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β-Carotene enhances the expression of inflammation-related genes and histone H3 K9 acetylation, K4 dimethylation, and K36 trimethylation around these genes in juvenile macrophage-like THP-1 cells

Shinnnosuke Kondo [a], Rina Suzuki [b], Yuki Nakashima [b], Kazuki Mochizuki [b,*]

[a] *Department of Integrated Applied Life Science, Integrated Graduate School of Medicine, Engineering, and Agricultural Sciences, University of Yamanashi, 4-4-37 Takeda, Kofu, Yamanashi, 400-8510, Japan*

[b] *Laboratory of Food and Nutritional Sciences, Department of Local Produce and Food Sciences, Faculty of Life and Environmental Sciences, University of Yamanashi, Kofu, Yamanashi, Japan*

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ABSTRACT

β-Carotene is converted into vitamin A in the body and can remove reactive oxygen species. However, it is still unclear whether β-carotene alters the expression levels of inflammation-related genes in macrophages and how this is regulated. In the present study, we investigated whether the administration of β-carotene under hyperglycemic conditions altered the expression level of inflammation-related genes and whether any observed differences were associated with changes in histone modifications in juvenile macrophage-like THP-1 cells. THP-1 cells (from a human monocytic leukemia cell line) were cultured in low glucose (5 mM), high glucose (25 mM), or high glucose (25 mM) + β-carotene (5 μM) media for 1 day, and mRNA expression levels of genes related to oxidative stress and inflammation, and histone modifications were determined by mRNA microarray and qRT-PCR analyses, and chromatin immunoprecipitation assays, respectively. The expression of inflammation-related genes, such as *IL31*RA*, CD38,* and *NCF1B,* and inflammation-associated signaling pathway genes, such as *ITGAL, PRAM1,* and *CSF3R*, were upregulated by β-carotene under high-glucose conditions. Under these conditions, histone H3 lysine 4 (K4) demethylation, H3K36 trimethylation, and H3K9 acetylation around the *CD38*, *NCF1B*, and *ITGAL* genes were higher in β-carotene-treated cells than in untreated cells. Treatment of juvenile macrophage-like THP-1 cells with β-carotene under these high glucose conditions induced the expression of inflammation-related genes, K9 acetylation, and K4 di- and K36 trimethylation of histone H3 around these genes.

1. Introduction

Recent studies have suggested that one of the pathogenic mechanisms underlying cardiovascular disease progression, including ischemic heart disease, arrhythmia, and hypertension, is the formation of atherosclerosis by innate leukocytes, including monocytes and macrophages [1]. Macrophages are known to induce the secretion of proinflammatory cytokines and chemokines that amplify the immune response by mobilizing leukocytes, such as monocytes, to blood vessel walls and tissues with bacterial infection or injury [2]. In addition, vascular endothelial cells, which are activated by proinflammatory cytokines that are secreted from innate leukocytes, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, express numerous cell surface adhesion molecules such as vascular cell adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1, P-selectin, and E-selectin. Therefore, the activation of monocytes allows the adhesion of innate leukocytes to the blood vessel wall and infiltration of innate leukocytes into each tissue [3]. The development of atherosclerosis by the infiltration and activation of macrophages is known to be facilitated by oxidative stress in the blood vessels [4]. When monocytes infiltrate the vessel wall, they differentiate into macrophages, some of which accumulate cholesterol and transform into foam cells in the vessel wall, resulting in the progression of atherosclerosis [5]. Recent studies have suggested that oxidative stress and the production of reactive oxygen species (ROS) are involved in the activation of innate leukocytes, including monocytes and macrophages. The removal of ROS by

Abbreviations: BC, β-carotene; ChIP, chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; HG, high glucose; LG, low glucose; ROS, reactive oxygen species.

* Corresponding author. Faculty of Life and Environmental Sciences, University of Yamanashi, 4-4-37 Takeda, Kofu, Yamanashi, Japan.
E-mail address: mochizukik@yamanashi.ac.jp (K. Mochizuki).

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chromium in juvenile macrophage-like U937 cells co-treated with hydrogen peroxide reduced TNF-α protein secretion into the culture medium and lipid peroxidation levels in these cells [6]. These findings suggest that ROS induced by hyperglycemia may be involved in the activation of innate leukocytes, such as monocytes and macrophages, and in the enhancement of proinflammatory cytokine expression in these cells.

Carotenoids, which are a major class of natural food coloring additives, are thought to play important roles in maintaining health by providing vitamin A and antioxidants to the body. Among carotenoids, α-, β-, and γ-carotene, and β-cryptoxanthin are considered as provitamin A because they are converted into vitamin A in the body. Carotenoids such as lycopene and lutein are not converted to vitamin A. In general, overconsumption of micronutrients can lead to adverse effects. Among carotenoids, numerous foods contain β-carotene, which is easily converted into vitamin A. Because this conversion occurs when the amount of vitamin A in the body is insufficient, the risk of vitamin A overload disorders caused by β-carotene intake is considered to be low. In addition, carotenoids, including β-carotene, are thought to have the benefits of inhibiting numerous diseases including lifestyle diseases because they remove ROS such as singlet oxygen [7].

β-Carotene generates radicals and induces oxidative damage when it is oxidized [8]. Furthermore, β-carotene administration to patients with type 2 diabetes (n = 20) at a daily dose of 60 mg for 3 weeks reduced low-density lipoprotein oxidation and slowed the progression of atherosclerosis [9], suggesting that β-carotene can have a preventive effect on the development of atherosclerosis in patients with diabetes.

However, β-carotene is easily consumed as a supplement and accumulates in the body as a fat-soluble substance [10]. Generally, fat-soluble food factors can easily lead to excessive disorders. Indeed, a meta-analysis summarizing 50 randomized controlled trials that studied supplemental intake of antioxidants, including vitamins and β-carotene, in subjects with cardiovascular diseases (myocardial infarction, angina pectoris, stroke, and/or transient ischemia) did not affect the subsequent onset and risk of vascular-related death [11]. A previous cross-sectional study of 1312 men and 1544 women using National Health and Nutrition data demonstrated that the concentration of C-reactive protein (CRP) was positively associated with dietary β-carotene content and serum β-carotene concentration [12]. CRP is an inflammation-related protein released by the liver, and higher CRP concentrations are associated with the onset of fatty liver or liver injury [13]. Therefore, it is possible that excessive intake of β-carotene supplements is not effective or can even enhance atherosclerosis in patients with diabetes.

