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The estimation of the relative gene expression in the hypothalamus and liver of agouti mice using quantitative reverse transcription PCR

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Abstract

Female C57Bl/6J mice have a mutation in the A^y locus associated with overexpression of ASIP1, an antagonist of the types 3 and 4 melanocortin receptors. This mutation is characterized by the development of the melanocortin-type obesity. After treatment of the agouti mice with metformin (MF) in the different doses for 10 days, the hypothalamus and liver sections of wild-type mice, untreated agouti-mice and MF-treated agouti-mice were dissected, and the expression of the genes encoding the components of the hypothalamic signaling systems and the liver proteins were estimated by a quantitative reverse transcription PCR (qRT-PCR). The total RNA was isolated using ExtractRNA Reagent. The reverse transcription reaction was carried out using MMLV RT kit. The polymerase chain reaction was performed using the qPCRmix-HS SYBR + LowROX, and the amplified signals were detected with the Applied Biosystems® 7500 Real-Time PCR System (“Life Technologies, Thermo Fisher Scientific Inc.”, Waltham, Massachusetts, USA). The primers for the genes of signal proteins were selected using the Primer-BLAST web application. The genes encoding 18S rRNA and hypoxanthine-guanine phosphoribosyl transferase (Hprt) were used as an endogenous control. Relative expression was calculated using the ddCt method compared with the control group.

Materials

For qRT-PCR of signaling and effector proteins, the following reagents were used:

1. ExtractRNA Reagent (TRIzol analogue) (“Evrogen”, Moscow, Russia)
2. MMLV RT kit (“Evrogen”, Moscow, Russia)
3. qPCRmix-HS SYBR+LowROX ("Evrogen", Moscow, Russia)
4. Set of the forward and reverse primers for mice genes encoding the leptin receptor (*Lepr*), types 3 and 4 melanocortin receptors (*Mc3r* and *Mc4r*), pro-opiomelanocortin (*Pomc*), agouti-related peptide (*Agrp*), neuropeptide Y (*Npy*), BAX (*Bax*), BCL-2 (*Bcl-2*), interleukin 1β (*IL1beta*), TNF-α (*TNFalpha*), 18S RNA (*18SRna*) and hypoxanthine-guanine phosphoribosyl transferase (*Hprt*).

Before start

Primers were selected using literature and the web application Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Then, a verification of the efficiency of the primers was carried out at the specified annealing temperature. The *18S Rna* and *Hprt* genes were used as an endogenous controls.

Preparation of samples

1 In the end of experiment animals were decapitated, and the hypothalamus and liver sections were removedand frozen immediately at -80°C before the analysis and then used for qRT-PCR

2 Total RNA was isolated from the sections of the hypothalamus and liver using the ExtractRNA Reagent (TRIzol analogue) (“Evrogen”, Moscow, Russia) according to the manufacturer‘s protocol. (http://evrogen.com/)

3 The samples containing 1 µg of RNA were reverse-transcribed to cDNA using the MMLV RT kit (“Evrogen”, Moscow, Russia) and the random oligodeoxynucleotide primers were used according to the manufacturer‘s protocol. (http://evrogen.com/)

Preparation of reaction mixture in the 96-well plate

4 The 51µl of distillated water add to a 200 µl PCR-tube.

5 Add 15µl of qPCRmix-HS SYBR+LowROX ("Evrogen", Moscow, Russia).

6 Add 6µl of the forward and reverse primer mixture until 0.4 µM of each.

7 Add 3µl sample containing 30 ng of cDNA or distilled water in control sample.

8 Mix the solution by pipetting and quick spin.

9 Dispend the PCR tube containing the reaction mixture (75 µl) into three wells (25 µl in each) in the 96-well plate. Cover the plate with the seal for qPCR plates.

10 Briefly mix and centrifuge 96-well plate in the centrifuge/vortex for the PCR plates and confirm that reaction mixture is at the bottom of the wells, with no bubbles.

