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Molecular study of ovine glucose 6-phosphate dehydrogenase gene expression in respect to different energy intake

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ABSTRACT

Glucose 6-phosphate dehydrogenase (G6PD) plays an important role in a ruminant's metabolism catalyzing the first committed reaction in the pentose phosphate pathway as it provides necessary compounds of NADPH for the synthesis of fatty acids. The cloning of ovine (*Ovis aries*) G6PD gene revealed the presence of two cDNA transcripts (oG6PD$_A$ and oG6PD$_B$), with oG6PD$_B$ being a product of alternative splicing and with no similarity to any other previously reported G6PD transcript. Here, we attempt to study the effect of energy balance in ovine G6PD transcript expression, trying simultaneously to find out any potential physiological role of the oG6PD$_B$ transcript. Changes of energy balance that lead to synergistic changes in the expression of both transcripts, but in opposite directions and not in a proportional way. Negative energy balance favours the presence of the oG6PD$_B$ transcript leading to a significant increase of its expression, compared to oG6PD$_A$ expression ($P<0.05$). In contrast, positive energy balance leads to a significant increase of oG6PD$_A$ compared to oG6PD$_B$ expression ($P<0.05$). In either condition oG6PD$_B$ expression is unchanged. Regression analysis showed that there is an energy balance threshold where the expression of both transcripts shows no change.

1. Introduction

During the past decades, the increasing interest in obesity and in animal products with low fat content, as fat-related diseases have become a major problem in the developed countries, has led to a more focalized study of adipose tissue. (McLaren, 2007). The use of farm animals in biomedical research issues requires the knowledge of differences between these species and humans (Bergen and Mersmann, 2005). In addition, studying the lipid biology of these species, applies knowledge to animal production and animal health. A major difference in lipogenesis between human and ruminant species is that in the ruminant's lipogenesis occurs mainly in adipose tissue in contrast to human, where it occurs predominately in the liver (Vernon, 1981; Naficov and Beitz, 2007).

The biosynthesis of fatty acids requires considerable amounts of reducing equivalents in the form of NADPH, for the reduction of acetyl-CoA to fatty acids (Rogdakis, 1982). Several enzymes are responsible for NADPH production, including cytosolic NADP isocitrate dehydrogenase (IDH, *EC 1.1.1.42*), cytosolic NADP malic enzyme (ME, *EC 1.1.1.40*) and the first two enzymes of pentose phosphate shunt, glucose-6-phosphate dehydrogenase (G6PD, *EC 1.1.1.49*) and 6-phosphogluconate dehydrogenase (6PGD, *EC 1.1.1.44*). The last two are recognized as the main suppliers in ruminants providing 50–80% of the required NADPH for fatty acid synthesis (Vernon, 1981).

Among the four NADPH-producing enzymes, G6PD is the rate-limiting enzyme of the shunt catalyzing the first committed reaction. Apart from producing necessary compounds of NADPH, it also, plays a crucial role in cell status controlling the redox potential via the glutathione reductase system (Tian et al., 1999). At molecular level, G6PD has been well studied in many eutherian species. Complete characterized sequences have been already published for human, mouse and rat genes (Persico et al., 1986; Chen et al., 1991; Zollo et al., 1993; Ho et al., 1988), while many studies have shown the regulation of gene expression in respect to various stimuli (Kletzien et al., 1994).

Concerning ovine G6PD, several studies have been conducted on enzymatic level showing the reaction of enzyme activity to different stimuli (Thomson and Butterfield, 1988; Panopoulou et al., 1989; Rogdakis et al., 1997). Recently the ovine G6PD has been cloned and characterized (Laliotis et al., 2007a,b, 2008) elucidating the presence of two mRNA G6PD transcripts (oG6PD$_A$ and oG6PD$_B$). oGPD$_B$ is the result of a splicing event leading to a transcript with 30 extra nucleotides in total. This extra segment causes a frameshift in the polypeptide chain resulting in changes around the area of the substrate binding site as an enlarged binding "pocket" is observed. It has been hypothesized that the oG6PD$_B$ transcript could probably result in a low glucose 6-phosphate dehydrogenase activity (Laliotis

Abbreviations: aa, amino acids; bp, base pair; BW, body mass; cDNA, complementary to RNA; dNTPs, deoxyribonucleotides triphosphate; G6PD, glucose 6-phosphate dehydrogenase; IDH, isocitrate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; kDa, kilodalton; ME, malic enzyme; MJ, megajoule; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NEL, net energy lactation; PCR, polymerase chain reaction; RT, reverse transcription.

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et al., 2007a), as analogous events have already been previously reported in sheep (Maronpot, 1972; Calabrese et al., 1980). Thus, the presence of a novel alternative transcript of ovine G6PD mirrors a potentially novel role in metabolism. In order to ascertain the above, more focused physiological experiments should be conducted. In this study, we report the effect of dietary energy level in ovine G6PD transcripts, and in parallel elucidate any potentially physiological role of the oG6PD$_B$ transcript.