In an *in vitro* experiment using RW264.7 macrophages, it was reported that 2 μM β-carotene treatment reduced the inflammatory response of cells stimulated with the carcinogen phorbol 12-myristate 13-acetate, whereas the 20 μM β-carotene treatment exerted an upregulation of *IL-8* and *TNF-α* in lipopolysaccharide-stimulated cells [14]. However, it is not clear whether the expression of proinflammatory cytokines in hyperglycemia-derived macrophages or monocytes is repressed or promoted by β-carotene administration.

Recently, gene expression changes involved in the development of lifestyle diseases, such as type 2 diabetes and its complications, which are caused by long-term practice of certain lifestyle factors, have been implicated in the imprinting of chromatin. For example, during the United Kingdom Prospective Diabetes Study, patients with type 2 diabetes mellitus who received intensive glucose therapy had a lower risk of microvascular complications than those who received conventional dietary therapy. A 10-year follow-up study showed that patients who underwent intensive glucose control targeting HbA1c that was initiated early in the course of diabetes had a lower risk of diabetic complications and a significant reduction in the risk of myocardial infarction and death from any cause compared with patients who received typical care [15]. The effects of therapies remembered by the body are known as legacy effects. Legacy effects persist for a longer time; therefore, these effects could be imprinted on chromatin as epigenetic memories, such as DNA methylation and histone modification. High glucose levels increase the expression of proinflammatory cytokines such as *IL-6, IL-12p40,* macrophage inflammatory protein-1α (*MIP-1α*), and *MIP-1β* in macrophages, and induction of these genes was associated with a reduction in histone H3 lysine 9 (K9) trimethylation levels around these genes [16]. Regarding β-carotene, a report demonstrated that hypomethylation of the CpG promoter/enhancer of retinol binding protein 4 (*Rbp4*) and hypermethylation of the *Pcna* promoters were observed in the white adipose tissue of 21-day-old β-carotene-treated rats [17], and co-treatment with β-carotene and arachidonic acid altered methylation of the CpG island of the connexin 43 (*Cx43*) gene promoters in human umbilical vein endothelial cells [18]. However, it is still unclear whether histone modifications are involved in the increased expression of proinflammatory cytokines in macrophage-like cells treated with β-carotene under high-glucose conditions.

In the present study, we investigated whether administration of the antioxidant food ingredient β-carotene to juvenile macrophage-like THP-1 cells under hyperglycemic conditions alters the expression of inflammation-related and antioxidant-related genes and whether these alterations are associated with changes in histone modification around these genes.

2. Materials and methods

2.1. Cell culture

THP-1 cells (a human monocytic leukemia cell line) were provided by American Type Culture Collection (Manassas, VA, USA). Cells were incubated with 10% fetal calf serum, 2 mM L-glutamine, antibiotic-antimycotic mixed stock solution (Nacalai Tesque, Kyoto, Japan), 1 M HEPES (pH 7.4), 1% non-essential amino acids, and RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) with 10 mM glucose at 37 °C, 5% carbon dioxide, and 95% atmospheric partial pressure in an incubator (Forma Direct-Heat CO_2 Incubator, Thermo Fisher Scientific, Waltham, MA, USA). Ten days after incubation, the culture medium was replaced with RPMI 1640 medium containing low glucose (5 mM). After five days of incubation, cells were divided into three groups as follows: the low-glucose RPMI 1640 medium (5 mM) + dimethyl sulfoxide (DMSO) (LG) group, high-glucose RPMI 1640 medium (25 mM) + DMSO (HG) group, and high-glucose RPMI 1640 medium + β-carotene (5 μM) (BC) group. β-carotene was dissolved in DMSO and administered to cells. After 1 day of culture, THP-1 cells cultured in each medium were collected for total RNA and chromatin immunoprecipitation (ChIP).

2.2. Real-time RT-PCR

After removing the supernatant by centrifugation at 3300×*g* for 3 min, 500 μL of lysis solution (4 M guanidine thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sodium N-laurylsarcosine, 0.1% 2-mercaptoethanol) was dispensed, stirred, and stored at −80 °C until total RNA extraction. Total RNA was extracted using the acidified guanidine thiocyanate method, as described previously [19]. Total RNA samples (100 ng) were converted into cDNA by reverse transcription using SuperScript III reverse transcriptase (Invitrogen, Waltham, MA, USA) in accordance with the manufacturer's instructions. For the quantitative analysis of mRNA levels, qRT-PCR was performed using a Light-Cycler Instrument System (Roche Molecular Biochemicals, Penzberg, Germany). qRT-PCR was carried out using gene-specific primers, cDNA, and SYBR Green I Master (Roche Molecular Biochemicals). The CT values of each gene determined by qRT-PCR were converted into signal intensities based on the ΔΔCT method, which recognizes a difference of one CT value as a two-fold difference between samples [20]. RNA levels were normalized to *TF2B* levels. The primer sequences are shown in Table S1.

2.3. mRNA microarray analysis

Total RNA was extracted from three samples from each group and used for microarray analysis. The samples were randomly selected using a random number table. Aliquots containing 100 ng of total RNA were individually converted to cDNA, fractionated, and labeled using the Gene ChIP® Poly-A RNA Control Kit, WT Amplification Kit, and Gene ChIP® WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA), according to the manufacturer's instructions. Hybridization, washing, and staining were performed using the Affymetrix® HuGene2.1 ST Array Strip and a GeneAtlas® Hybridization Wash and Stain Kit for WT Assay Strips (Affymetrix), according to the manufacturer's instructions. After washing, the HuGene2.1 ST Array Strip was analyzed using the GeneAtlas Imaging Station (Affymetrix). Data analysis was performed using the Expression Console (Affymetrix), Transcriptome Analysis Console (Affymetrix), and Excel (Microsoft) software. Significant differences ($p < 0.05$) between BC and LG or between BC and HG were assessed by analysis of variance in the Transcriptome Analysis Console. Gene ontology analysis was not performed because the number of detected genes, whose expressions were upregulated by β-carotene, was relatively small. Instead a PubMed search of published literature was used to classify gene functions into the following categories: metabolism, which included metabolic enzymes and nutrient transporters; immune response, which included typical genes related to inflammation and leukocytes; signal transduction, which included hormones, their membrane receptors, second messenger and kinase cascades under the membrane receptors; protein synthesis, which included ribosomal proteins and elongation factors for proteins; stress/apoptosis, which included protein degradation cascades, specifically ubiquitin cascades, and heat shock proteins; transcription/chromatin, which included transcriptional factors, non-coding RNAs, chromatin regulators, and unknown.

