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Original Article

Differential gene expression profile analysis in corticosterone-treated PC12 cells

Jing-Jie Zhao[1], Ping Zhang[2], Li Li[1], Shu-Xing Chen[3,5], Allison Joines[3], Donald Wu[3], Megan Wong[3], Justin Shahan[3], Teresa Golden[3], Ning Wu[3], Ming-Zhen Li[4]

[1]Department of Traditional Chinese Medicine, Beijing Friendship Hospital, Capital Medical University, Beijing, China; [2]Department of Research and Development, Beijing Center for Physical and Chemical Analysis, Beijing, China; [3]Department of Biological Sciences, Southeastern Oklahoma State University, Durant, OK 74701, USA; [4]Shenzhen Ruipuxun Academy for Stem Cell & Regenerative Medicine, Shenzhen, China; [5]School of Food and Bioengineering, Henan University of Science and Technology, Luoyang 471023, Henan, China

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Abstract: Major depressive disorder (MDD) is a highly prevalent psychiatric disorder that has been ranked as the 4th leading cause of disability worldwide. Past clinical and laboratory evidence has confirmed that abnormalities of the hypothalamic-pituitary-adrenal (HPA)-axis are involved in MDD development. In this study, we took advantage of corticosterone treatment of PC12 cells as a model to identify genes regulated by HPA-axis hormones. Next-generation RNA-Seq technology was utilized to explore genome-wide differentially expressed gene profiles between control and corticosterone treated PC12 cells. 1,274 genes with at least two-fold expression level change were identified. Gene ontology analysis showed that the top enriched biological processes included response to glucocorticoid signaling, apoptosis, cell division/DNA replication, and neuron projection/axon guidance, highly consistent with phenotypes of PC12 cells treated with corticosterone. Taken together, RNA-seq data is reliable and comprehensive, thus providing a valuable resource for understanding underlying mechanisms of glucocorticoid-induced neuron malfunction.

Keywords: Corticosterone, PC12 cells, RNA-Seq, gene expression profiling

Introduction

Hypothalamic-pituitary-adrenal (HPA)-axis refers to a set of direct stimulus and feedback between the hypothalamus, pituitary gland, and adrenal glands. Glucocorticoid is released by the adrenal cortex and plays critical roles in regulating inflammation and controlling stress [1]. In past years, many studies have reported the involvement of human HPA-axis (the glucocorticoid signaling) in the etiology of major depressive disorder (MDD), a highly prevalent psychiatric disorder which has become the 4th leading cause of disability worldwide [2-5]. Previous studies have demonstrated that even though optimal doses of glucocorticoids enhance neural plasticity and spatial memory behavior, higher doses or sustained treatment with low doses can induce neuronal cell toxicity [6]. Long term use of synthetic glucocorticoids in clinics for immune suppression increases risk of MDD [7]. Recent studies have also found that glucocorticoids could significantly increase the amount of mitochondrial DNA and reduce the length of telomeres in an experimental animal model, both of which are consequences of stress and represent molecular markers for MDD [8-10].

Glucocorticoids impact the nervous systems in many aspects including stress response in neurons [11], neurotransmitter synthesis [12], neuronal survival [13], and neuronal differentiation [14]. Corticosterone, the main HPA-axis glucocorticoid hormone in rodents, plays a similar role in regulating animal stress responses. As shown in previous animal experiments, corticosterone is an adaptation-related biomarker during chronic stress [15].

The PC12 cell line, derived from pheochromocytoma of the rat adrenal medulla, is a widely used *in vitro* model for investigation of neuronal differentiation, neuro-secretion, and other neuro-biochemical and -biological events [16-18]. Nerve growth factor (NGF)-treated PC12 cells