11 Place the 96-well plate into the Applied Biosystems® 7500 Real-Time PCR System (“Life Technologies, Thermo Fisher Scientific Inc.”, Waltham, Massachusetts, USA).

PCR analysis

12 The following qPCR amplification protocol is used: an initial denaturation at 95 °C for 5 min; a three-step amplification andquantification program consisting of 38 cycles of 95 °C for 30 s, 55–58 °C for10 s (annealing temperature depends on the specific pair of the primers), and 72 °C for 30 s; and the ABI Melt Curve program to verify the presence of a single peak and the absence of primer-dimer formation in eachtemplate-containing reaction. The expression of each gene from all the studied groups of animals is determined on the same plate.

	No	Gene	Localization	Primer	NCBI Reference Sequence	Temperature of annulling
	1	*Pomc*	For 5′–3′	CAGTGCCAGGACCTCACC	NM_008895.4	55
			Rev 5′–3′	CAGCGAGAGGTCGAGTTTG		
	2	*Agrp*	For 5′–3′	ACAACTGCAGACCGAGCAGAA	NM_001271806.1	58
			Rev 5′–3′	CGACGCGGAGAACGAGACT		
	3	*Npy*	For 5′–3′	CGGGAGAACAAGTTTCATTTCC	NM_023456.3	56
			Rev 5′–3′	ACAGAAAACGCCCCCAGAAC		
	4	*Lepr*	For 5′–3′	GCATGCAGAATCAGTGATATTTGG	NM_146146.2	56
			Rev 5′–3′	CAAGCTGTATCGACACTGATTTCTTC		
	5	*Mc3r*	For 5′–3′	CAAGGAGATTCTCTGCGGCT	NM_008561.3	56
			Rev 5′–3′	TCCCGTCTGAGCGTTGTTTT		
	6	*Mc4r*	For 5′–3′	GGGTCGGAAACCATCGTCAT	NM_016977.4	56
			Rev 5′–3′	TGCAAATGGATGCGAGCAAG		
	7	*Bax*	For 5′–3′	TGGAGCTGCAGAGGATGATTG	NM_007527.3	56
			Rev 5′–3′	GAAGTTGCCGTCAGAAAACATG		
	8	*Bcl2*	For 5′–3′	GTGGATGACTGAGTACCTGAAC	NM_009741.5	55
			Rev 5′–3′	GAGACAGCCAGGAGAAATCAA		

	9	*IL1beta*	For 5′–3′	TCCAGGATGAGGACATGAGCAC	NM_008361.4	56
			Rev 5′–3′	GAACGTCACACACCAGCAGGTTA		
	10	*TNFalpha*	For 5′–3′	TATGGCCCAGACCCTCACA	NM_013693.3	56
			Rev 5′–3′	GGAGTAGACAAGGTACAACCCATC		
	11	*Hprt*	For 5′–3′	AGCCGACCGGTTCTGTCAT	NM_013556.2	57
			Rev 5′–3′	GGTCATAACCTGGTTCATCATCAC		
	12	*18S rRNA*	For 5′–3′	GGGAGCCTGAGAAACGGC	NR_003278.3	57
			Rev 5′–3′	GGGTCGGGAGTGGGTAATTT		

13 qRT-PCR amplification continues about 2 hours.

14 The melting curve analysis should show the presence of a single peak and the absence of the primer dimers in each reaction containing the sample.

Data analysis

15 Data analysis is performed using the 7500 Software v2.0.6 and the Expression Suite Software v1.0.3. All the data are presented as relative mRNA level of the target gene (RQ). The calculation of the expression level of the target gene produced by the method of ΔΔCT (Schmittgen, Livak, 2008):

$\Delta CT = CT_{\text{gene of interest}} - CT_{\text{endogenous control}}$

$\Delta\Delta CT = \Delta CT_{\text{sample or control}} - \Delta CT_{\text{control mean}}$

$RQ = 2^{-\Delta\Delta CT}$

To calculate the RQ values for the control and experimental groups, the mean ΔCT control is calculated, which is subtracted from all the control and experimental ΔCT.