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Neuropeptide Y Regulates Leptin Signaling Pathway via Receptor Y2 Assessed by Microarray in Human Macrophages

Hidesuke Kaji[1*], Mio Okada[1], Maiko Mori[1], Akiko Hamaue[1] and Masayo Nagai[1]

[1]Division of Physiology and Metabolism, University of Hyogo, Japan.

Authors' contributions

This work was carried out in collaboration between all authors. Author HK designed the study, performed most of the experiments and wrote the manuscript. Authors MO, MM, AH and MN also performed some experiments reported in the manuscript. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aims: It has been repored that neuropeptide Y receptor Y2 (Y2R) is involved in stress- and high caloric diet-induced metabolic syndrome in mice. Our previous report indicated the association between SNPs of *Y2R* gene upstream and plasma HDL-cholesterol levels in healthy subjects (rs6857530: GG<GA<AA or rs6857715: TT<TC<CC). Human macrophage differentiated from THP-1 cells revealed the luciferase activity when transfected with pGL3 -Basic vector inserted by promoter region of *Y2R* containing rs6857530AA plus rs6857715CC but not by rs6857530GG plus rs6857715TT. This study was carried out to clarify the mechanism of the association between SNPs of *Y2R* gene upstream and plasma HDL-cholesterol levels.
Methodology: *Y2R* gene upstream included heterozygous rs6857530 (G/A) and rs6857715 (T/C) in macrophage/THP-1 cells by direct sequencing. Thus, the experiment was carried out to investigate the effect of potent Y2R antagonist BIIE0246 on gene expression in cultured macrophage/THP-1 cells using microarray.

**Corresponding author: E-mail: hidesuke_kaji@cnas.u-hyogo.ac.jp;*

Results: BIIE0246 up-regulated 60 transcripts (>2.0-fold) and down-regulated 55 entities (<0.5-fold) among 60,000 probe sets. It is of interest that *leptin receptor* gene was up-regulated (2.83-fold) by BIIE0246. BIIE0246-induced up- and down-regulation of gene expression indicated 6 and 1 gene ontology terms, respectively ($P<0.001$), and 7 pathways including leptin signaling and 1 pathway, respectively ($P<0.01$).

Conclusion: BIIE0246 regulated several genes and pathways including leptin signaling in macrophage/THP-1 cells. Taken together with the previous report by others, NPY might stimulate cholesterol efflux through Y2R by inhibiting leptin signaling in macrophage/THP-1 cells. Results were consistent with our previous reports indicating higher plasma HDL-cholesterol levels in subjects with Y2R expressive SNPs rs6857530AA plus rs6857715CC in macrophage.

Keywords: Neuropeptide Y receptor Y2; BIIE0246; human macrophage; leptin signaling pathway; microarray; high density lipoprotein cholesterol.

1. INTRODUCTION

Neuropeptide Y (NPY), originally isolated from porcine brain [1], is generally known to stimulate appetite through hypothalamic NPY receptor Y1 [2]. NPY is released under stress from sympathetic nerve terminal. Kuo et al have reported that NPY receptor Y2 (Y2R) is involved in stress- and high caloric diet-induced metabolic syndrome in mice [3]. Our previous report indicated the association between single nucleotide polymorphisms (SNPs) of *Y2R* gene promoter and metabolic phenotypes. These SNPs were significantly associated with plasma high density lipoprotein-cholesterol (HDL-C) levels in healthy subjects [4]. Plasma HDL-C levels were significantly different in subjects with each SNPs (rs6857530: GG<GA<AA, rs6857715: TT<TC<CC).

