



Molecular Characterization and Expression Analysis of the Peroxisome Proliferator Activated Receptor Delta (*PPAR δ*) Gene before and after Exercise in Horse

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ABSTRACT: While athletic abilities such as speed, endurance and recovery are important in the horse, genes related to these abilities have not been extensively investigated. Here, we characterized the horse peroxisome proliferator-activated receptor delta (*PPAR δ*) gene and analyzed the expression of *PPAR δ* during exercise. *PPAR δ* is a known regulator of β -oxidation, muscle fiber transformation, and running endurance. Through evolutionary analysis using the synonymous and non-synonymous mutation ratio, it was revealed that positive selection occurred in the horse *PPAR δ* gene. Two important domains related to nuclear hormone receptors, C4 zinc finger and ligand binding domain, were also found to be conserved well in horse *PPAR δ* . Horse *PPAR δ* was expressed ubiquitously in many tissues, but the expression level was various depending on the tissues. In the skeletal muscle, *PPAR δ* increased about 2.5 folds after 30 min of exercise. Unlike in muscle, the increase of *PPAR δ* expression was observed at 60 min but not 30 min of exercise in leukocytes. This finding might be useful for testing the endurance of horse using blood samples. Conclusively, the horse *PPAR δ* gene is evolutionarily conserved well and can be used as a biomarker of endurance in horse. (**Key Words:** Horse, Peroxisome Proliferator-activated Receptor Delta, Exercise, Endurance)

INTRODUCTION

Many economic traits including speed, robustness, and endurance of racing horses are related to exercise. Previously, whole transcriptome analysis using RNA-sequencing in six thoroughbred horses before and after

exercise was performed to identify exercise-related genes (Park et al., 2012). Differentially expressed gene analysis of skeletal muscle and blood using RNA-sequencing of selected genes regarded as associated with economically important traits of horses has become an interest for the purposes of selection and improvement.

Peroxisome proliferator-activated receptors (*PPAR*) are members of the nuclear receptor superfamily and was first identified in xenopus (Krey et al., 1993). *PPARs* can be activated and cooperate with thyroid hormone and retinoid X receptors by the formation of a heterodimer complex for transcriptional regulation of target genes (Wahli et al., 1995). *PPARs* play essential roles in the regulation of cellular differentiation, development, metabolism (carbohydrate, lipid, protein), and tumorigenesis of higher organisms (Berger and Moller, 2002; Belfiore et al., 2009). Among the *PPARs*, *PPAR δ* has an important role in fat metabolism in muscle; in knockout mice studies, it has been shown that it activates oxidation of fatty acid in muscle

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while increases conversion of glucose to fatty acid in liver (Lee et al., 2006). The overexpression of *PPAR δ* resulted in an increased oxidative metabolism and hyperplasia causing the change of fiber composition in mouse muscle (Luquet et al., 2003). Together with its metabolic role in muscle, *PPAR δ* acts as an important regulator of satellite cell proliferation and muscle regeneration (Angione et al., 2011).

According to previous studies, expression of *PPAR δ* mRNA and protein is increased in the skeletal muscle after endurance exercise (Luquet et al., 2003; Mahoney et al., 2005). *PPAR*'s expression in skeletal muscle is related to the late induction of exercise-induced responses during endurance training, such as increased oxidative capacity of type I muscle fibers and mitochondrial biogenesis (Wang et al., 2004). The overexpression of *PPAR δ* in mouse skeletal muscle induces the up-regulation of gene expression related to slow fiber contractile proteins, mitochondrial biogenesis, and β -oxidation. Therefore, the rapid and regulated changes of *PPAR δ* expression could improve physical performance.

Despite extensive and numerous studies on the function and expression of the *PPAR δ* gene in various species, it has not been researched in horses. The objective of this study was to characterize the horse *PPAR δ* gene at the molecular level by performing bioinformatics and the real-time polymerase chain reaction (RT-PCR) analysis, and to investigate the transcriptional regulation of *PPAR δ* gene after exercise and the possibility of *PPAR δ* as a biomarker.

