRVTGTLVLAVFSAVLGSLQFGYNIGVINAPQKVIEQSYNETW
Human        1 ............................................................
Bovine       1 ................R.........G...............................
Rat          1 ............................................................A..
Mouse        1 ..........D.....R.....................................A..

Equine      61 LGRQGPEGPSSIPPGTLTTLWALSVAIFSVGGMISSFLIGIISQWLGRKRAMLVNNALAV
Human       61 ............................................................V...
Bovine      61 .........G.................................................FS.....
Rat         61 ......G..D...Q.....................................A..V...
Mouse       61 ......G..D...Q.....................................A..V...

Equine     121 LGGSLMGLADTAASYEMLILGRFLIGAYSGLTSGLVPMYVGEIAPTHLRGALGTLNQLAI
Human      121 .........NA..............................................
Bovine     121 ...T.....KA............F...................................
Rat        121 ...A.....NA.....I.....................................
Mouse      121 ...A.....NA.....I.....................................

Equine     181 VIGILIAQVLGLESMLGTATLWPLLLGITVLPALLQLVLLPFCPESPRYLYISRNLEGPA
Human      181 ..............L....S.......L.....................IQ......
Bovine     181 .T.....................................M....L..........I.......
Rat        181 .....V.....................A..........L.............I.......
Mouse      181 .....V.....................AL.........I.............I.......

Equine     241 RKSLKRLTGWADVSGVLAELKEEKRKLERERPLSLLQLLGSRVHRQPLVIAVVLQLSQQL
Human      241 .....................D.....................T.....I..........
Bovine     241 ..............E..............S.........HT........I........
Rat        241 ..............DA.....D.....................T.....I..........
Mouse      241 ..............DA.....D..........M.........T.....I..........

Equine     301 SGINAVFYYSTSIFEKAGVGQPAYATIGAGVVNTVFTLVSVFLVERAGRRTLHLLGLAGM
Human      301 ...............T.........................L...................
Bovine     301 ...............S...EK.......................H...........
Rat        301 ...............L...E.....................L...................
Mouse      301 ...............S.........................L...................

Equine     361 CGCAILMTVALLLLERVPAMSYVSIVAIFGFVAFFEIGPGPIPWFIVAELFSQGPRPAAM
Human      361 ............................................................
Bovine     361 .A.....................................C.....................
Rat        361 ..................S.....................................
Mouse      361 ............................................................

Equine     421 AVAGFSNWTCNFIIGMGFQYVADAMGPYVFLLFAVLLLGFFIFTFLRVPETRGRTFDQIS
Human      421 .........S............E...................................
Bovine     421 ..............................................K.............
Rat        421 ............V.....................................
Mouse      421 ............V.............................K.............

Equine     481 AAFHRTPSLLEQEVKPSTELEYLGPDEND
Human      481 ............................
Bovine     481 .V.......................H.
Rat        481 .T.R.......................
Mouse      481 ...R........................
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Figure 2—Alignment of the deduced amino acid sequence of equine GLUT-4 with known complete sequences of other mammals. Numbers on the left represent amino acids beginning with methionine. Periods indicate amino acids identical to those in equine GLUT-4.

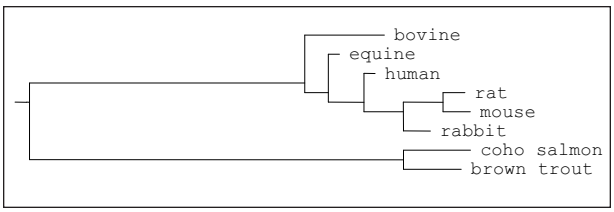

Figure 3—Phylogeny dendrogram calculated for known complete GLUT-4 amino acid sequences of various vertebrates. Two branches are evident; 1 represents mammalian GLUT-4, and the other represents lower vertebrates.

Results

Characterization of equine GLUT-4 cDNA—Equine GLUT-4 nucleotide and deduced amino acid sequences were determined (**Figure 1**).[r] The 1,623 bp of sequenced cDNA contained 7 bp of 5Â **untranslated region (UTR)**, 1,530 bp of open reading frame (from 8 to 1,537 inclusive), and 86 bp of 3Â UTR. Similarly to other species, the equine GLUT-4 cDNA encoded 509 amino acids. The calculated molecular weight of the resulting protein was 54.8 kd, and the isoelectric point was 7.17. The nucleotide and deduced amino acid sequences of equine GLUT-4 were compared with other mammals for which complete mRNA sequences are available (**Figure 2**). Analysis of the amino acid sequence deduced from equine GLUT-4 cDNA suggested a secondary structure consisting of 12 transmembrane helices, which was identical to other species. A phylogeny dendrogram for known complete GLUT-4 amino acid sequences was constructed (**Figure 3**), and revealed that equine GLUT-4 was closely related to that of other mammals. Nucleotide and deduced amino acid sequences of equine GLUT-4 were > 85% identical to that of other mammalian species with known GLUT-4 cDNA sequences (**Table 2**).