2.4. Chromatin immunoprecipitation (ChIP) assay

Target cells were incubated with fixation solution (1% formaldehyde, 4.5 mM HEPES pH 8.0, 9 mM NaCl, 0.09 mM EDTA, and 0.04 mM EGTA) in culture medium for 15 min at 37 °C. The reaction was terminated by adding glycine to a final concentration of 150 mM. After washing with 2% bovine serum and 0.05% NaN_3 in 1 × PBS(−), the samples were sonicated in SDS lysis buffer [50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 1% SDS, and cOmplete Mini (one tablet/10 mL of SDS lysis buffer; Roche Molecular Biochemicals)] until the sample sizes were 200–500 bp. One-tenth of the sample volume was diluted in dilution buffer (50 mM Tris-HCl pH 8.0, 167 mM NaCl, 1.1% Triton X-100, 0.11% sodium deoxycholate) and stored at 4 °C as the input fraction. Cell fixation and ChIP assays were performed as described previously using specific antibodies: against mono-, di-, and trimethylation of H3K4 and H3K36; anti-acetyl-histone H3 K9; NF-κB p65 (Millipore, Billerica, MA, USA); and control rabbit IgG (Sigma-Aldrich). Protein–DNA–protein G Sepharose complexes were obtained by immunoprecipitation, and the input fractions were dissociated in 200 μL of ChIP direct elution buffer (10 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.5% SDS, and 300 mM NaCl) at 65 °C overnight. After the removal of RNA and protein by treatment with RNase A, proteinase K, and phenol/chloroform extraction, the precipitated DNA was subjected to qPCR using primers corresponding to the indicated sites in the promoter/enhancer and gene bodies. The CT values of the ChIP and input signals detected by qPCR were converted into signal intensities using the ΔΔCT method, which calculated a difference of 1 CT value as a two-fold difference between samples [20]. All ChIP signals were normalized to the corresponding input signals. The formula used was as follows: $2^{(\text{CT input} - \text{CT acetylated histone})}$. The sequences of the PCR primer pairs are shown in Table S2.

2.5. Statistical analysis

The results are expressed as mean ± SEM. The significance of differences for qRT-PCR and ChIP among groups was determined by Tukey–Kramer's test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Comparison of mRNA levels between THP-1 cells with high glucose and those treated with β-carotene using microarray analyses

We performed a mRNA microarray analysis to compare cells in the HG group with those in the BC group. The numbers of upregulated genes (1.5-fold or more) in the BC group compared with those in the LG group and in HG group were 182, 114, and 72, respectively. The numbers of downregulated genes (−1.5-fold or less) in the BC group compared with those in the LG group and HG group were 367, 97, and 80, respectively (Fig. 1). A list of up- and downregulated genes in the BC group compared with those in the LG and HG groups is provided in Tables S3 and S4. Among the upregulated genes, we demonstrated that 32 genes were related to metabolism, immune responses, and signal transduction (Table 1). The metabolism-related genes with upregulated expression levels in the BC group compared with that in the HG group were *ECH1, FBP1, B4GALT5*, and *PFKFB3.* The immune response genes with upregulated expression levels in the BC group compared with that in the HG group were *IL31*RA*, CD38, ITGAL, ITGAX, NCF1B, NCF1C, CD1D, CSF3R*, and *TRBY6-5*. The signal transduction genes with upregulated expression in the BC group compared with that in the HG group were *FGR*, *PLCB2*, *GRN*, *SULF2*, *SCUBE1*, *OR2A1*, *SLC43A2*, *TBC1D2*, *PRAM1*, *SPDYE2B*, *HIPK2*, *MERTK*, *PSAP, SPDYE2*, *TTLL4*, *LRG1*, *OGFR*, and *PPP2R3B* (Table 1).

3.2. Confirmation of upregulation of metabolism-, inflammation- and signal transduction-related genes in the BC group by qRT-PCR

qRT-PCR was performed for the genes identified in the mRNA microarray analyses whose expression levels were upregulated in the BC group compared to the HG group as well as on related genes. Among genes related to metabolism, the expression levels of *FBP1* and *PFKFB3* were higher in the BC group than in the HG group and that of *FBP1* was higher in the BC group than in the LG group (Fig. 1 A). The expression levels of inflammation-related genes such as *IL31*RA*, NCF1B,* and *CD38* were higher in the BC group than in the HG group and in the LG group (Fig. 1 B). Expression levels of signaling pathway genes such as *ITGAL, FGR, PLCB2, SULF2, SLC43A2, PRAM1, PSAP, LRG1*, and *CSF3R* were higher in the BC group than in the HG group and LG group (Fig. 2).

3.3. Histone modifications of several immune response genes whose expression levels were upregulated in the BC group determined by ChIP assay

Next, we performed ChIP assays to examine histone modifications around the immune response genes *CD38*, *ITGAL*, and *NCF1B* in THP-1 cells treated with or without β-carotene under high-glucose conditions using specific antibodies: against mono-, di-, and trimethylation of H3K4 and H3K36, anti-acetyl-histone H3 K9, and NFκB p65. The average of normal IgG was significantly lower than that of other histone levels. The average values of normal IgG for *CD38*, *NCF1B* and *ITGAL* in each region of each group were <0.26%, <0.21%, and <0.11%, respectively (Fig. S1). The dimethylation of H3K4 and trimethylation of H3K36 and AcH3K9 around *CD38* were higher in the BC group than in the HG group and/or in the LG group (dimethylated H3K4: +500 bp, +1000 bp, and +2000 bp; trimethylated H3K36: +2000 bp and over 2000 bp), whereas monomethylated H3K4 at 1000 bp of NFκB at +500 bp and over 2000 bp was lower in the BC group than in the HG group and/or the LG group (Fig. 3). The dimethylation of H3K4 and trimethylation of H3K36 and