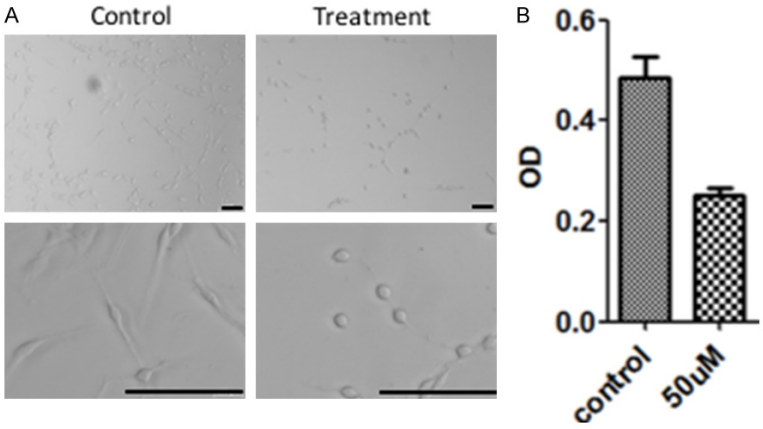

Figure 1. Effect of corticosterone treatment on PC12 cell survival and neurite growth. PC12 cells were treated with vehicle control or 50 μM corticosterone for 24 hours. A. Microscopic images of PC12 cell neurites. B. MTT assay results. There was a significant difference in the number of live cells between control and treatment groups ($P < 0.01$).

differentiate into chromaffin-like cells with noradrenergic phenotype through activation of distinct molecular signaling pathways [18, 19]. Differentiated cells have many properties similar to those of sympathetic neurons such as proliferation cessation, neurite outgrowth, and electrical activity [19]. With an especially great abundance of glucocorticoid receptors and typical features of brain neurons, PC12 cells exhibits an excellent cellular model for investigating underlying molecular mechanisms by which glucocorticoids impact neuronal cell survival, differentiation, and morphogenesis [20].

Although solid evidence has demonstrated that HPA-axis hormones directly respond to unpredictable stress by elevating extracellular glucocorticoid levels and, thereafter, affect neuronal cell morphology and function, the underlying molecular mechanisms still require further investigation. Our study applied next-generation RNA-Seq technology to comparatively study differentially expressed gene profiles between corticosterone treated and control PC12 cells, aiming to identify all responsive genes and decipher the underlying mechanisms of glucocorticoid-induced depression.

Materials and methods

Cell culture

PC12 cells were obtained from the Cell Bank of Chinese Academy of Sciences (Beijing, China). They were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Grand Island, NY, USA) and supplemented with 5% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA), 5% horse serum (Life Technologies, Grand Island, NY, USA), 100 μg/ml streptomycin, and 100 U/ml penicillin in a water-saturated atmosphere of 5% CO_2 at 37°C. Cells were seeded in a 60 mm dish at a density of 1×10^6 cells/dish for all experiments and cultured in serum-free medium for 12 hours before drug treatment.

Corticosterone treatment

Corticosterone was purchased from SIGMA-ALDRICH (Saint Louis, MO, USA). As described in previous studies [21], PC12 cells with 50% confluency were incubated with 50 μM corticosterone for 24 hours.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay

After corticosterone treatment, MTT assay was performed to detect cell viability [22]. In brief, PC12 cells were incubated with 5 mg/ml MTT (15 μl/well) at 37°C for 4 hours. The cultural medium was removed after incubation and cells were dissolved in dimethyl sulfoxide (1:1,000). Formazan reduction products were measured by obtaining the absorbance at 570 nm in a microplate reader. Six replicates were performed in each group. Data were collected from three independent experiments.

RNA-sequencing

Total RNAs of control and corticosterone treated PC12 cells were extracted by using TRIzol (Life Technologies, Grand Island, NY, USA). The quantity and quality of extracted RNAs were evaluated using NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA, USA), and RNA electropherograms with calculation of RNA integrity number (RIN), respectively (Schroeder et al. 2006). RNA samples were subjected to next generation RNA-sequencing if their RIN > 7 and

Table 1. Mapped transcriptome reads of paired-end RNA-Seq in both control and corticosterone treated PC12 cells