We have previously reported that *Y2R* gene expression was cell type- and SNP type-dependent [5]. Human hepatoma cell HepG2 revealed the luciferase activity when transfected with pGL3-Basic vector inserted by promoter region of *Y2R* containing rs6857530GG plus rs6857715TT but not by rs6857530AA plus rs6857715CC. Thus, we have investigated the effect of potent Y2R antagonist BIIE0246 [6] on transcriptome profiling by using microarray in HepG2 [7]. BIIE0246-induced down-regulation of gene expression were significantly involved in 44 pathways in which sterol responsive element binding protein signaling was included. Moreover, BIIE0246-induced up-regulation of gene expression significantly involved 22 pathways in which HDL metabolic pathway was included. These results suggested that Y2R blockade was beneficial to protect atherosclerosis in subjects with Y2R expressive SNPSs rs6857530GG and rs6857715TT in liver.

On the other hand, we have previously reported that the luciferase activity was detected in human macrophage derived from monocytic leukemia cell THP-1 when used pGL3-Basic including *Y2R* gene promoter with rs6857530AA plus rs6857715CC but not with rs6857530GG plus rs6857715TT [5]. To clarify the underlying mechanism of the association between *Y2R* gene SNPs and plasma HDL-C levels, the effect of BIIE0246 on transcriptome profiling in macrophage/THP-1 cells was investigated by using microarray.

2. MATERIALS AND METHODS

2.1 Cell Culture

THP-1 cells were purchased from Health Science Research Resources Bank (Osaka Japan) and cultured in RPMI-1690 with 5% fetal bovine serum at 37℃ under atmosphere of 5% CO_2 as described previously [5]. THP-1 cells were differentiated to macrophage by adding 160 nM phorbol 12-myristate 13-acetate (LC Laboratories, Woburn, MA) as described previously [5].

2.2 SNP Typing

DNA was extracted from cultured THP-1 cells by phenol-chloroform method as described previously [7]. The 5'-flanking region of *Y2R* gene was amplified by PCR using thermal cycler (Techgene, Techne, St. Louis, Mo) with forward primer: ttcgtgtcccatagctttcc and reverse primer: tctagctgggcggtccctgtg. PCR products were directly sequenced by Sanger method as described previously [7] to identify the type of *Y2R* gene SNP rs6857530 and rs6857715.

2.3 Y2R Antagonist

Macrophages, differentiated from THP-1 cells, were cultured for 24 h in the presence of 100 nm human NPY (ABGENT) with or without 1μM BIIE0246 (TOCRIS bioscience).

2.4 Microarray Analysis

RNA was isolated from 4 replicates of each cell group by total RNA purification kit (Biosynthesis). RNA integrity number (RIN), ideally higher than 7.0, was 9.0 in control group and 9.2 in BIIE0246-treated cell group assessed by Bioanalyzer (Agilent Technologies). A260/A280 of isolated RNA was 1.87 in control cell group and 1.88 in BIIE0246-treated cell group assessed by spectrophotometer NanoDrop ND-1000 (NanoDrop Technologies). A260/A230 of isolated RNA was 2.01 in both control and BIIE0246-treated cell group, suggesting that DNA was not contaminated.

Total RNA was reverse-transcribed to complementary DNA, which was transcribed to Cy3-labeled complementary RNA (cRNA). This cRNA was hybridized to SurePrint G3 Human GE 8x60K v2 Microarray (Agilent Technologies, Inc.) for 17 h. Array was washed and scanned by SureScan microarray scanner (G2600D). GeneSpring GX (Agilent Technologies) was used for the analyses of scatter plot, hierarchical cluster, gene ontology (GO), pathway and Natural Language Processing (NLP) network. Hierarchical clustering analysis was performed by using clustering algorithm and the results were shown by heat map. GO analysis referenced AmiGO 2 online database. The ratio of regulated genes vs. total genes in the GO term was considered statistically significant when *P*-value was less than 0.001. WikiPATHWAYS online database was referenced in pathway analysis (Single Experiment Analysis), in which the ratio of regulated genes vs. total genes was considered statistically significant when *P*-value was less than 0.01. NLP network analysis was carried out by using algorithm of direct interaction in simple analysis.

3. RESULTS

3.1 SNP Typing

Y2R gene upstream from macrophage/THP-1 cells demonstrated heterozygous rs6857530 (G/A) and rs6857715 (T/C) by direct sequencing.