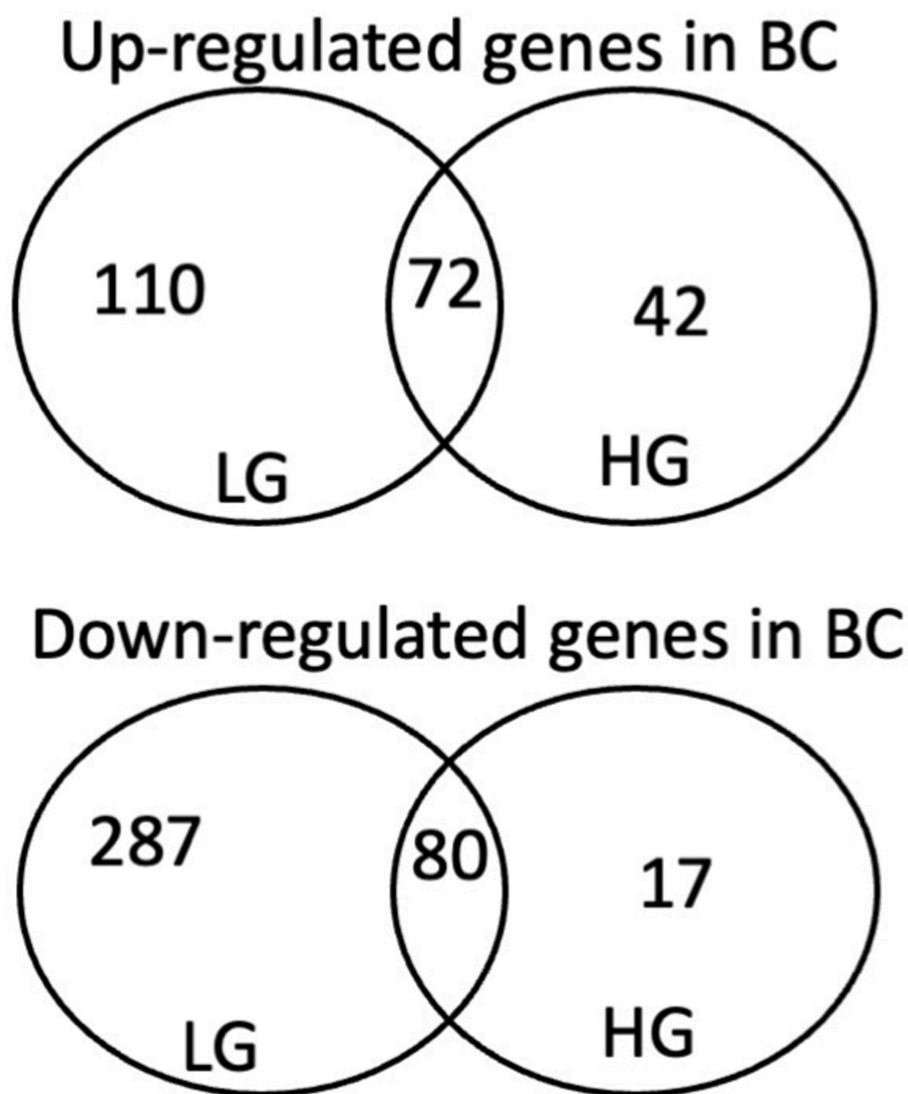

Fig. 1. Venn diagrams of up- and downregulated gene expressions in BC compared with LG and/or HG.

AcH3K9 around *NCF1B* were also higher in the BC group than in the HG and/or LG groups (dimethylated H3K4: +500 bp, +1000 bp, and +2000 bp; trimethylated H3K4: +2000 bp and +500 bp; trimethylated H3K36: +2000 bp; AcH3K9: +500 bp, +1000 bp, and 2000 bp), whereas dimethylated H3K36 at +500 bp was lower in the BC group than in the HG and LG groups (Fig. 4). The dimethylation of H3K4 and trimethylation of H3K36 and ACH3K9 around *ITGAL* was higher in the BC group than in the HG and LG groups (dimethylated H3K4: +500 bp and +1000 bp; trimethylated H3K36: +2000 bp and +5000 bp; AcH3K9: +500 bp, +1000 bp, and over 2000 bp), whereas monomethylated H3K4 (500 bp, +2000 bp, and +5000 bp), dimethylated H3K4 (over 2000 bp), and dimethylated H3K4 (+2000 bp) were lower in the BC group than in the HG and LG groups (Fig. 5) (see Fig. 6).

4. Discussion

Recent studies have suggested that antioxidant food ingredients can reduce oxidative stress and inflammation. For example, a systematic review of several studies suggested that consumption of antioxidant foods is effective in reducing inflammation [21]. However, the antioxidant β-carotene can oxidize other molecules if it itself is oxidized [8]. These results indicate that anti-inflammatory effects are not always beneficial to the body. Therefore, in this study, we conducted microarray analyses and qRT-PCR on macrophage-like THP-1 cells to determine whether β-carotene reduces or induces inflammation under high-glucose conditions in these cells. We found that β-carotene treatment under high-glucose conditions resulted in upregulated expression levels of metabolism-related genes (*FBP1* and *PFKFB3*), genes related to ROS production (*NCF1B*, *PLCβ2*, and *PRAM-1*), and genes involved in inflammation (*IL31*RA*, FGR, CD38, SULF2*, and *CSF3R*). Furthermore, the expression levels of these genes were not significantly upregulated by high-glucose conditions alone when compared to cells cultured in low-glucose conditions. Regarding metabolism-related genes, FBP1 is an important enzyme in glycogenesis that is involved in the irreversible reaction of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate [22], and PFKFB3 is involved in the glycolytic system to synthesize fructose-2,6-bisphosphate as an allosteric activator of PFK-1 [23]. Although FBP1 is an enzyme involved in glycogenesis, that expression of FBP1 in natural killer cells inhibits the glycolytic system and increases ROS levels [24]. However, excessive activation of PFKFB3 accelerates ROS production and increases autophagy [25]. As previously mentioned, β-carotene generates radicals and induces oxidative damage when it oxidizes itself [8]. Studies have also shown that increased expression levels of PFKFB3 protein are an early and persistent fibrogenic marker in hepatic stellate cells [26]. Regarding genes related to ROS production, NCF1 is an important component of

Table 1
Genes upregulated in β-carotene-treated THP-1 cells under hyperglycemic conditions.

