The effect of divergent selection on adrenocortical activity in Large White pigs on gene expression after ACTH, LPS and social stress challenges

Elena Terenina, Claire Dugué, E. Kulikova, Darya Bazovkina, Laure Gress, Gaëlle Lefort, Yvon Billon, Catherine Larzul, Pierre Mormède

The effect of divergent selection on adrenocortical activity in Large White pigs on gene expression after ACTH, LPS and social stress challenges.

E. Terenina[1], C. Dugué[1], E. Kulikova[2], D. Bazovkina[2], L. Gress[1], G. Lefort[1], Y. Billon[3], C. Larzul[1] & P. Mormede[1]

[1] GenPhySE, INRA, INPT, ENSAT, Université de Toulouse, 31326 Castanet-Tolosan, France elena.mormede@inra.fr (Corresponding Author)
[2] Department of Behavioral Neurogenomics, Siberian Branch of the Russian Academy of Sciences, 630090 Novosibirsk, Russia
[3] GenESI, INRA, Le Magneraud, 17700 Surgères, France

Keywords: genetic selection, cortisol, social stress, ACTH, LPS, robustness, gene expression

Introduction

The genetic potential of animals is usually not fully expressed in commercial conditions, due to the limiting influence of the environment. Robustness is a specific quality of an individual to express a high production potential in a wide variety of environmental conditions and is now a major specific breeding goal in the context of sustainable farm animal breeding. Various strategies are available to increase robustness, and we have suggested that the reinforcement of the neuroendocrine stress responses may favour the processes of adaptation and dampen the negative consequences of the environment (Mormede & Terenina, 2012). The hypothalamic-pituitary-adrenocortical (HPA) axis is the main neuroendocrine system involved in adaptation to stress and is strongly influenced by genetic factors (Mormede et al., 2011a; Mormede & Terenina, 2012). It is therefore a primary way for the selection of more robust animals (Mormede *et al.*, 2011b). In order to further investigate the potential of this approach, a divergent selection was carried out in the Large White pig breed based on the plasma cortisol level measured one hour after injection of ACTH (Larzul *et al.*, 2018). In the third generation of selection, the expression in blood of candidate genes playing a role in HPA axis activity and stress responses was tested at 4 time points (0, 1h, 4h, and 24h) after different challenges: ACTH or LPS injection, and social stress.

Material and methods

Animals and experimental procedures

The experimental protocol was accepted by the ethics Committee in animal experimentation Poitou-Charentes (decision of 21/01/2013). The selection experiment and the breeding conditions are presented in Larzul et al. (2018). Briefly, two divergent lines were selected on the plasma cortisol level one hour after ACTH injection at 6 weeks of age, 2 weeks after weaning. Thirty-two animals of the 3rd generation of selection were studied. The post-ACTH plasma cortisol level was 2.16 times higher in the high line than in the low line, the line difference being about 5 genetic standard deviations for the selection criterion.

At 6 weeks of age, each animal received an ACTH stimulation test by the injection in the neck muscles of 333 µg of synthetic 1-39 ACTH diluted in 1 mL of 0.9% saline.

At 7 weeks, the social stress test was performed. Unfamiliar animals were placed for one hour in a novel environment in groups of 8 according to the protocol described by Turner et al. (2009). The groups consisted of animals from 4 different breeding groups. The number of skin lesions, indicative of aggressive interactions, was counted before and after the test. Animals were video recorded for 1 h following mixing. The measures per animal were the

number and duration of fights, and the status of aggressor or attacked for each fight.

At 8 weeks, each animal was injected in the neck muscles with LPS (E. coli serotype 055:B5, Sigma-Aldrich, Saint Quentin Fallavier, FR) at a dose of 15 µg/kg body weight. Injections occurred from 10:00 to 11:00 AM and social stress from 8:00 to 12:00 to avoid nycthemeral variations. Blood samples were collected before the injection or the beginning of social encounter (t = 0) and 1 hour (t = +1), 4 hours (t = +4) and 24 hours (t = +24) later. At each time, two blood samples (on heparin and EDTA) were taken by jugular vein puncture. An aliquot (400 µL) of EDTA blood was mixed with the same volume of DL buffer (Macherey-Nagel), frozen at –20 °C for 4 h and then at –80 °C until analysis for gene expression.