3.2 Microarray Analysis

Microarray showed the effect of Y2R antagonist BIIE0246 on transcriptome profiling in cultured macrophage/THP-1 cells. Scatter plots of transcriptome were shown in macrophages cultured in NPY alone (control) and NPY plus BIIE0246 (Fig. 1a). X-and Y-axis is control and BIIE0246-treated gene signal values, respectively. In order to correct chip variation, each value is expressed as the ratio relative to 0 that is $\log_2$ value of 75 percent shift normalization. Two-fold up-regulation, no change and 2-fold down-regulation were shown as upper, middle and lower diagonal line, respectively. In 60,000 spots, BIIE0246-induced up-regulation of gene expression (>1.5-fold) were 317 entities, and down-regulation of gene expression (<0.67-fold) were 244 entities with analyzable signal. Among them, BIIE0246 up-regulated 60 entities (>2.0-fold) and down-regulated 55 entities (<0.5-fold) (Table 1). Hierarchical clustering was performed among top 100 entities regulated by BIIE0246. Control group and BIIE0246-treated group revealed different pattern in dendrogram of functionally related and co-regulated cluster shown by heatmap (Fig. 1b).

GO analysis identified 6 GO terms in BIIE0246-upregulated genes (>1.5-fold) and 1 GO term in BIIE0246-downregulated genes (<0.67-fold) (*P*<0.001) (Table 2).

Pathway analysis identified 7 pathways in BIIE0246-induced up-regulation of gene expression (>1.5- fold) and 1 pathway in BIIE0246-induced down-regulation (<0.67-fold) (*P*<0.01) (Table 3).

NLP network analysis was shown in Fig. 2. This analysis also revealed that 3 genes such as *leptin receptor* (*LEPR), erb-b2 receptor tyrosine kinase 2 (ERBB2),* and *interleukin-1B* (*IL-1B)* that were involved in leptin signaling pathway.

4. DISCUSSION

Macrophage/THP-1 cells revealed the luciferase activity when transfected by pGL3-Basic vector including *Y2R* gene promoter with rs6857530AA plus rs6857715CC but not with rs6857530GG plus rs6857715TT [5]. Therefore, the 5'-flanking region of *Y2R* in macrophage/THP-1 cells was directly sequenced and resulted in heterozygous rs6857530 (G/A) and rs6857715 (T/C), suggesting that *Y2R* gene was expressed in macrophage/THP-1 cells.

Thus, we analyzed the effect of BIIE0246 on transcriptome profiling in macrophage/THP-1 cells by using microarray. BIIE0246 highly up-regulated several genes such as *potassium channel subfamily member 3 (KCNK3), CD27, G-protein coupled receptor 3 (GPR3) and highly down-regulated several genes such as macrophage stimulating 1 pseudogene 2 (MST1P2), potassium voltage-gated channel subfamily A regulatory beta subunit 3 (KCNAB3), laminin subunit alpha 3 (LAMA3), cytochrome P450 family 21 subfamily A member 2 (CYP21A2)*. These genes did not have the function related to HDL metabolism.