MATERIALS AND METHODS

Bioinformatic analysis of horse peroxisome proliferator-activated receptor delta gene

The coding DNA and amino acid sequences of *PPAR δ* of various species (horse, human, chimpanzee, mouse, rat, cow, pig, chicken, dog, and cat) were retrieved from Ensembl (<http://www.ensembl.org/>). The amino acid sequences were aligned using ClustalW in the BioEdit software. The protein domains were predicted by using the SMART domain search program (<http://smart.embl-heidelberg.de/>). Phylogenetic analysis data was obtained by using the Neighbor-Joining method (Saitou and Nei, 1987) in MEGA software (Tamura et al., 2011). The parameters were as follows: pairwise-deletion, 1000 bootstrap replications, and Kimura 2. Pairwise deletion was chosen to retain all such sites initially, excluding them as necessary in the pairwise distance estimation. The group within tree was used for 1000 bootstrap replications to derive the reliability value. Substitutions of nucleotides were obtained by using the Kimura 2-parameter model. We also showed synonymous substitutions per site (Ks) and non-synonymous substitutions per site (Ka) in *PPAR δ* gene for individual variation.

Horse blood and tissue samples

Blood and muscle samples were collected from three thoroughbred horses, which were 2 to 4 year-old geldings and maintained at Ham-an Racing Horse Resort & Training Center. Samples were collected before and after 30 min and 60 min of trotting. Exercise was performed about 13 km/h of speed on a treadmill. The National Institute of Subtropical Agriculture, Rural Development Administration, Republic of Korea, provided three Jeju horses which were used for tissue sampling. Skeletal muscle, kidney, thyroid, lung, appendix, colon, spinal cord and heart tissues were kept in a liquid nitrogen tank until RNA extraction. Animal care and sample collection were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Pusan National University (PNU-2014-0644 and PNU-2014-0645).

RNA extraction and cDNA synthesis

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from tissue samples and leukocytes according to the manufacturer's manual. In order to prevent contamination of genomic DNA, all RNA samples were treated with RNase-free DNase (Qiagen, Venlo, Netherlands). Next, the RNA samples were quantified by using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The cDNA was synthesized from the each RNA sample by moloney-murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) in a reaction containing oligo-dT primers and RNase inhibitor (Promega, USA) for 4 hours incubating at 37°C.

Real time-quantitative polymerase chain reaction analysis

Firstly, PCR was conducted to verify the removal of genomic DNA and the synthesis of cDNA. The *PPAR δ* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) primer sets for PCR were designed by using the PRIMER3 software (<http://frodo.wi.mit.edu/primer3/>). The sequences of *PPAR δ* primers were 5'- AGC AAC CTC TTC CTC AAC GA-3' for forward primer and 5'- GAG GCT GCG TAG GAA CTC AC-3' for reverse primer. The PCR product size of *PPAR δ* was expected to be 150 bp. The PCR conditions consisted of an initial step of 94°C for 10 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and a final step of 72°C for 10 min. The PCR products were analyzed by electrophoresis on a 2.0% Seakem LE agarose gel.

To quantify the *PPAR δ* gene in horse tissues and leukocytes, RT-quantitative polymerase chain reaction (qPCR) was conducted by using CFX-96 real-time PCR (BioRad, Hercules, CA, USA). Each reaction was executed in 25 μ L of a total volume containing 14 μ L of SYBR green

master mix, 2 µL of forward primer (5 pmol), 2 µL of reverse primer (5 pmol), 5 µL of distilled water, and 2 µL of the cDNA (50 ng/µL). The PCR conditions were as follows: a pre-denaturation step at 94°C for 3 min, 39 cycles at 94°C for 10 s, 60°C for 30 s, and 72°C for 30 s, followed by a final step at 72°C for 10 min. Dissociation was accomplished in conditions where temperature increased from 60°C to 95°C for over 25 min (linear increase of 0.5°C). All samples were measured in triplicate to ensure reproducibility, and the expression levels were calculated from Ct value by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The *GAPDH* gene was used for reference.