Gene expression analysis

The RNA isolation and purification was done according to the manufacturer's instructions using the Nucleospin RNA Blood kit (Macherey-Nagel, FR) followed by DNase treatment. The quality of each RNA sample was checked through the Bioanalyser Agilent 2100 (Agilent Technologies, Massy, FR) and low-quality RNA preparations were discarded (RIN < 8). Total RNA (1 µg) was reverse-transcribed as previously described (Bonnet et al., 2011). Ninety candidate genes were chosen for functional reasons or because they have been associated with HPA axis activity in transcriptomic studies (Sautron et al., 2015). Primer sequences for genes were designed using Primer3plus software (primer3plus.com). Pre-amplified samples were analyzed with a 96x96 Dynamic Array IFC (Fluidigm) following the protocol defined by Spurgeon et al. (2008), with some modifications. All measurements were performed on the same plate. Each gene was tested twice for each sample. Four dilution points containing a pool of all samples were used to determine PCR efficiency. Data were analyzed using BioMark Gene Expression Data Analysis software (Fluidigm) to obtain Ct values. The Pfaffl method (Pfaffl, 2001) was applied to compute the relative expression of each gene.

Statistical analyses

For ACTH and social stress, a linear model was performed to reveal the genes differentially expressed according to the line (high, low) and the time point (0, 1, 4, 24h):

$$expr_i = \beta_0 + \beta_1 t + \beta_2 line_i + \beta_3 t * line_i + \varepsilon_i$$

As blood cell composition was found to vary over time after LPS injection, we used the lymphocytes/ granulocytes (L/G) ratio as a covariate in the analysis of the response to LPS.

Results and discussion

Differentially expressed genes are summarized in Table 1.

Response to ACTH

Thirteen genes (out of 82) were differentially expressed in response to ACTH, among them thirteen genes were up-regulated at T1 and eleven were up-regulated at T4. No gene was differentially expressed at T24. No effect of the line was found. No gene expression was correlated with the level of cortisol measured one hour after the ACTH injection or four hours after the LPS injection.

Response to LPS

Thirty-four genes (out of 77) were differentially expressed in response to LPS, among them 23 genes were up-regulated at T1, 13 genes were down-regulated at T1, 1 gene was up-

regulated at T4, 21 genes were down-regulated at T4, and 4 genes were down-regulated at T24. No effect of the line was found. Eight genes (*PSAP, S100A9, stefinA8, FAS, TNFAIP6, HEXA, S100A12, CARD6, CCL4*) were correlated to the cortisol level measured one hour after ACTH injection. No gene expression was correlated with the level of cortisol measured four hours after the LPS injection.

Table 1. Genes whose expression varies significantly at the different time points.

Gene	Gene Description	ACTH	LPS	Social Stress
AKAP13	A-kinase anchoring protein 13			T4
ALOX15	arachidonate 15-lipoxygenase		T1	
ALOX5AP	arachidonate 5-lipoxygenase activating protein		T1 T4	T4
ASPH	aspartate beta-hydroxylase	T4		T4
C2H19orf59			T1 T4 T24	T4
CARD6	caspase recruitment domain family member 6		T1	
CASP6	caspase 6	T4		T4
CCL4	C-C motif chemokine ligand 4		T1 T4	
CD24	CD24 molecule		T1 T4	T4
CD79A	CD79a molecule			T4
CERS4	ceramide synthase 4	T4	T4	T4
CHI3L1	chitinase 3 like 1		T24	
CHIT1	chitinase 1			T4
CKB	creatine kinase B			T4
CNDP2	carnosine dipeptidase 2		T1 T4	
COMT	catechol-O-methyltransferase	T4		T4
CRHR1	corticotropin releasing hormone receptor 1			T4
CRISPLD2	cysteine rich secretory protein LCCL domain containing 2	T1		T1
CSF2RA	colony stimulating factor 2 receptor alpha subunit		T4	
DUSP2	dual specificity phosphatase 2		T1	
FAS	Fas cell surface death receptor		T4	
GALK2	galactokinase 2			T4
GNG10	G protein subunit gamma 10		T4	
HHEX	hematopoietically expressed homeobox			T1
HSPA4	heat shock protein family A (Hsp70) member 4	T1		
HTT	huntingtin			T1
IL7R	interleukin 7 receptor			T4
CXCL8	Interleukin-8		T1	
JAK2	Janus kinase 2		T4	
LDLR	low density lipoprotein receptor			T1 T4
MAOA	monoamine oxidase A	T4		T4
MEGF9	multiple EGF like domains 9	T4	T1	T4
MXD1	MAX dimerization protein 1		T1 T4	T4
NME6	NME/NM23 nucleoside diphosphate kinase 6	T4		
PADI4	peptidyl arginine deiminase 4		T1	
PDPN	podoplanin		T1	
PIM1	Pim-1 proto-oncogene, serine/threonine kinase			T4
PSAP	prosaposin	T4	T1	T4
RAB31	RAB31, member RAS oncogene family		T1 T4 T24	
RGS2	regulator of G protein signaling 2		T4	T1 T4
RNF2	ring finger protein 2	T4		T1 T4
S100A12	S100 calcium binding protein A9		T1 T4	
S100A9	S100 calcium binding protein A12	T4	T1 T4	T4
SCARB1	scavenger receptor class B member 1		T4	
SLA	Src like adaptor		T1 T4	T4
SOD2	superoxide dismutase 2		T4	
STEFIN A8	Cystatin-A8		T1 T4	T4
TANK	TRAF family member associated NFKB activator		T4	
TIAM1	T-cell lymphoma invasion and metastasis 1	T4	T1	
TMBIM6	transmembrane BAX inhibitor motif containing 6		T4	
TNFAIP6	TNF alpha induced protein 6		T1 T4	
VNN2	vanin 2		T1 T24	
XAF1	XIAP associated factor 1		T1	
XCL1	X-C motif chemokine ligand 1			T4