2. Materials and methods

2.1. Animal treatment

A total of seven (7) randomly selected lactating ewes (*Ovis aries*) of the Chios breed were used. After weaning (45 days) the animals were housed at the experimental farm of the Agricultural University of Athens. The experimental design was as follows (Fig. 1): ewes were fed individually twice per day, at about 7:00 and 16:00 h on an alfalfa hay (4.14 MJ/kg) and on a concentrated diet. The concentrated diet consists of a basal (7.1 MJ/kg) and a lactation (7.3 MJ/kg) ration. Until the end of the second experimental week the amount of diet was set in such level that a negative energy balance was formed according to ewe's requirements (0.4 kg alfalfa hay, 0.5 kg basal ratio, 1 kg lactation ratio). The next two weeks, the amount of diet was increased to create a positive energy balance (0.4 kg alfalfa hay, 0.5 kg basal ratio, 1.5 kg lactation ratio). Water was freely available. Moreover, body mass (BW) and milk yield were recorded weekly. Once per week, samples of milk from each ewe were also collected to determine the milk fat content (Gerber method).

At the end of the first (1st), second (2nd) and fourth (4th) experimental weeks, samples of the subcutaneous adipose tissue from the tail region were taken from each ewe by biopsy. Prior to biopsy the ewes were fasted but with free access to water. At the day of sampling ewes were anesthetized with the use of Tiletamine and Zolazepam (Zoletin 50) in 15–20 mg/kg BW. Samples of adipose tissues were immediately frozen in liquid N_2 and stored at −80 °C for further RNA extraction.

2.2. Energy balance determination

The energy balance was estimated weekly for each ewe according to the following equitation:

$$\textit{Energy balance} = \textit{feed diet energy} - \textit{ewe's energy requirements} = \textit{feed diet energy} - (\textit{Maintenace Requirements} + \textit{Milk energy content})$$

Maintenance requirements were calculated using the following equation: $E_R = 0{,}23BW^{0.75}$, while milk energy content was estimated in MJ of net energy according to the following equation (Mavrogenis and Papachristoforou, 1988):

$$E = [91.17M\,(4.97 + f)]\,0.00418,$$

where

E	milk energy (MJ)
M	milk yield (L/day)
f	milk fat content (%)

2.3. RNA isolation and cDNA synthesis

Samples from the tail subcutaneous adipose tissue were taken by biopsy. Total RNA from the ovine adipose tissue was extracted using "Rneasy Lipid Tissue Kit" (Qiagen) according to the manufacturer's protocol. The eluted RNA was treated twice with Dnase*I* (1500 units) in order to exclude any possibility of DNA traces.

For first-strand cDNA synthesis a two step RT-PCR procedure was followed using 1 μg of the eluted total RNA. After an initial denaturation of RNA at 65 °C for 5 min, an annealing step at 37 °C for 1 h was followed by adding reverse transcriptase buffer, 0.5 mM of each dNTPs, 1 μM of oligo$(dT)_{17}$ primer, 10 U RNAse Inhibitor (Invitrogen) and 4 U of Omniscript Reverse Transcriptase (Qiagen) in a total volume of 20 μL.

2.4. Real-time PCR

Relative levels of mRNA were quantified with real-time quantitative RT-PCR using fluorescent TaqMan® technology. Two custom designed TaqMan® gene expression assays for oG6PD$_A$ and oG6PD$_B$ transcripts using FAM as a reporter dye were used (Applied Biosystems). oG6PD assays were designed according to the differences noted in a previous study (Laliotis et al., 2007). A TaqMan® Eukaryotic 18 S rRNA Endogenous Control using VIC as a reporter dye (Applied Biosystems) was used in multiplex reactions as a reference to normalize the amount of sample RNA. All probes had minor grooved binders (MGB) as quencher (Table 1).

The PCR was performed in the 7500 Real-time PCR System (Applied Biosystems) using the TaqMan Universal PCR Master Mix with AmpErase® UNG (Applied Biosystems), according to the manufacturer's protocol. Each multiplex reaction for the quantification of either oG6PD$_A$ or oG6PD$_B$ and 18 S rRNA, was performed in a total volume of 20 μL and contained: 90 ng RNA equivalents as well as 700 nM forward primer of G6PDA or G6PDB, 700 nM reverse primer of oG6PD$_A$ or oG6PD$_B$, 700 nM of 18 S rRNA primesr, 200 nM of oG6PD$_A$ or oG6PD$_B$ TaqMan probe and 200 nM of 18 S TaqMan probe. The reactions were performed in MicroAmp 96-well plates capped with MicroAmp optical film (Applied Biosystems). Thermal cycling parameters were as follows: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C

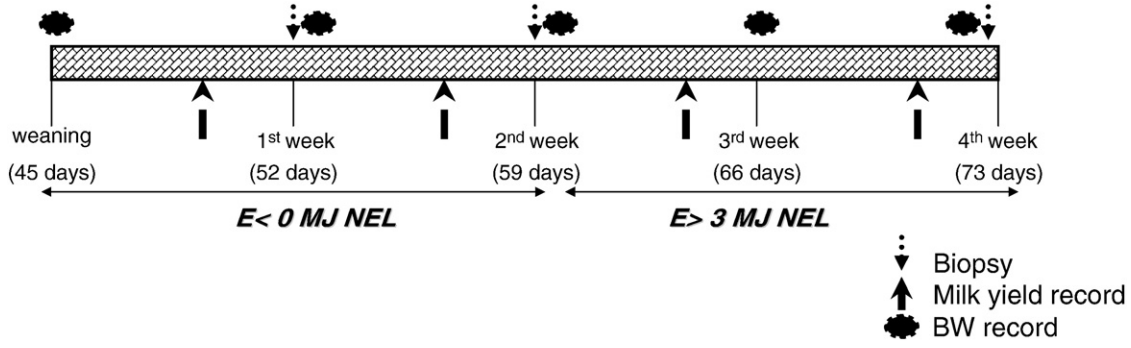

Fig. 1. Experimental design of treatments.