Statistical analysis of gene expression

The significance (p<0.05) of data was determined using Student’s t-test or analysis of variance method. The difference among each value was determined by Tukey’s test with alpha = 0.05. All data values are presented as mean ±standard error of mean.

RESULTS AND DISCUSSION

The horse *PPARδ* gene consisted of eight exons with a total length of 1,644 bp. The translation of *PPARδ* starts in the third exon and stops at the last exon. In comparing the nucleotide sequences in various species (human, chimpanzee, mouse, rat, cow, pig, chicken, dog, cat), the synonymous substitution (Ks) ranged from 0.363 to 1.330, whereas the non-synonymous substitution (Ka) ranged from 0.407 to 1.553 in horse (Table 1). It is of note that the lowest (0.407) and highest (1.553) differences of Ka of horse *PPARδ* were obtained when horse compared with cat and horse compared with mouse respectively. Human and chimpanzee are of similar sequence and/or amino acid, showing the lowest Ks value (0.018). The average Ka value was 1.056 and that of Ks was 0.914, resulting to a Ka/Ks ratio greater than 1. This indicates that non-synonymous

substitutions occurred faster than synonymous substitutions. These results are based on the assumption that the protein-coding regions in the human genome undergo positive selection during evolution (Bustamante et al., 2005). Results indicate that positive selection occurred in the *PPARδ* genes during evolution in horses. This ratio also suggests possible evolution of the *PPARδ* gene in various species.

To characterize horse *PPARδ* protein, amino acid sequences of *PPARδ* from various species were aligned and analyzed. The *PPARδ* amino acid sequences are highly conserved between the species except for the N-terminal of the sequence (Figure 1A). In domain search using the SMART program, it was revealed that horse *PPARδ* protein has two highly conserved domains, C4 zinc finger and ligand binding domains of nuclear hormone receptors. In a phylogenetic analysis with the Neighbor-Joining method, horse *PPARδ* is clustered in the same clade with dog and chicken while having a large genetic distance from mouse and rat (Figure 1B).

Next, we performed RT-PCR analysis using eight different tissues from three horses to examine the gene expression pattern. All horse tissues expressed the *PPARδ* gene ubiquitously, and *PPARδ* was highly expressed in thyroid and lung while relatively low in skeletal muscle (Figure 2). It is of note that *PPARδ* was highly expressed in thyroid cells unlike other *PPAR* genes such as *PPARα* and *PPARγ* (Bookout et al., 2006). High expression of the *PPARδ* in thyroid and lung also corresponds to a previous expression study in xenopus (Krey et al., 1993). In human, the expression of *PPARδ* in primary thyroid cells was induced by serum, thyroid-stimulation hormone, and cyclic AMP (3'-5'-cyclic adenosine). *PPARδ* is up-regulated in human thyroid tumors and induces cell proliferation through the cyclin E1. In this sense, increased expression of *PPARδ* and Ki-67 is known as an *in situ* proliferation marker of benign and malignant human thyroid tumors

Table 1. Synonymous (Ks) and non-synonymous (Ka) substitutions per site in the *PPARδ* gene of various species

Ks	Ka									
	Horse	Human	Chimpanzee	Mouse	Rat	Cow	Pig	Chicken	Dog	Cat
Horse	-	0.859	0.855	1.553	1.521	0.507	0.897	1.452	0.439	0.407
Human	0.766	-	0.021	1.508	1.471	0.484	0.987	1.620	0.892	0.686
Chimpanzee	0.769	0.018	-	1.513	1.488	0.480	0.993	1.627	0.877	0.681
Mouse	1.330	1.266	1.262	-	0.258	1.020	1.555	1.522	1.565	1.174
Rat	1.292	1.245	1.245	0.246	-	0.957	1.505	1.537	1.514	1.062
Cow	0.500	0.445	0.443	0.905	0.847	-	0.534	1.577	0.507	0.622
Pig	0.798	0.874	0.875	1.325	1.271	0.512	-	1.61	1.024	0.692
Chicken	1.245	1.400	1.400	1.280	1.334	1.331	1.253	-	1.606	1.447
Dog	0.407	0.795	0.783	1.291	1.244	0.491	0.889	1.338	-	0.433
Cat	0.363	0.593	0.597	1.038	0.964	0.622	0.593	1.252	0.394	-