Sample groups		Total reads number	Left mapped reads		Right mapped reads		Total mapped reads	
			Number	%	Number	%	Number	%
Control	1	14,926,691	13,644,136	91	13,137,946	88	14,352,417	96
	2	14,856,540	13,635,007	92	13,188,946	89	14,236,841	96
	3	16,523,068	14,961,440	91	14,374,881	87	15,687,269	95
Total control		46,306,299	42,240,583	91	40,701,773	88	44,276,527	96
Treated	1	17,667,615	16,009,459	91	15,489,222	88	16,781,244	95
	2	14,021,101	12,807,465	91	12,324,597	88	13,403,884	96
	3	11,814,166	10,673,710	90	10,256,811	87	11,179,928	95
Total treated		43,502,882	39,490,634	91	38,070,630	88	41,365,056	95

total RNA quantity > 400 ng. Six sequencing libraries (3 replicates for both control and corticosterone treatment groups) were constructed by using NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) with poly (A) messenger RNA selection. The libraries were then used to build clusters on the Illumina flow cell, according to manufacturer protocol. Sequencing was performed using Illumina HiSeq 2500 System (Illumina, San Diego, CA, USA) with a 125 bp paired-end sequencing strategy.

RNA-Seq data analysis

The quality of raw sequencing reads was evaluated using FastQC package. Adapters and low quality reads were removed by using Trimmomatic [23]. Cleaned reads were mapped into rat reference genome and transcriptome downloaded from the UCSC Genome Browser (http://www.genome.ucsc.edu) using Tophat2 with no more than 5 mismatches for each alignment [24, 25]. Gene expression levels were determined by the number of fragments per kilobase of exon per million mapped reads (FPKM) using Cufflinks [26] Cross comparison and data merging were performed using Cuffmerge, resulting in a comprehensive transcriptome database for each group. Differentially expressed genes with Log2 (fold change of FPKM) were determined by cufflinks and identified using Circos, version 0.66 (http://circos.ca/) [27].

Functional annotation analysis of identified target genes

Identified differentially expressed genes were subjected to functional annotation analysis using DAVID (https://david.ncifcrf.gov/summary.jsp) to identify enriched biological processes and KEGG pathways.

Results

Corticosterone treatment reduced PC12 cell viability and neurite growth

To examine the effects of corticosterone on cell viability and neurite growth, we incubated PC12 cells with 50 μM corticosterone or vehicle control for 24 hours. In contrast to control cells which showed extended outgrowth of neurites, neurites of corticosterone treated cells were much shorter, or had even disappeared (**Figure 1A**). MTT assay revealed that corticosterone reduced cell viability by 50% (**Figure 1B**), consistent with the images observed (**Figure 1A**). These data demonstrated that corticosterone treatment inhibited PC12 cell growth and neurite extension.

Identification of differentially expressed genes by RNA-Seq

To decipher underlying molecular mechanisms of the effect of glucocorticoids on PC12 cell growth, we performed RNA-seq to identify genome-wide differentially expressed genes between control and corticosterone treated PC12 cells. A total of 46,306,299 and 43,502,882 reads were obtained for the control and corticosterone treated groups, respectively, covering around 95% of total mapped transcriptome (**Table 1**). Clustering assembly of sequencing data for both control and treatment groups generated 33,737 records with 89.9% of multiple exon transcriptomes compared to a total of 35,891 rat known genes. Differential gene expression analysis using FPKM identified

Table 2. Top 5 enriched biological processes for upregulated genes induced by corticosterone treatment