Function	Gene symbol	Gene name	Gene ID	BC/HG	*P*	BC/LG	*P*
Metabolism	*ECH1*	enoyl-CoA hydratase 1, peroxisomal	BC014786	4.06	<0.001	3.32	0.004
	FBP1	fructose-1,6-bisphosphatase 1	NM_000507	1.79	0.007	1.98	0.001
	B4GALT5	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 5	NM_004776	1.53	<0.001	1.54	0.000
	PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	NM_001145443	1.5	0.016	1.63	0.007
Immune response	*IL31*RA	interleukin 31 receptor A	NM_001242636	2.74	<0.001	2.69	0.000
	CD38	CD38 molecule	NM_001775	2.3	<0.001	2.26	0.000
	ITGAL	integrin alpha L	NM_001114380	2.14	<0.001	2.21	0.000
	ITGAX	integrin alpha X	NM_000887	2.01	0.027	2.28	0.019
	NCF1B	neutrophil cytosolic factor 1B pseudogene	NR_003186	1.89	0.012	1.86	0.007
	NCF1C	neutrophil cytosolic factor 1C pseudogene	NR_003187	1.84	<0.001	2.11	0.001
	CD1D	CD1d molecule	NM_001766	1.8	<0.001	1.71	0.000
	CSF3R	colony stimulating factor 3 receptor	NM_000760	1.56	<0.001	1.73	0.000
	TRBV6-5	T cell receptor beta variable 6–5; T cell receptor beta joining 2-5	BC110303	1.55	0.009	1.59	0.000
Signal transduction	*FGR*	FGR proto-oncogene, Src family tyrosine kinase	NM_001042729	2.07	<0.001	2.15	0.000
	PLCB2	phospholipase C, beta 2	NM_001284297	1.96	<0.001	2.06	0.000
	GRN	granulin	NM_002087	1.83	<0.001	1.96	0.000
	SULF2	sulfatase 2	NM_001161841	1.79	0.010	1.71	0.023
	SCUBE1	signal peptide, CUB domain, EGF-like 1	NM_173050	1.73	<0.001	1.82	0.001
	OR2A1	olfactory receptor, family 2, subfamily A, member 1	NM_001005287	1.73	0.007	1.59	0.008
	SLC43A2	solute carrier family 43 (amino acid system L transporter), member 2	NM_001284498	1.72	0.002	1.85	0.000
	TBC1D2	TBC1 domain family, member 2	NM_001267571	1.7	0.002	1.82	0.000
	PRAM1	PML-RARA regulated adaptor molecule 1	NM_032152	1.7	<0.001	1.77	0.000
	SPDYE2B	speedy/RINGO cell cycle regulator family member E2B; speedy/RINGO cell cycle regulator family member E6; speedy/RINGO cell cycle regulator family member E2	NM_001166339	1.69	0.001	1.82	0.001
	HIPK2	homeodomain interacting protein kinase 2	NM_001113239	1.69	<0.001	1.57	0.000
	MERTK	MER proto-oncogene, tyrosine kinase	NM_006343	1.67	0.001	1.62	0.000
	PSAP	prosaposin	NM_001042465	1.65	<0.001	1.8	0.000
	SPDYE2	speedy/RINGO cell cycle regulator family member E2; speedy/RINGO cell cycle regulator family member E2B	NM_001031618	1.64	<0.001	1.83	0.001
	TTLL4	tubulin tyrosine ligase-like family member 4	NM_014640	1.64	0.002	1.71	0.001
	LRG1	leucine-rich alpha-2-glycoprotein 1	NM_052972	1.62	<0.001	1.82	0.000
	OGFR	opioid growth factor receptor	NM_007346	1.58	0.010	1.74	0.012
	PPP2R3B	protein phosphatase 2, regulatory subunit B, beta	ENST00000381625	1.54	0.005	1.65	0.011
	RCSD1	RCSD domain containing 1	NM_05286	1.51	<0.001	1.58	0.000

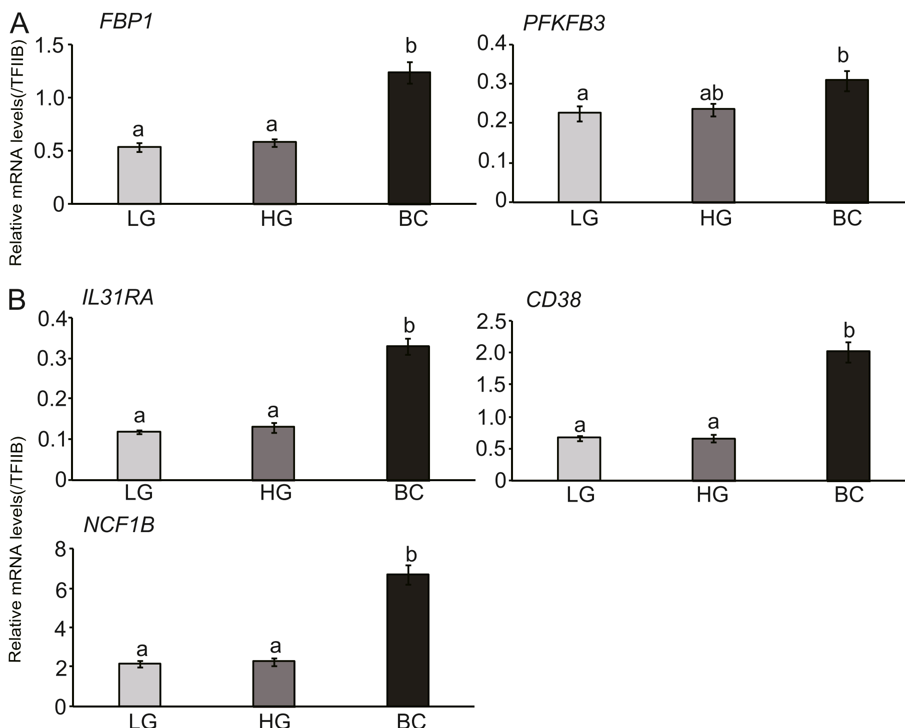

Fig. 2. mRNA expression of metabolism- and inflammation-related genes in THP-1 cells treated with low glucose or high glucose with/without β-carotene.
mRNA levels in THP-1 cells treated with low glucose (LG), high glucose (HG), and/or β-carotene (BC) were quantified using qRT-PCR. A) metabolism and B) inflammation. LG, low glucose; HG, high glucose; BC, high glucose with β-carotene. Values are presented as the mean ± SEM (n = 6). The values were normalized against *TFIIB*. Significant differences are indicated by different alphabets ($p < 0.05$).