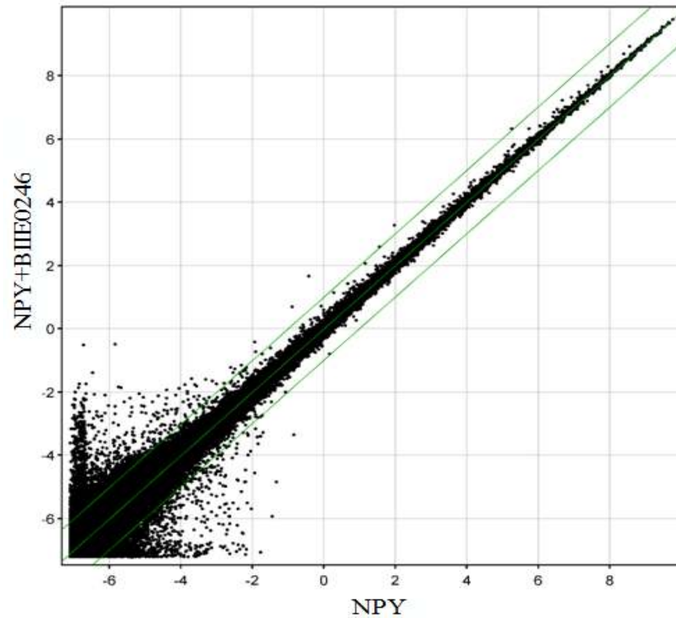

Fig. 1a. Effect of Y2R antagonist BIIE0246 on gene expression in macrophage derived from THP-1 cells was compared by scatter plots. Signal values are expressed as log2 after 75% shift normalization

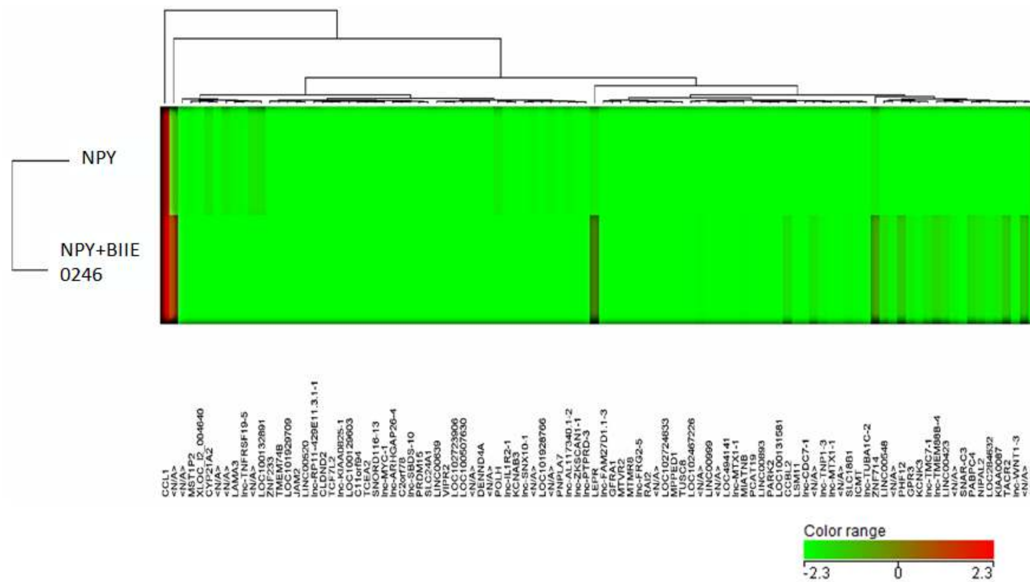

Fig. 1b. Hieralchical clustering of top 100 regulated genes were shown by dendrogram with heatmap. The grade of up- and down-regulaion is shown by red and green color gradation, respectively, as indicated by color range (signal log ratio)

Table 1. Up (>2) and down (<0.5) regulated genes by BIIE0246 in macrophage derived from THP-1 cells