GO Term ~ Biological Process	Genes	N (%)	*P*-Value
GO:0071549 Cellular response to dexamethasone stimulus	ICAM1, ASS1, SERPINF1, ABCB1B, SERPINE1, FOXO1, BCL2L1, TRIM63, AQP1, ASL, ERRFI1, DDIT4	12 (1.9)	3.02E-06
GO:0045766 Positive regulation of angiogenesis	PRKCA, HYAL1, RAMP2, LGALS3, C3, PGF, AQP1, PTGIS, ADM, HIPK2, SERPINE1, RHOB, ZC3H12A, RRAS, THBS1, CTSH	16 (2.5)	6.97E-06
GO:0001666 Response to hypoxia	CAV1, ALAD, AHCY, PGF, ALDOC, BCL2L1, CITED2, PTGIS, KDM3A, LIMD1, PAK1, ICAM1, RAMP2, CYP1A1, CRYAB, VHL, CST3, UBE2B, PRKCD, DDIT4, ITPR2, PENK, ADM, ABCB1B, PSEN2	25 (3.9)	1.10E-05
GO:0042594 Response to starvation	ZFP36, ADSSL1, SORBS1, ADM, ULK1, SERPINE1, NUCB2, PDK4, AK3, FSTL1	10 (1.6)	5.24E-05
GO:0051384 Response to glucocorticoid	CXCL1, BCKDHA, CAV1, ALAD, ASS1, C3, IL6R, TRIM63, CTSL, DUSP1, ADM, S100B, SULT1A1, LCAT, PIK3R1	15 (2.4)	1.27E-04

Table 3. Other interesting enriched biological processes for upregulated genes induced by corticosterone treatment

GO Term ~ Biological Process	Genes	N (%)	*P*-Value
GO:0006915 Apoptotic process	STEAP3, ADAMTSL4, ALDOC, FOXO1, RTKN, FOXO3, BCL2L1, SQSTM1, ZC3H12A, RHOB, PAK1, DPEP1, AATK, PRKCA, SGK1, CST3, NR4A1, PIM3, PRKCD, DDIT4, PLEKHF1, ARRB1, SIAH3, CTSH, ADAM15	25 (3.9)	0.001
GO:0070373 Negative regulation of ERK1 and ERK2 cascade	CAV1, SPRY1, DUSP1, VRK3, ARRB1, NDRG2, TNIP1, ERRFI1, RGS14	9 (1.4)	0.001
GO:0043407 Negative regulation of MAP kinase activity	CAV1, SPRY1, DUSP1, PRKCD, FEM1A, RGS14	6 (0.9)	0.014
GO:0007399 Nervous system development	ENC1, MAP1B, CSRP1, MAFK, GAS7, SLC7A5, NUMBL, CNTF, TPP1, TNR, GPSM1, ZC3H12A, NDRG2, DCLK1	14 (2.2)	0.015
GO:0007409 Axonogenesis	OGN, RAB3A, KLF7, ULK1, MAP1B, PAK1, SLIT1, DCLK1, NUMBL	9 (1.4)	0.04

1,274 genes with at least 2-fold change in the corticosterone treated group compared to that of the control group (FDR < 0.05), including 627 downregulated and 647 upregulated genes (data not shown).

Gene ontology (GO) enrichment analysis of identified target genes

We next performed GO analysis to identify key biological processes in which differentially expressed genes were participating. Among the top five biological processes identified for the 647 upregulated genes (**Table 2**), two were directly related with glucocorticoid signals: Cellular response to dexamethasone stimulus and response to glucocorticoid. The data confirmed that RNA-seq was very successful. Consistent with glucocorticoid functions, the remaining three were all related with stress including one process in regulation of angiogenesis, one in response to hypoxia, and one to starvation. Furthermore, we also found many other intriguing processes (**Table 3**). For instance, genes involved in apoptosis were significantly enriched, which could explain why and how corticosterone treatment inhibits PC12 cell survival. Other interesting biological processes included negative regulation of ERK1 and ERK2 cascade, negative regulation of MAPK cascade, nervous system development, and axon extension involved in axon guidance. MAPK pathway has been reported to be involved in neuron axon branching and cellular stress (REF). Therefore, it is highly possible that MAPK signaling is one of the pathways mediating the inhibitory effect of corticosterone on PC12 cell neurite outgrowth.

In contrast to upregulated genes involved in multiple biological processes and molecular functions, all of the top five enriched biological processes from downregulated genes were involved in inhibition of DNA replication and cell division (**Table 4**). This finding indicates that besides inducing apoptosis, inhibiting cell proliferation is another mechanism contributing to impaired cell survival and growth caused by corticosterone treatment.