Plasma glucose, serum immunoreactive insulin, and muscle glycogen—Results of plasma substrates and hormones, muscle glycogen, and whole body glucose turnover rates in this study are reported in greater detail elsewhere.[28] In brief, feeding type after exercise significantly affected plasma glucose and

serum insulin concentrations after exercise (comparison of main effects: corn vs unfed ($P < 0.001$), hay vs unfed ($P = 0.03$), and corn vs hay [$P = 0.07$; **Table 3**]). Feeding 2 meals of cracked corn that provided approximately half the daily energy requirements immediately and 4 hours after exercise resulted in an approximately 20% increase in plasma glucose concentrations, verses unfed horses (corn feeding compared with feed withholding from 2 to 8 hours after exercise [$P < 0.01$]). Peak serum insulin concentration was higher in corn-fed horses, compared with feed withholding (at 2 hours, corn vs unfed [$P < 0.01$]). Three consecutive days of exercise decreased muscle glycogen concentrations by 67 ± 3% of the initial values (prior to exercise vs other time points after exercise [$P < 0.001$]). Muscle glycogen concentration at 24 hours was higher than at 0 hours in grain-fed horses ($P = 0.04$), whereas nonsignificant increases were observed in the other trials.

Muscle GLUT-4 mRNA—Muscle glycogen-depleting exercise, but not feeding type, significantly altered GLUT-4 mRNA levels in skeletal muscle (main effects: time [$P < 0.001$], treatment [$P = 0.4$], and interaction [$P = 0.9$]; **Figure 4**). All horses had a higher number of GLUT-4 transcripts in skeletal muscle at 4 hours after exercise, compared with prior to exercise (both with and without normalization for β-actin expression). Expression of GLUT-4 gene gradually decreased and by 24 hours after exercise, was similar to that prior to exercise ($P = 0.3$). β-Actin mRNA concentrations in skeletal muscle had an inverse relationship with GLUT-4 mRNA concentrations. β-Actin gene expression was lower at 4 hours postexercise, compared with prior to and 24 hours after exercise (pre vs 4 hours [$P = 0.002$] and 4 vs 24 hours [$P = 0.03$]). For this reason, GLUT-4 mRNA concentrations were reported with and without normalization for β-actin gene expression.

Table 2—Nucleotide and deduced amino acid (aa) sequence identity (%) between equine GLUT-4 (National Center for Biotechnology Information [NCBI] accession No. AF531753) and the homologous sequences of several other mammals.

Species	NCBI accession No.	Identity cDNA	Identity aa
Human	NM_001042.1	92	96
Mouse	AB008453.1	88	94
Rat	X14771.1	88	94
Cattle	D63150	90	90
Rabbit	AY339876	91	94
Pig*	AF141956	91	93
Dog*	AJ388533	91	94
Sheep*	AB005283	86	94
Goat*	AB005284	85	94
Buffalo*	AF254423	92	96

*Partial sequences. Identity calculated by use of the corresponding partial equine GLUT-4.

Table 3—Mean ± SEM plasma glucose, serum immunoreactive insulin, and muscle glycogen concentrations before a glycogen-depleting exercise protocol (Pre) in horses and after the third consecutive day of exercise (0, 2, 4, 8, and 24 hours).[28] For 8 hours after exercise, horses had feed withheld or were fed 2 meals of corn (approx 2.2 kg and approx 7.4 Mcal digestible energy each meal; n = 6) or 2 isocaloric meals of hay (approx 3.4 kg each meal; 5). All horses were fed hay from 8 to 24 hours after exercise.

	Time					
Variable	Pre	0 h	2 h	4 h	8 h	24 h
Glucose (mM)						
Not fed	4.7 ± 0.2	4.3 ± 0.3	4.7 ± 0.3	4.8 ± 0.2	4.9 ± 0.3	5.3 ± 0.2
Hay	4.8 ± 0.1	4.7 ± 0.2	4.9 ± 0.0*	5.1 ± 0.1	5.1 ± 0.3	5.2 ± 0.3
Corn	4.9 ± 0.2	4.6 ± 0.4	5.5 ± 0.2†	5.6 ± 0.2†	5.6 ± 0.2†	5.1 ± 0.2
Insulin (pM)						
Not fed	51.9 ± 8.6	36.1 ± 1.0	39.0 ± 1.9	40.9 ± 3.6	37.1 ± 1.0	44.3 ± 3.9
Hay	58.9 ± 13.4	36.4 ± 0.2	53.9 ± 5.5‡	58.9 ± 11.2	54.7 ± 7.8	54.8 ± 12.8
Corn	69.4 ± 22.4	37.0 ± 0.4	79.9 ± 9.3†	64.4 ± 12.7	62.9 ± 17.1	46.8 ± 4.9
Glycogen (mmol · kg^{-1}[dw])						
Not fed	499 ± 32§	171 ± 19	ND	231 ± 48	222 ± 32	260 ± 45
Hay	556 ± 35§	205 ± 37	ND	265 ± 41	237 ± 28	231 ± 21
Corn	563 ± 53§	170 ± 56	ND	226 ± 60	247 ± 23	294 ± 29‖