Up-regulation		Down-regulation	
Fold change	**Gene symbol**	**Fold change**	**Gene symbol**
5.67	KCNK3	0.160	
5.59		0.192	XLOC_014512
4.60	CD27	0.197	
4.44	GPR3	0.207	MST1P2
4.34		0.269	XLOC_001595
4.34	LINC00548	0.270	KCNAB3
4.11	XLOC_009740	0.273	XLOC_010309
4.10	SNAR-C3	0.283	LAMA3
3.73	XLOC_000293	0.289	CYP21A2
3.59	NIPAL2	0.301	XLOC_006188
3.56	LOC284632	0.323	LOC101928766
3.51	LSM11	0.324	
3.50	LOC100131581	0.333	LOC100132891
3.42		0.338	LINC00639
3.40		0.340	
3.39	KIAA0087	0.341	DENND4A
3.25	THBS3	0.344	
3.23	PABPC4	0.348	XLOC_007645
3.22	LINC00893	0.348	
3.08		0.351	PNPLA7
3.04	PARK2	0.378	C2orf78
2.96		0.387	
2.95	ICMT	0.387	XLOC_006491
2.94	PCAT19	0.394	XLOC_006019
2.84		0.401	VIPR2
2.83	**LEPR**	0.402	
2.78	SLC18B1	0.414	SLC24A1
2.76	LINC00423	0.415	SNORD116-13
2.75	LINC00999	0.416	
2.64	PHF12	0.419	
2.63		0.424	POLH
2.58		0.428	C11orf94
2.53	XLOC_009080	0.429	
2.48	MIATNB	0.430	LOC100507630
2.44	RAI2	0.432	TCEA2
2.43	CCL1	0.441	LOC100129603
2.40	MTVR2	0.441	
2.38		0.441	
2.38	LOC102467226	0.446	XLOC_006923
2.33	TACR2	0.450	PRDM15
2.28	GFRA1	0.454	TMEM74B
2.28	ZNF714	0.455	CLDND2
2.24	TUSC8	0.455	JAM2
2.23		0.467	TCF7L2
2.22		0.470	LOC101929709
2.22	LOC102724833	0.477	ZNF233
2.20	XLOC_000017	0.480	LINC00520
2.18	MPPED1	0.481	LOC388282
2.15	XLOC_007368	0.482	RIBC2
2.13	XLOC_004229	0.494	**HSD11B2**
2.13	CCBL2	0.494	XLOC_012694
2.13		0.494	LIG4
2.11	MTMR8	0.496	FLJ37201
2.08	HCN2	0.496	NR4A2
2.07	ADAMTSL2	0.498	LOC730102
2.07	TP73		
2.05	LOC100128374		
2.02	EMC1		
2.02	LINC00847		
2.01	NPTX1		