NADPH oxidase, which is known to be involved in ROS production [27]. *NCF1* expression has also been shown to be upregulated in THP-1 cells by lipopolysaccharide [28]. PRAM1 participates in a signaling pathway downstream of cytoskeletal rearrangements, linking actin remodeling to the assembly of the NADPH oxidase complex (required for ROS production) and exocytosis of granules [29]. Our results, along with those of

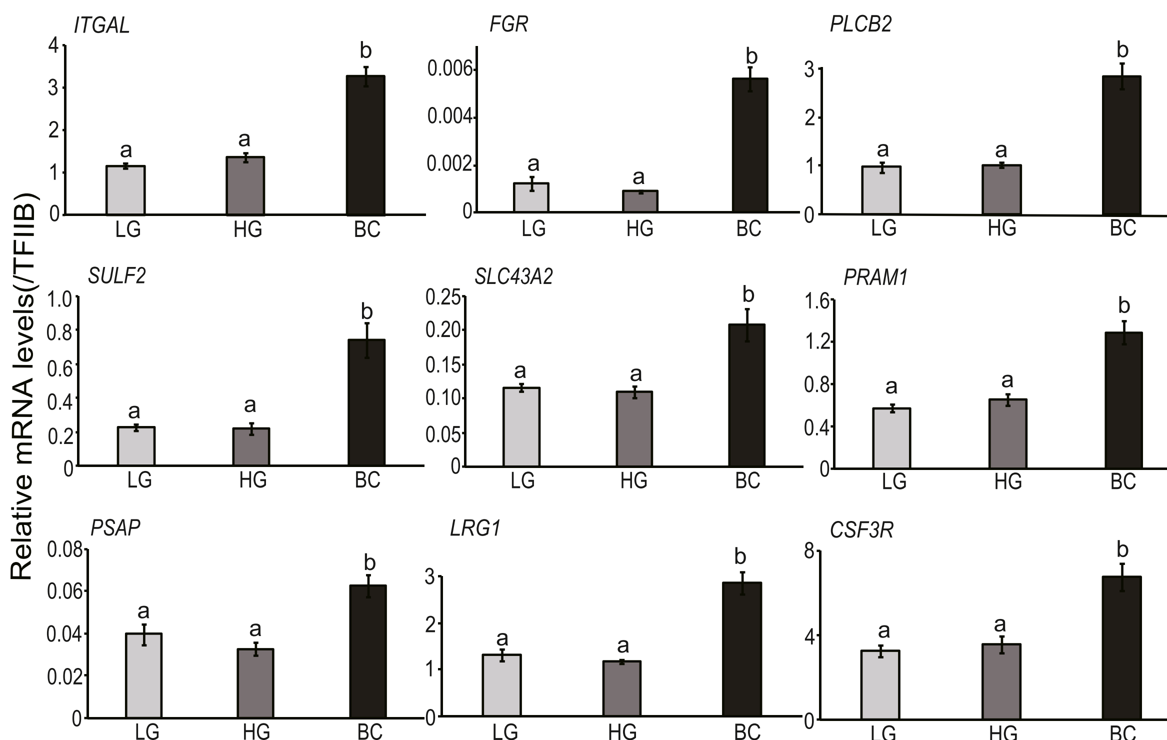

Fig. 3. mRNA expression levels of signaling pathway genes in THP-1 cells treated with low glucose or high glucose with/without β-carotene.
mRNA levels in THP-1 cells treated with low glucose (LG), high glucose (HG), and/or β-carotene (BC) were quantified using qRT-PCR. LG, low glucose; HG, high glucose; BC, high glucose with β-carotene. Values are expressed as the mean ± SEM (n = 6). The values were normalized against *TFIIB*. Significant differences are indicated by different alphabets ($p < 0.05$).

previous studies, suggest that the upregulation of gene expression levels by β-carotene under high-glucose conditions may contribute to inflammation and oxidative stress in THP-1 cells by generating ROS. Interestingly, we found that the expression levels of inflammation-related genes such as *IL31*RA*, FGR, CD38, SULF2*, and *CSF3R* were significantly upregulated by β-carotene under high-glucose conditions. IL-31RA is a receptor for IL-31, which induces the secretion of proinflammatory cytokines such as IL-1β, IL-6, and IL-18 [30]. CSF3R is an associated cytokine receptor in granulocytes, monocytes, and macrophages [31]. In addition, a significant increase in the expression of *CD38*, which contributes to leukocyte migration [32], was induced by β-carotene treatment in a high-glucose medium. FGR is known to activate macrophages [33]. SULF2 has a protective effect against inflammation (granulocyte colony-stimulating factor and interferon-γ-inducible protein 10) [34]. These results suggest that β-carotene may enhance inflammation by upregulating the expression levels of proinflammatory cytokines and genes involved in ROS production, and that β-carotene induces the expression of anti-inflammatory cytokines such as *SULF2*.

The mechanism underlying the upregulation of gene expression by β-carotene under high-glucose conditions has not yet been elucidated. Expression levels of inflammation-related genes, including those mentioned earlier, are regulated by nuclear transcription factors such as NF-κB [35]. Recently, high-glucose stimulation has been reported to promote histone acetylation by producing acetyl-CoA via glycolysis [36]. In addition, the glucosamine synthesis pathway in the glycolytic subcircuit promotes *O*-linked β-N-acetylglucosamine modification, which activates the histone-modifying enzyme mixed lineage leukemia 5 (MLL5) by increasing protein stasis [37]. In this study, we examined histone modifications in areas surrounding the immune response genes *CD38, ITGAL*, and *NCF1B* using a ChIP assay. The mono- and dimethylation of H3K4 around *NCF1B* and other genes was higher in cells cultured under low-glucose conditions than in those cultured under high-glucose conditions. However, histone H3K4 dimethylation, H3K36 trimethylation, and H3K9 acetylation of areas around all these three genes were higher in β-carotene-treated cells than in untreated cells, both under high-glucose conditions. H3K4 leads to transcriptional activation by inducing acetylation of histone H3 via histone acetyltransferase [38]. Furthermore, acetylated H3K9 is known to be involved in the formation of euchromatin by competing with methylated H3K9 and binding to heterochromatin protein 1, which induces heterochromatin formation [39]. Therefore, treatment in THP-1 cells with a dose of 5 μM of β-carotene, which can be observed in blood after supplement intake, promoted the transcription of genes involved in immune responses by inducing histone acetylation and histone H3K4 dimethylation. Regarding histone H3K36, we previously considered that H3K36 methylation is related to transactivation because H3K36 methylation is positively associated with active chromatin [40]. However, recent studies have demonstrated that H3K36 methylation plays a significant role in terminating transcriptional elongation in the gene body region in active chromatin by recruiting a DNA methyl-transferase, DMNT3b [41, 42]. Therefore, trimethylation of histone H3K36 around *CD38, ITGAL*, and *NCF1B* likely corresponds to the active region of chromatin, and the upregulation of trimethylation of histone H3K36 around these genes may terminate the excessive transcription elongation step in active chromatin. It is still unclear whether β-carotene alters histone modifications around inflammation-related genes. Previous studies have shown that the histone deacetylase sirtuin 1 (SIRT1) is involved in the reduction of oxidative stress. With resveratrol treatment, SIRT1 knockdown in retinal stem cells increased ROS production and further enhanced oxidative stress-induced cell damage [43]. In contrast, deficiency of ASH-2, an enzyme of histone H3K4me3, in intestinal tissues and gonads of *Caenorhabditis elegans*, induces oxidative stress tolerance [44]. Therefore, increased oxidative stress may enhance the acetylation of histone H3K9 and the methylation of H3K4 around inflammation-related genes in THP-1 cells. However, this issue should be investigated further in future studies. In addition, DNA is coiled over histones, and DNA and histones are incorporated into the chromatin when dramatic changes affect the chromatin structure. In particular,