PPARδ, peroxisome proliferator-activated receptor delta.

The numbers displayed in the table are the Ka and Ks substitutions between the corresponding species.

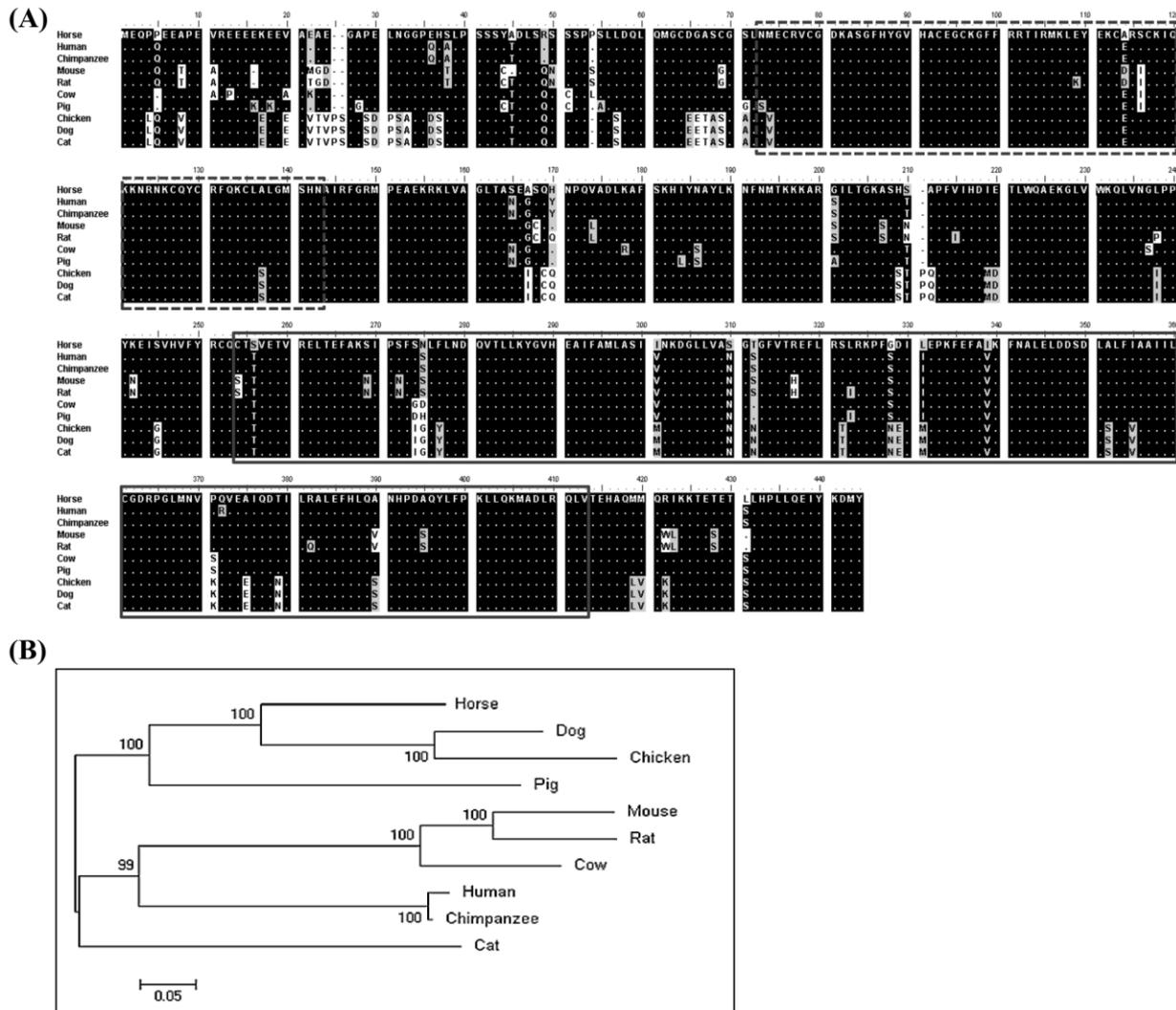


Figure 1. Analysis of horse *PPARδ* amino acid sequences. (A) Comparison of amino acid sequences of horse *PPARδ* in various species. In SMART domain prediction, it is revealed that C4 zinc finger (dashed box) and ligand binding (solid box) domains of nuclear hormone receptors were conserved in horse *PPARδ*. (B) Phylogenetic tree of *PPARδ* in various species. *PPARδ*, peroxisome proliferator-activated receptor delta.

(Zeng et al., 2008), where the mean expression of native *PPARδ* was increased by 2-fold to 5-fold and correlated with that of the *in situ* proliferation marker Ki67 in six different classes of benign and malignant human thyroid tumors.

To identify the relation between exercise and *PPARδ* gene expression levels, the expression pattern of the gene was analyzed in skeletal muscle and leukocytes of horse. In skeletal muscle, *PPARδ* was highly expressed about 2.5 fold at 30 min of exercise (Figure 3A, $p < 0.001$). In leukocytes, expression levels of *PPARδ* were analyzed by RT-qPCR with the samples obtained before and after 30 min and 60 min of exercise (Figure 3B). Unlike skeletal muscle, which showed higher expression at 30 min of exercise, the expression of *PPARδ* increased slowly and was significantly higher at 60 min of exercise in leukocytes. It is well known that endurance exercise increases expression of *PPARδ* gene

in the muscle of mouse and human (Luquet et al., 2003; Wang et al., 2004; Mahoney et al., 2005). This expression pattern of *PPARδ* was also observed in horse muscle and even in the leukocytes although it took a longer time than in muscle. The increase of *PPARδ* expression in horse muscle might induce the oxidative metabolism to supply the energy required for endurance exercise. As the leukocytes are relatively feasible method of collecting samples from the living horse, analysis of *PPARδ* expression in leukocytes may be a useful tool for indirectly scoring the muscle fatigue.

CONCLUSION

In conclusion, the horse *PPARδ* gene is well conserved evolutionarily and has functional domains found in other animals. The expression of *PPARδ* gene is regulated by