*Hay feeding significantly ($P < 0.05$) lower than corn feeding. †Corn feeding significantly ($P < 0.05$) higher than not fed. ‡Hay feeding significantly ($P < 0.05$) higher than not fed. §Significantly ($P < 0.001$) greater muscle glycogen concentration before exercise than all other time points after exercise in all trials.‖ Significantly ($P = 0.04$) greater muscle glycogen concentration at 24 hours than that at 0 hours in grain-fed horses. ND = Not done. dw = Dry weight.

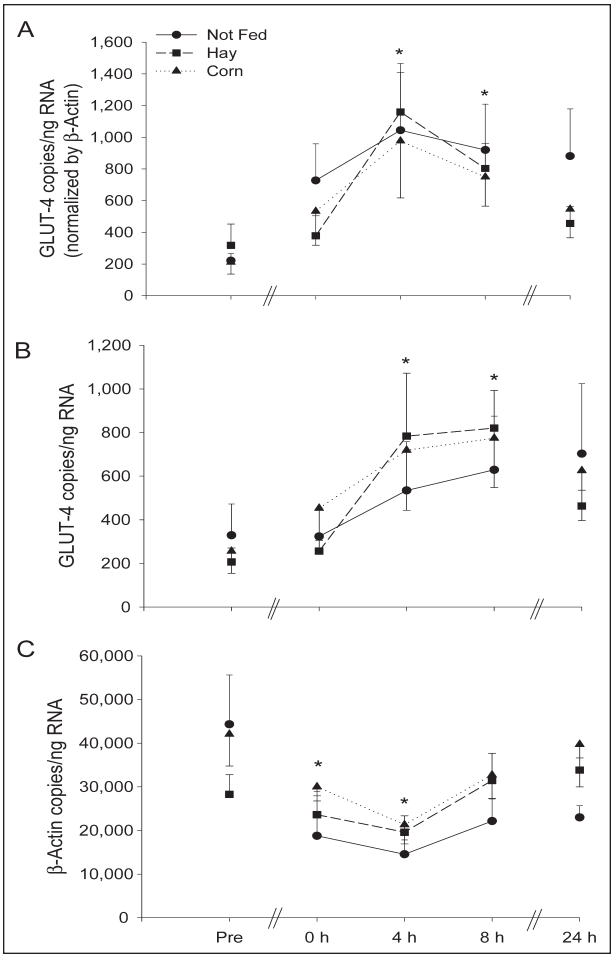

Figure 4—Mean ± SEM number of muscle GLUT-4 transcripts normalized for β-actin gene expression (A), number of muscle GLUT-4 transcripts without correction for β-actin gene expression (B), and number of muscle β-actin transcripts (C) before 3 consecutive days of exercise (Pre) and immediately (0 hour) and 4, 8, and 24 hours after exercise in horses. Treatments were grain feeding (n = 6 horses) or isocaloric hay feeding (5) immediately and 4 hours after exercise or feed withholding (6). *Significantly ($P < 0.05$) different, compared with prior to exercise.

Discussion

The results reported here confirm that the equine GLUT-4 gene has a high degree of homology with that in other mammals. In agreement with our hypothesis, equine GLUT4 gene expression in skeletal muscle increased in the hours after exercise. However, ingestion of carbohydrates as starch-rich meals did not enhance GLUT-4 gene expression in muscle, compared with isocaloric fiber-rich meals or feed withholding.

Previous studies in humans and rodents revealed that expression of GLUT-4 mRNA is subject to tissue-specific, hormonal, and metabolic regulation. In general, exercise and carbohydrate supplementation result in increased GLUT-4 gene expression in skeletal muscle, measured as mRNA concentrations or total GLUT-4 protein content.[31] Conversely, diabetes and fasting decrease GLUT-4 mRNA concentrations in adipose tissue, and diabetes, but not fasting, decreases GLUT-4 mRNA in skeletal muscle.[7] To our knowledge, this is the first study that reveals an increase in GLUT-4 mRNA in equine skeletal muscle associated with glycogen-depleting exercise. In contrast, a previous study[32] conducted by our laboratory did not reveal an increase in GLUT-4 protein or mRNA in equine muscle after a single bout of exercise. The apparent discrepancy may be attributable to differences in the exercise protocols, degree of muscle glycogen depletion (30% depletion in the previous study vs > 60% in the present study), and analytical techniques (northern blot analysis with a human GLUT-4 cDNA probe in the previous study vs real-time RT-PCR with equine-specific primers in the present study).