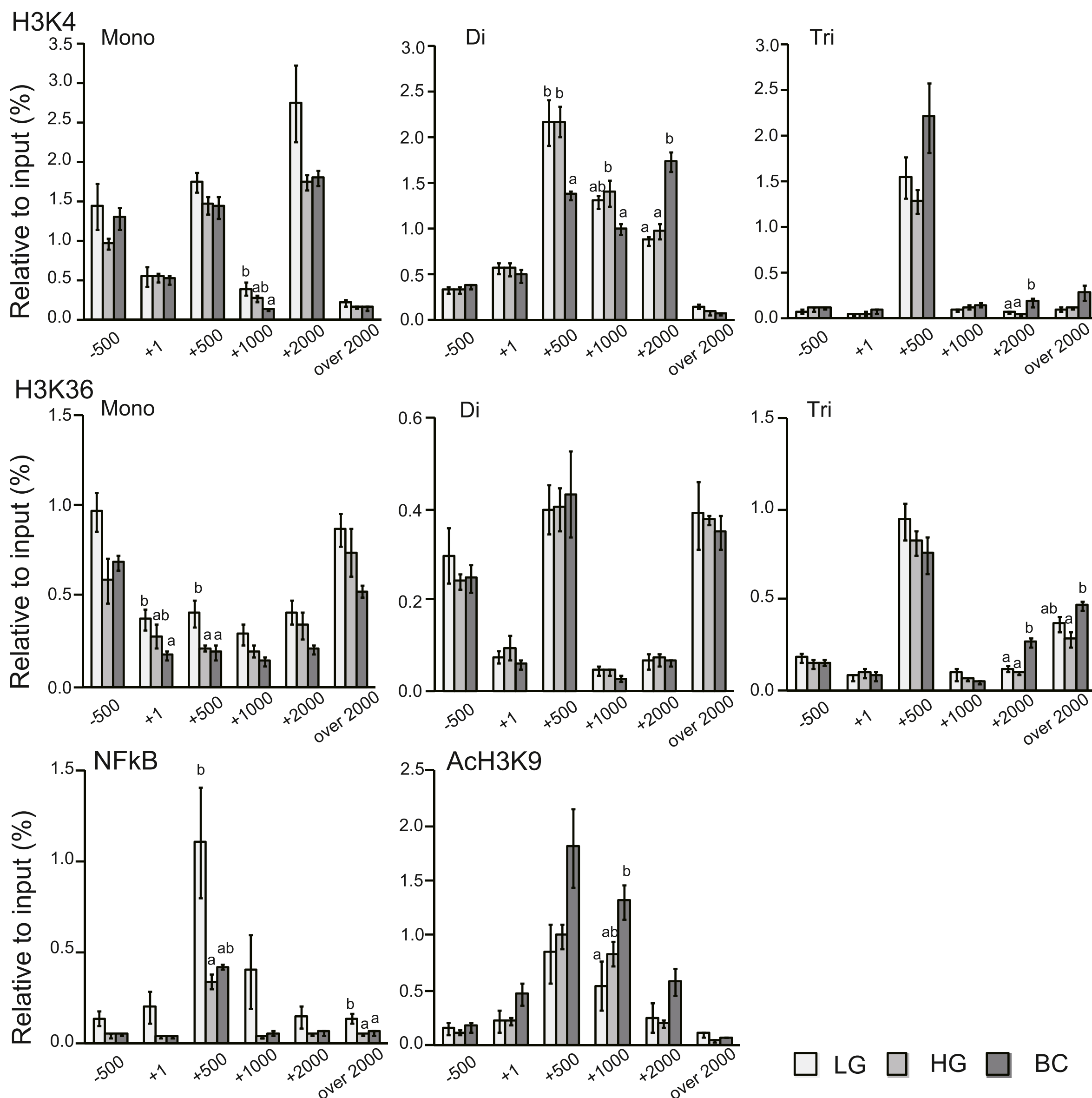

Fig. 4. Methylated histones H3K4 and K36, and acetylated H3K9 around the *CD38* gene in THP-1 cells treated with low glucose or high glucose with/without β-carotene.
ChIP signals for anti-acetyl-histone H3K4, anti-acetyl-histone H3K36, anti–NF–κ B, and anti-AcH3K9 were quantified using qRT-PCR in THP-1 cells treated with low glucose (LG), high glucose (HG), and/or β-carotene (BC). LG, low glucose; HG, high glucose; BC, high glucose with β-carotene. Data are expressed as mean ± SEM for six plates per group. Significant differences are indicated by different alphabets ($p < 0.05$).

several studies demonstrated that a variant of histone H3, histone H3.3, is highly expressed in active chromatin and is easily modified by transactivation-related histone modifications, such as acetylation and H3K4 methylation [45], rather than histone H3.1. In this study, we did not examine the deposition of histone H3, particularly between histone H3.3 and histone H3.1. This must be examined in further studies.