We also analyzed differentially expressed genes for enrichment of KEGG pathways (**Table**

Table 4. Top 5 enriched biological processes for downregulated genes induced by corticosterone treatment

GO Term~ Biological Process	Genes	N (%)	*P*-Value
GO:0051301 Cell division	CKS1B, KIFC1, FZR1, ANAPC15, AURKA, PTTG1, KIF2C, SPC25, CCNE1, NCAPH, CDC45, CDCA8, CDCA2, SKA3, CDCA4, CDCA3, CDC7, KIF14, CDC6, CDK1, RAN, DSN1, LIG1, CCNF, NUF2, KIF18B, CDC20, SPDL1, BIRC5, KNSTRN, CDK2, MCM5, NCAPD2, CDC25B, CCNB1, CCND1, TIMELESS, SPAG5, CKS1L, CKS2, BUB1B, CENPW, CENPT, HAUS8, UBE2S	45 (7.3)	7.20E-27
GO:0006260 DNA replication	TICRR, POLA1, POLA2, RPA1, RPA2, MCM8, MCM7, PRIM2, NS5ATP9, ORC1, FEN1, RECQL4, GINS1, LIG1, NASP, GINS3, POLE, FAM111A, GINS4, RBBP7, BRCA1, MCM6, RFC3, RFC2, RRM2, POLD1, RRM1, POLD2	28 (4.5)	1.91E-20
GO:0006270 DNA replication initiation	CDC7, CDC6, GINS4, POLA1, TOPBP1, MCM2, POLA2, MCM10, MCM3, MCM4, MCM5, MCM6, CCNE1, CDC45, MCM7, ORC6	16 (2.6)	4.06E-18
GO:0007059 Chromosome segregation	KIF11, NEK2L1, NEK2, CENPF, NDC80, BIRC5, ESPL1, CENPE, PMF1, PTTG1, RCC1, KNSTRN, BRCA1, SPC25, HJURP, SPAG5, BUB1, CDCA2, CENPW, SKA3, CENPT, CDK5RAP2, TOP2A	23 (3.7)	3.68E-17
GO:0000070 Mitotic sister chromatid segregation	NEK2L1, NEK2, DSN1, KIF18A, NUSAP1, KIF18B, ESPL1, KNSTRN, CDCA8, MAD2L1, SPAG5, CENPA, PLK1, NSL1, CIT	15 (2.4)	1.37E-15

Table 5. Top 5 enriched KEGG pathways for upregulated genes induced by corticosterone treatment

KEGG pathway	Genes	N (%)	*P*-Value
rno04512 ECM-receptor interaction	COL4A2, COL4A1, ITGA10, SDC4, LAMB2, LAMA5, ITGA5, TNR, ITGA7, COL6A2, COL6A1, SV2B, LAMC1, THBS1	14 (2.2)	1.95E-05
rno04510 Focal adhesion	PRKCA, COL4A2, CAV1, COL4A1, PGF, ITGA10, FLNC, VAV2, PXN, LAMB2, ITGA5, LAMA5, TNR, ITGA7, COL6A2, COL6A1, PAK1, LAMC1, THBS1, PIK3R1	20 (3.1)	2.50E-04
rno00280 Valine, leucine and isoleucine degradation	BCKDHA, BCAT1, MCCC2, ALDH6A1, IVD, MCCC1, AOX1, ALDH2, AUH	9 (1.4)	8.18E-04
rno01230 Biosynthesis of amino acids	BCAT1, GLUL, ASS1, ALDOC, SDSL, ENO2, PFKM, PSAT1, ASL, GPT2, PC	11 (1.7)	8.71E-04
rno01100 Metabolic pathways	SAT1, GDA, ALAD, OGDHL, QARS, ACSS2, AUH, MCCC2, PTGIS, ST3GAL5, ST3GAL4, XYLT1, MCCC1, AGPAT4, GPT2, HYAL1, ALDH6A1, PLD1, CYP1A1, FAXDC2, NDUFC2, PNPLA2, PFKM, LPIN1, ACADVL, GLUL, PLCE1, PRDX6, PLA2G2A, GAA, KDSR, INPP4A, MPST, BCAT1, XDH, AHCY, ASS1, GALNT7, ALDOC, CERS6, CTPS2, ASL, EXTL1, MTMR2, CSAD, IVD, ENO2, ALDH4A1, PNPO, BDH2, RGD1566085, BCKDHA, ADSSL1, PLB1, SDSL, UAP1L1, AMPD3, TST, POLD4, MLYCD, ATP6V1E1, AOX1, ALDH2, GAMT, PSAT1, DCXR, PC, NNMT	68 (10.7)	0.002