Response to social stress

Thirty-two genes (out of 76) were differentially expressed after the social stress challenge,

among them 6 genes were up-regulated at T1, 26 genes were up-regulated at T4. No gene was differentially expressed at T24. No effect of the line was found. No gene expression was related to the level of cortisol measured one hour after the ACTH or four hours after the LPS injection.

Four differentially expressed genes (*PSAP, MEGF9, CERS4* and *S100A9*) are the same for the three tests. These genes are linked to each other via *FOS* (Proto-Oncogene Fos, AP-1 transcription factor subunit) and *TP53* (tumor protein p53) which also acts as transcription factors. It has been described that *FOS* expression is associated with stressful conditions. *TP53* encodes the p53 tumor protein, which responds to various cellular constraints to regulate target genes that induce cell cycle arrest, activation of apoptosis or changes in metabolism.

Conclusion

This experience aimed at the study of responses to various stress challenges in the third generation of selection of two lines of pigs genetically selected on the basis of cortisol levels measured one hour after injection of ACTH. The data presented here show that the direct stimulation of the HPA axis, inflammatory stress and social stress induce different gene expression profiles.

In conclusion, we have demonstrated that there are specific biomarkers indicative of an ACTH-stimulated, LPS-stimulated and social stress-stimulated response. Furthermore, these responses persist for prolonged periods of time and at significant expression levels, making them good candidate markers for the study of mechanisms involved during different stress challenges. More specifically, four genes (*MEGF9, S100A9, CERS4* and *PSAP*) differentially expressed in the three tests could be used for the overall study of stress response.

Acknowledgements

With the collaboration of the technical teams of the experimental unit GenESI Magneraud[2]. We thank PEGASE INRA Saint-Gilles (more specifically Raphaël Comte) for cortisol assay, Lisa Bluy for technical assistance and Frédéric Martins and Emilie Bonin for Fluidigm analysis. With the financial support of the Agence Nationale de la Recherche, program ANR BIOADAPT, project SUSoSTRESS (ANR-12-ADAP-0008).

List of References

Bonnet A., Bevilacqua C., Benne F., Bodin L., Cotinot C., Liaubet L., Sancristobal M., Sarry J., Terenina E., Martin P., Tosser-Klopp G., Mandon-Pepin B, 2011.Transcriptome profiling of sheep granulosa cells and oocytes during early follicular development obtained by laser capture microdissection. BMC Genomics. 12: 417.

Larzul, C., E. Terenina, Y. Billon & P. Mormede, 2018. Genetic determinism of adrenocortical activity in pigs. 11th WCGALP, Auckland, New Zealand, February 11-16, 2018.

Mormede P., A. Foury, P. Barat, J.B. Corcuff, E. Terenina, N. Marissal-Arvy & M.P. Moisan, 2011a. Molecular genetics of hypothalamic-pituitary-adrenal axis activity and function. Ann. N.Y. Acad. Sci. 1220: 127–136.

Mormede P., A. Foury, E. Terenina & P.W. Knap, 2011b. Breeding for robustness: the role of cortisol. Animal 5: 651–657.

Mormede P. & E. Terenina, 2012. Molecular genetics of the adrenocortical axis and breeding

for robustness. Dom. Anim. Endocrinol. 43: 116–131.
Pfaffl M.W., 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. May 1; 29(9): e45Sautron V., Terenina E., Gress L., Lippi Y., Billon Y., Larzul C., Liaubet L., Villa-Vialaneix N., Mormede P, 2015. Time course of the response to ACTH in pig: biological and transcriptomic study. BMC Genomics. 16(961):PMC4650497.
Spurgeon S.L., Jones R.C., Ramakrishnan R, 2008. High throughput gene expression measurement with real time PCR in a microfluidic dynamic array. PLoS ONE. 3(466):e1662.