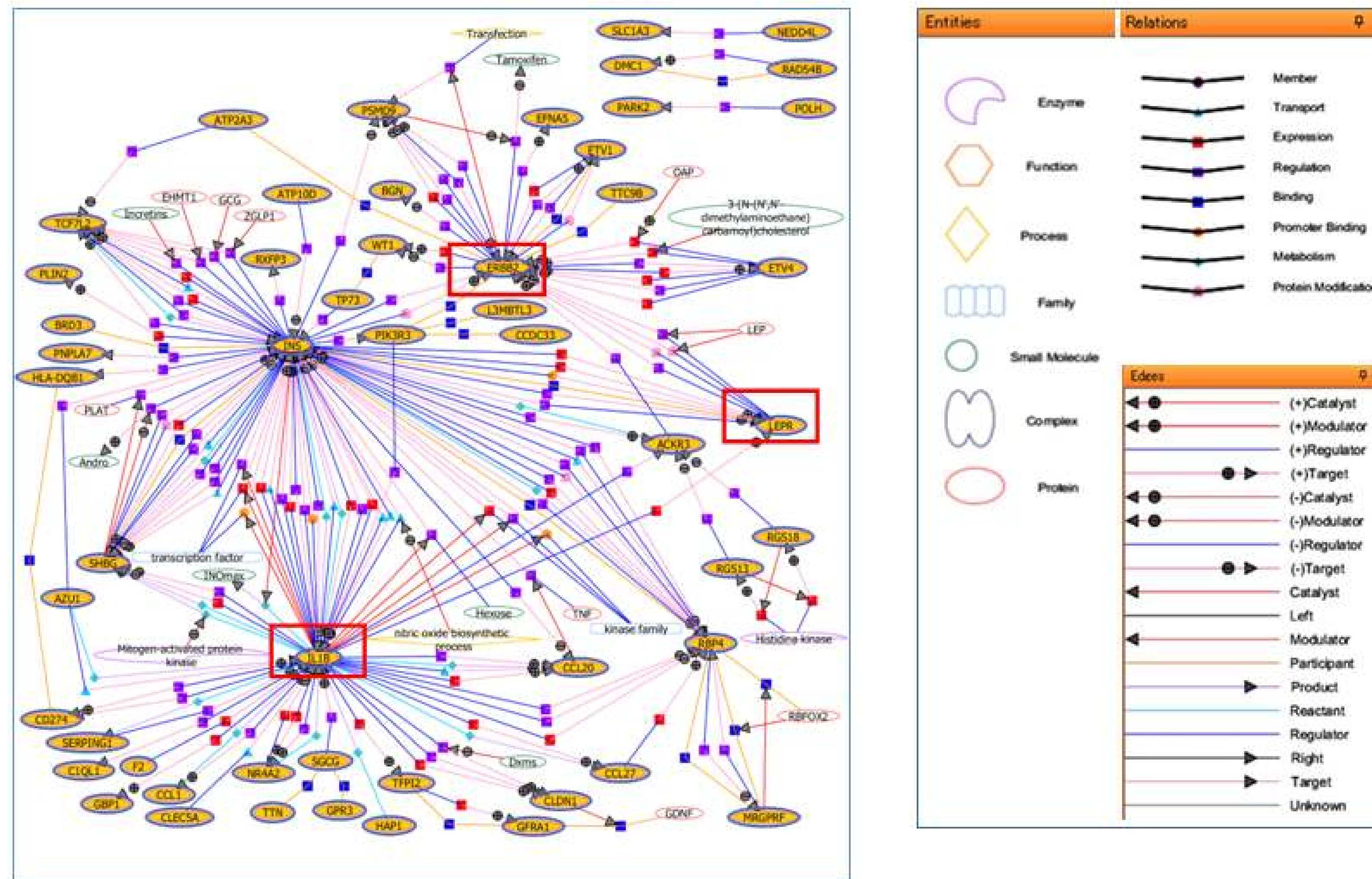

Fig. 2. NLP network analysis of BIIE0246-regulated genes (shown in orange ellipse) in macrophages/THP-1 cells. BIIE0246-induced up-regulation of genes that were involved in leptin signaling pathway were shown in red box. Entities, relations and edges were defined as shown in left legends

Table 2. Gene ontology analysis in BIIE0246-regulated genes (*P* <0.001) in macrophage dreived from THP-1 cells

Go accession	Go term	*P*-value	Count in selection	Count in total
	Upregulated genes (>1.5)			
GO:0072284	Metanephric S-shaped body morphogenesis	0.00026	2	3
GO:0038032	Termination of G-protein coupled receptor signaling pathway	0.00046	4	39
GO:0072050	S-shaped body morphogenesis	0.00051	2	4
GO:0033058	Directional locomotion	0.00051	2	4
GO:0009894	Regulation of catabolic process	0.00051	19	877
GO:0023021	Termination of signal transduction	0.00061	4	42
	Downregulated genes (<0.67)			
GO:0003071	Renal system process involved in regulation of systemic arterial blood pressure	0.00026	3	22
GO:0005496	Steroid binding	0.00085	4	74

Table 3. Pathways related to BIIE0246-regulated genes (*P* <0.01) in macrophage derived from THP-1 cells

Pathway	*P*-value	Matched entities	Pathway entities
Upregulated genes (>1.5)			
Hs Calcium Regulation in the Cardiac Cell WP536 72097	0.0028	5	149
Hs_Myometrial_Relaxation_and_Contraction_Pathways_WP289_72107	0.0033	5	156
Hs Vitamin B12 Metabolism WP1533 70117	0.0041	3	53
Hs_Leptin_signaling_pathway_WP2034_71413	**0.0071**	**3**	**61**
Hs_TP53_Network_WP1742_71700	0.0079	2	22
Hs Nicotine Activity on Dopaminergic Neurons WP1602 69773	0.0079	2	21
Hs Folate Metabolism WP176 72364	0.0084	3	67
Downregulated genes (<0.67)			
Hs_Glucocorticoid_&_Mineralcorticoid_Metabolism_WP237_72079	0.00065	**2**	**9**

Genetic studies clarified new loci for regulating HDL metabolism [8-10]. On the other hand, functional studies have reported that ATP binding cassette transporters A1 (ABCA1) and G1 (ABCG1), and scavenger receptor class B type I (SR-BI) play major roles in cholesterol export from macrophages [11]. The present study indicated that BIIE0246 failed to change mRNA levels of these molecules.

