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EVALUATION OF GENE EXPRESSION IN ENDURANCE HORSES BY REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

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Introduction

The knowledge of molecular mechanisms of stress response is necessary in athlete horse management to obtain high performance and to preserve the animals from the so called ‘‘overtraining syndrome’’.
Endocrinal and immunological alterations, in response to a number of different exercise protocols, are well documented but to better define the relationship among exercise, immune response and gene regulation we decided to analyze detailed transcript profiles of endurance horses.
In animal species for which little genomic information is available, a DNA fragment analysis based method, such as cDNA-AFLP (Amplified Fragment Length Polymorphisms), provides an appropriate tool for genome-wide expression research.
In our previous studies, the cDNA-AFLP technique was applied to two Arab horses under different stressing conditions to visualise variations in transcriptional profiles.
Database search analysis (BLAST, National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) of 49 differentially expressed cDNA-AFLP fragments evidenced sequence similarity with genes known to be important in normal cell growth, proliferation and metabolism (see Verini-Supplizi et al., 2003). The similarities of four messengers were particularly interesting for our aims because they strongly correlated to physical stress.
In the present study, we applied RT-PCR to these four fragments to confirm the gene modulations and RACE-PCR to obtain a full-length cDNA clones.
Moreover, we analysed the expression of the genes considered in two other horses during an endurance race (160 Km) to confirm the variations observed.

Materials and methods

Four horses were tested during a 160 Km European Endurance Riding Championship. Blood samples were collected just before the ride and at 0, 24 and 48 hours after the competition. Peripheral blood mononuclear cells (PBMCs) were isolated by the Ficoll-Hypaque method (Amersham Pharmacia Biotech). Total RNA was isolated by TRIzol reagent (Life Technology).

Retrotrancription Polymerase Chain Reaction (RT-PCR)
3µg of total RNA derived from the lymphocytes of 4 horses were subjected to RT-PCR with the specific primers of four cDNA-AFLP fragments that had high sequence similarity with messengers modulated by the stress. cDNA was synthesised utilising a Smart cDNA kit *(Clontech BD Bioscience)* and amplification of *Equus caballus* βActin cDNA fragment (accession number GI: 2661135 of the National Center for Biotecnology Information) was used as an internal control of the reactions.
Amplification products were separated on 2% agarose gel electrophoresis and then blotted on Hybond N membrane *(Amersham Pharmacia Biotech, Little Chalfont, UK).*
Probes were synthesized by PCR utilizing 5ng of plasmidic DNA,10 µci of ^{32}P-dCTP and specific primers of cDNA. Hybridations were performed at 55°C for 16h and filters exposed to KODAK X-AR film (Kodak BioMax Film) O/N at –70°C.

Rapid Amplification of cDNA ends (RACE)
5 µg of the total RNA pooled from the lymphocytes analysed with cDNA-AFLP and RT-PCR were submitted to Rapid Amplification of cDNA Ends PCR (RACE-PCR, BD Bioscience) to clone the full-length of the cDNA fragments. The resulting cDNAs were cloned (PCR4 topo for sequencing, InVitrogen) and sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit on the automatic DNA Sequencers 377 (Applied Biosystems). Resulting sequences of 3' and 5' RACE clones were aligned using Vector NTI software (Informax-InVitrogen) to obtain full-length cDNA. Entire clones were analyse with BLAST X program on National Centre for Biotechnology Information (NCBI) data base.

Results
The expression profile of four cDNA-AFLP fragments was confirmed by RT-PCR as shown in figure 1.

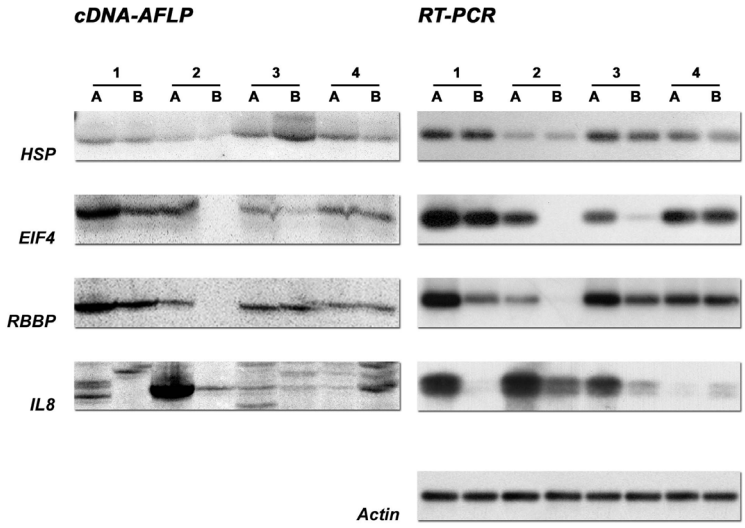

Fig. 1 Expression profile of cDNA-AFLP isolated transcripts detected by RT-PCR from lymphocytes total RNA of two endurance horses (A,B) at different time points. Lanes: **1**: at rest; **2**: immediately a.c. (after competition); **3**: 24 hours a.c. ; **4**: 48 hours a.c.. *Equus caballus* βActin was used as internal control.

Modulation of the same transcripts was also confirmed in the other two horses (C and D; figure 2).