Carbohydrate supplementation after exercise in rats results in enhanced muscle glycogen replenishment, increased muscle GLUT-4 protein content, and blunted increase in muscle GLUT-4 mRNA, compared with exercised rats after withholding of food.[33] Therefore, it was suggested that carbohydrate supplementation after exercise downregulates GLUT-4 transcription while simultaneously increasing GLUT-4 translation, which was mediated possibly by changes in circulating insulin concentrations. In contrast, results of a study[34] performed in sedentary rats infused with glucose and insulin revealed increased muscle GLUT-4 protein content and mRNA concentration. In our study, carbohydrate supplementation by ingestion of starch-rich meals in horses resulted in higher insulin concentrations, compared with unfed horses; however, muscle glycogen replenishment and GLUT-4 mRNA concentrations were not different regardless of the feeding state. In contrast to that seen in rodents, horses that receive carbohydrate supplementation by IV or oral glucose administration after exercise have decreased[20] or unchanged[32] muscle GLUT-4 protein content, compared with exercised horses that do not receive supplements. Therefore, regulation of GLUT-4 transcription and translation in horses in relation to exercise and carbohydrate supplementation bears some similarities but cannot be directly extrapolated from that seen in other species.

Transport of glucose through the sarcolemma appears to be the primary rate-limiting step in glucose uptake by muscle and muscle glycogen synthesis after exercise.[8,35] In the absence of stimuli such as exercise or insulin, > 90% of GLUT-4 remains inactive, sequestered in intracellular vesicles. Insulin and exercise can independently stimulate translocation of GLUT-4 from intracellular storage pools to the sarcolemma and T-tubules to facilitate glucose uptake.[36] Therefore, determination of total GLUT-4 protein content does not account for changes in the fraction of membrane-bound GLUT-4 versus inactive GLUT-4 sequestered intracellularly. One may speculate that carbohydrate supplementation after exercise in horses may not increase total GLUT-4 protein content or GLUT-4 mRNA but may increase the fraction of membrane-bound GLUT-4 that allows glucose to be transported into the sarcolemma. To determine translocation of GLUT-4 within the cell, separation of cellular components is required. This procedure was not performed in our study. The nucleotide and deduced amino acid sequence information and methodology for quantitative determination of GLUT-4 mRNA described in this study may serve in further studies of physiologic adaptations and pathophysiologic mechanisms involving glycogen metabolism, insulin resistance, and muscle energetics in horses.

a. RNAwiz, Ambion, Austin, Tex.
b. DNAfree, DNase treatment and removal reagents, Ambion, Austin, Tex.
c. Vector NTI Suite 5.5, Informax Inc, Frederick, Md.
d. Thermoscript RT-PCR system for first strand cDNA synthesis, Invitrogen Corp, Carlsbad, Calif.
e. Platinum *Taq* DNA polymerase, Invitrogen Corp, Carlsbad, Calif.
f. pCR2.1 TA cloning kit, Invitrogen Corp, Carlsbad, Calif.
g. DH5α chemically competent *E coli*, Invitrogen Corp, Carlsbad, Calif.
h. EcoRI, Invitrogen Corp, Carlsbad, Calif.
i. ABI PRISM 3700 DNA Analyzer, Applied Biosystems, Foster City, Calif.
j. Align Plus 4.0, Sci-ed Software, Durham, NC.
k. Kindly provided by Dr. D. W. Horohov, Louisiana State University, Baton Rouge, La. Equine β-actin cDNA (NCBI GenBank accession No. AF035774) was used to generate β-actin RNA standards via in vitro transcription.
l. Lightcycler system, Roche Diagnostics Corp, Mannheim, Germany.
m. QuantiTect SYBR green RT-PCR kit, Qiagen Inc, Valencia, Calif.
n. Lightcycler instrument, Roche Diagnostics Corp, Mannheim, Germany.
o. MaxiScript in vitro transcription kit, Ambion, Austin, Tex.
p. Angiocath (14 gauge, 5.25 inches), Becton-Dickinson, Franklin Lakes, NJ.
q. SPSS 12.0, SPSS Inc, Chicago, Ill.
r. Sequence information submitted to the NCBI GenBank database as accession No. AF531753. Available at: www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=45602823.

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