It is of interest that *LEPR* gene expression showed 2.8-fold up-regulation by BIIE0246. Leptin is an adipocytokine that suppresses appetite by stimulating the activity of proopiomelanocortin neuron and by inhibiting the activity of NPY/agouti-related peptide neuron on the arcuate nucleus [12]. Hongo et al. [13] have reported that leptin inhibits cholesterol efflux from macrophage through activation of acyl-coenzyme A: cholesterol acyltransferase (ACAT1). They also reported that cholesterol efflux from macrophage was inhibited by leptin in the absence of ACAT1 inhibitor K-604, but not in the presence of K-604. As leptin failed to change ABCA1, ABCG1 and SR-BI mRNA levels in human monocyte/macrophages, they considered that leptin suppressed HDL-mediated cholesterol efflux in human macrophages through enhanced cholesterol ester accumulation by ACAT1 up-regulation. Taken together with their reports, it is possible to assume that the up-regulation by BIIE0246 of leptin signaling pathway including *LEPR, ERBB2* and *IL-1B* stimulated ACAT1 activity with a decrease in cholesterol efflux from macrophages/THP-1 cells. Conversely, NPY might inhibit leptin action through Y2R and stimulate HDL-mediated cholesterol efflux. If it is correct, the results are consistent with our previous reports that plasma HDL-C is higher in subjects with Y2R expressive SNPs rs6857530AA plus rs6857715CC in macrophages [4,5].

Interestingly, *11β-hydroxysteroid dehydrogenase type 2* (*HSD11B2*) gene expression was down-regulated by BIIE0246 (0.494-fold). Moreover, *HSD11B2* was a down-regulated gene among 2 genes in glucocorticoid and mineralocorticoid metabolic pathway. HSD11B2 deficiency accelerates atherogenesis and proinflammatory changes in the aortic endothelium in *Apoe*-/- and *HSD11B2*-/- double knockout mice [14]. HSD11B2 is an enzyme that catalyze active cortisol to inactive cortisone [15]. HSD11B2 deficiency increased local cortisol (corticosteron in rodents) concentrations and activated mineralocorticoid receptor. Therefore, they concluded that HSD11B2 is atheroprotective. Although the present study was carried out in macrophage/THP-1 cells, *HSD11B2* down-regulation by BIIE0246 suggested that NPY action through Y2R in macrophage might be protective against atherosclerosis.

5. CONCLUSION

In conclusion, Y2R antagonist BIIE0246 regulated several gene expression in human macrophage/THP-1 cells assessed by microarray analysis. *LEPR*, *ERBB2, and IL1B* were BIIE0246-upregulated genes involved in leptin signaling pathway, suggesting that NPY might stimulate HDL-mediated cholesterol efflux via Y2R in macrophages. We have previously shown that plasma HDL-C levels were higher in healthy subjects with Y2R SNPs rs6857530AA plus rs6857715CC than those with Y2R SNPs rs6857530GG plus rs6857715TT. Luciferase activity of Y2R gene promoter with SNPs rs6857530GG plus rs6857715TT was detectable in HepG2 but not in macrophage/THP-1, whereas that with SNPs rs6857530AA plus rs6857715CC was detectable in macrophage/THP-1 but not in HepG2. Taken together with these findings, NPY might be a risk factor for atherosclerosis in subjects with *Y2R* SNPs rs6857530GG plus rs6857715TT, whereas NPY might be protective against atherosclerosis in subjects with *Y2R* SNPs rs6857530AA plus rs6857715CC. SNP type of *Y2R* gene promoter might be a good candidate for risk marker or therapeutic target of dyslipidemia or atherosclerosis.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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