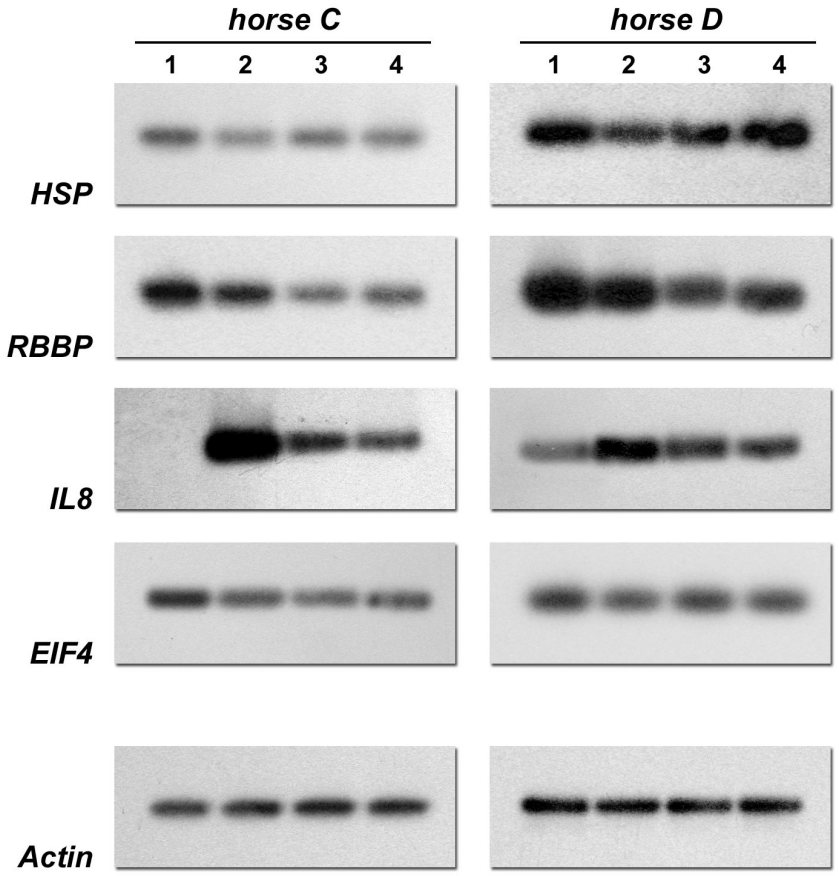

Fig. 2 Expression profile of four transcripts detected by RT-PCR from lymphocytes total RNA of two endurance horses (C,D) at different time points. Lanes: **1**: at rest; **2**: immediately a.c. (after competition); **3**: 24 hours a.c. ; **4**: 48 hours a.c.. *Equus caballus* βActin was used as internal control.

These fragments, showing a significant similarity with non sequenced genes in horses, were cloned full-length using Rapid Amplification of cDNA Ends PCR (RACE-PCR, BD Bioscience) and included in the GenBank database:
Equus caballus interleukin 8 mRNA, AY184956;
Equus caballus retinoblastoma binding protein 6 mRNA, AY184957;
Equus caballus heat shock protein 90 mRNA, AY383484;
Equus caballus eukaryotic translation initiation factor 4 gamma 3 mRNA, AY484519.

Discussion and Conclusion

Endurance horses represent a valid model to study the effects of exhaustive stress for their training procedure and agonistic longevity which are comparable to a human marathoner.

The semi-quantitative response in the cDNA-AFLP system seems to be broadly proportional to the input of cDNA and its sensitivity is confirmed by RT-PCR experiments in the horses A and B. Furthermore, the reliability of our cDNA-AFLP fragment database search results was confirmed by full-length cDNA multialignment analysis on the same database. Moreover, the same modulation of these genes was observed in horses C and D strengthening the hypothesis of their involvement in exercise stress-induced response.

The over-expression of gene encoding for IL-8 after the race confirm the human sport medicine results even if the most concentrate cytokine is IL-6 in blood after exercise. However, the transcription of interleukin-6 is a prerogative of muscle tissue; therefore it is not possible to isolate its mRNA from blood tissue.

The heat shock protein Hsp90 is down regulate by exercise in these four horses.

Hsp90 is a highly abundant protein in cells, understating its functional complexity, it is classified as a cellular molecular chaperone and its role is critical in numerous fundamental physiological processes. Its chaperone activities range from interacting with the specific proteins/chaperones involved in cell cycle control and hormone signaling, respectively. It has been demonstrated in human lymphocytes that its inhibition leads to an arrest of the G1-to–S phase of the cell cycle induced by cyclin D2 an so an retinoblastoma dependent arrest. It is interesting because our mRNA retinoblastoma binding protein 6 (a potential proliferation protein that binds RB protein) is expressed preferentially at rest. It might be possible that during physical stress the decrease in these two messengers induce a reduction in lymphoproliferative response and this would be a cause of stress-related immunodepression.

The eukaryotic initiation factor eIF4G is expressed predominantly at rest. It is a large modular protein which serves as a docking site for initiation factors and proteins involved in RNA translation. Thus, it is possible that also strenuous physical effort leads to an inhibition in transcription due to the activation of apoptotic pathways.

In conclusion we have visualised the same modulation of analysed genes in four horses during two different races. However, further studies are needed to confirm the involvement of these genes in stress-related response increasing the number of horses and testing different experimental conditions.

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