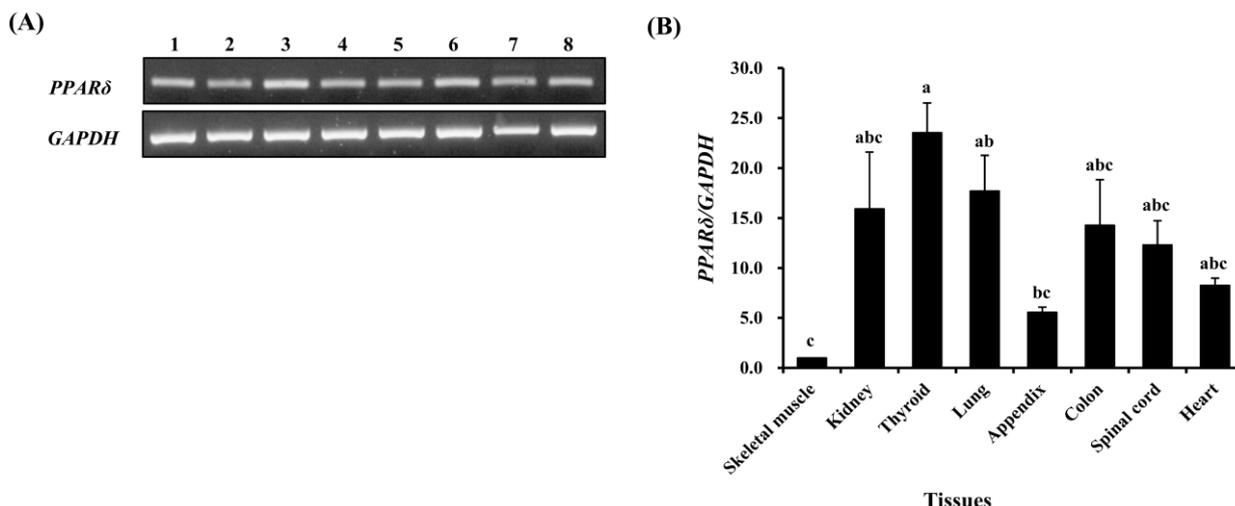


Figure 2. Analysis of horse *PPARδ* expression in various tissues of Jeju horse. (A) Expression of horse *PPARδ* gene in various tissues. 1: skeletal muscle, 2: kidney, 3: thyroid, 4: lung, 5: appendix, 6: colon, 7: spinal cord, and 8: heart. The bands are saturated by running the polymerase chain reaction for 35 cycles. (B) Determination of expression levels of horse *PPARδ* in horse tissues. Expression value was calculated using the $2^{-\Delta\Delta Ct}$ method. The values are presented by mean±standard error of mean. The expression levels of the gene were various in tissues (n = 3, p<0.01). The difference of each value was determined by Tukey’s test and the bars containing same letter on the top are not significantly different among them (alpha = 0.05). *GAPDH* was used for normalization. *PPARδ*, peroxisome proliferator-activated receptor delta; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

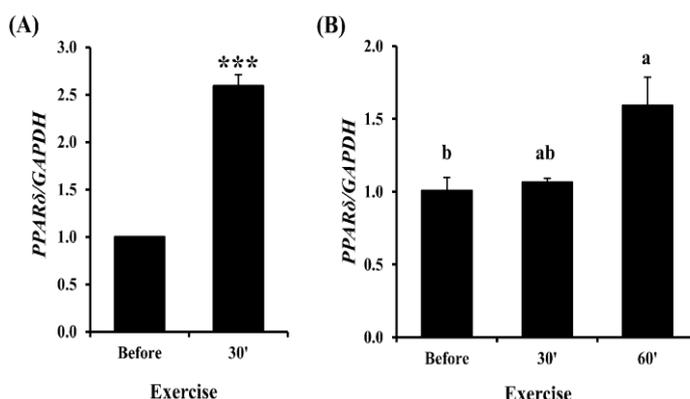


Figure 3. Analysis of *PPARδ* expression before and after exercise in skeletal muscle and leukocytes. (A) Expression level of *PPARδ* before and at 30 min after exercise in horse skeletal muscle. The expression of *PPARδ* increased about 2.5 fold at 30 min of exercise in skeletal muscle (n =5). *** means p<0.001. (B) Expression analysis of horse *PPARδ* before and after exercise in leukocytes. Expression value was calculated using the $2^{-\Delta\Delta Ct}$ method. The values are presented by mean±standard error of mean. The expression levels of *PPARδ* was significantly higher after 60 min of exercise than before exercise (n = 3, p<0.05). The difference of each value was determined by Tukey’s test and the bars containing same letter on the top are not significantly different (alpha = 0.05). *GAPDH* was used for normalization. *PPARδ*, peroxisome proliferator-activated receptor delta; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

endurance exercise in muscle and leukocytes, and this gene could be a useful biomarker to measure endurance and fatigue. In further studies, the analysis of gene expression related to oxidation, mitochondrial biogenesis, and enhancement of physical performance by *PPARδ* after exercise might be useful for understanding the relationship between *PPARδ* and athletic ability in horse.

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