5). The top two enriched pathways from upregulated genes were ECM-receptor interaction and focal adhesion, indicating that interaction and signaling between cells and the extracellular matrix are very important for the effects of corticosterone. Interestingly, we found that many metabolic pathways were enriched during treatment. It will be interesting to examine the effects and/or mechanisms of various metabolic pathways involved in corticosterone-induced impairment of cell survival and neurite growth.

Discussion

For our study, we obtained a total of 9G bps of transcriptomes by RNA-Seq, representing about 50X sequencing depth of rat transcriptome, based on final assembling results. Such sequencing depth provides a greater number of repeat sequence reads for individual genes which confers much better accuracy and reliability to the sequencing data.

GO analysis further confirmed that our RNA-seq data were very comprehensive and successful in identification of genes differentially expressed in response to corticosterone in PC12 cells. Top enriched biological processes from upregulated genes include responses to dexamethasone stimulus and glucocorticoids, exactly reflecting cellular responses to corticosterone treatment (**Table 2**). Our sequencing data also elegantly explained the phenotypes of

PC12 upon corticosterone administration. Consistent with the observation that corticosterone dramatically reduced live PC12 cell numbers, we identified highly enriched differentially expressed genes promoting apoptosis and inhibiting cell division, consistent with known functions and mechanisms of glucocorticoid signaling (**Table 3**) [28]. However, it is interesting that we also identified many downregulated genes involved in DNA replication and chromosome segregation (**Table 4**), which would suggest new functions/mechanisms of glucocorticoids that are worthy of further examination in the future. In accordance with the impairment of neurite outgrowth conferred by glucocorticoids, RNA-seq data also showed significant enrichment of genes involved in neuron projection and axon guidance. Many of them had not been identified as regulated by glucocorticoids before (**Table 3** and data not shown). For instance, SEMA4B and SEMA3F are well established molecules involved in axon guidance [29] but their roles in glucocorticoid signaling have hardly been studied.

Taken together, our RNA-seq data are highly reliable and provide a complete resource for investigating mechanisms of glucocorticoid signaling on neuronal cell survival, differentiation, and morphogenesis. We have previously demonstrated by quantitative PCR that expression levels of four MAPK pathway genes were significantly increased after corticosterone stimulation. In the future, it will be interesting to examine the roles of novel molecules and pathways and/or processes, aiming to identify new mechanisms of glucocorticoid signaling in neurons.

This study contributes to the understanding of underlying mechanisms of MDD since glucocorticoid signaling is a factor greatly involved in the development of MDD. Impairment of neuronal cell survival and neurite outgrowth caused by glucocorticoids would without doubt adversely affect neuronal cell function and signal transduction. Therefore, identifying underlying mechanisms would potentially shed new light in the development of therapeutic strategies for MDD.

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Disclosure of conflict of interest

None.

Address correspondence to: Ning Wu, Department of Biological Sciences, Southeastern Oklahoma State University, Durant, OK 74701, USA. Tel: +15-80-7452564; Fax: +1-580-7457459; E-mail: nwu@se.edu; Ming-Zhen Li, Shenzhen Ruipuxun Academy for Stem Cell & Regenerative Medicine, Shenzhen, China. Tel: +86-10-89777650; E-mail: mzh51-12@126.com; Li Li, Beijing Friendship Hospital, Capital Medical University, Beijing, China. Tel: +86-10-63138328; E-mail: liliqin_1999@yahoo.com

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