In this study, we found that β-carotene treatment under high-glucose conditions induced the expression of genes related to inflammation and ROS generation in THP-1 cells. However, differences in the expression of inflammation-related genes between cells cultured under low-glucose conditions and those cultured under high-glucose conditions were not significant. Treatment of THP-1 macrophage-like cells with 15 mmol/DL-glucose medium for 72 h induced the expression of inflammatory cytokine-related genes such as *TNF-α, IL-1β, MCP-1*, and *IFN-γ*, which was not observed with 5.5 mmol/DL-glucose medium [46]. The non-responsiveness of inflammation-related genes under high-glucose conditions in this study may be due to the relatively short (24 h) culture time. It is still unclear whether upregulation of mRNA expression levels by β-carotene is dependent on high-glucose culture conditions. This should be comprehensively investigated in future studies.

In this study, we measured the binding activity of NF-κB, a key transcription factor in inflammatory signaling, around inflammation-related genes, but we could not detect any significant differences between treatments. Therefore, it is necessary to study whether the binding activity of transcription factors that regulate genes involved in inflammation and oxidative stress, including STAT3, Nrf2, and NF-κB, is altered by high glucose and/or β-carotene signaling in THP-1 cells.

It is still unclear whether β-carotene directly upregulates the expression of genes related to ROS production, inflammation, metabolism, and histone modification around these genes in THP-1 cells treated with a high-glucose medium. The antioxidant β-carotene can oxidize other molecules if it itself is oxidized [8]. Therefore, oxidative β-carotene may have induced the expression of genes related to ROS production, inflammation, metabolism, and histone modification

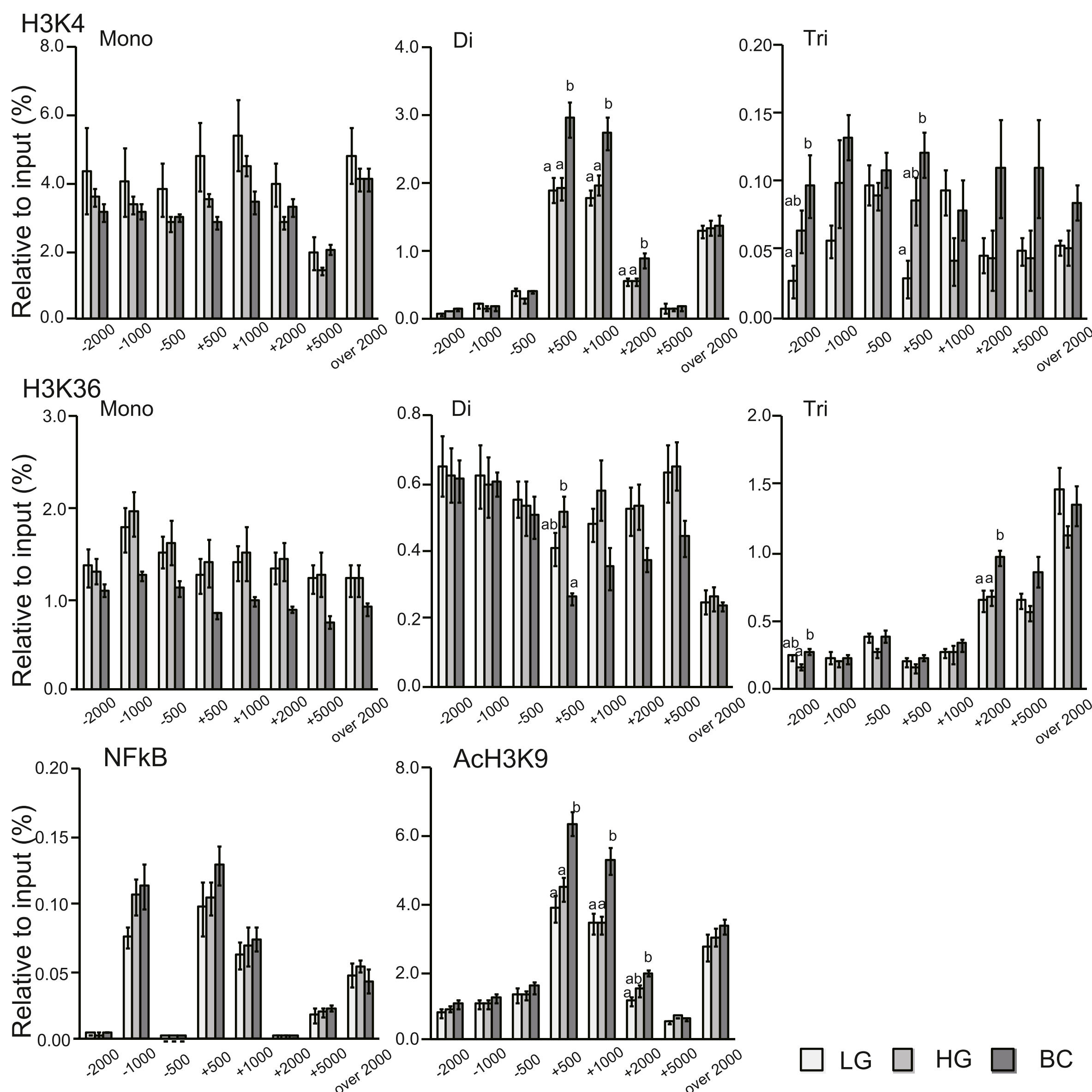

Fig. 5. Methylated histones H3K4 and K36, and acetylated H3K9 around the *ITGAL* gene in THP-1 cells treated with low glucose or high glucose with/without β-carotene

ChIP signals for anti-acetyl-histone H3K4, anti-acetyl-histone H3K36, anti–NF–κ B, and anti-AcH3K9 were quantified using qRT-PCR in THP-1 cells treated with low glucose (LG), high glucose (HG), and/or β-carotene (BC). LG, low glucose; HG, high glucose; BC, high glucose with β-carotene. Data are expressed as mean ± SEM for six plates per group. Significant differences are indicated by different alphabets ($p < 0.05$).

around these genes in THP-1 cells treated with a high-glucose medium. However, a previous study demonstrated that treatment of 3T3-L1 adipocytes with β-carotene, cultured in a similar medium with DMEM, reduced the hydrogen peroxide levels and induced expression of insulin-sensitivity genes in TNF-α treated cells [47]. Therefore, we cannot state that β-carotene is always oxidized in the medium and oxidative β-carotene induces the expression of genes related to ROS production, inflammation, and metabolism. In addition, β-carotene oxygenase 1 (BCO1), which is an enzyme that converts β-carotene to vitamin A, is expressed in the myeloid cells of mice [48]. Therefore, vitamin A, which is converted from β-carotene, may affect the induction of ROS production, inflammation, metabolism, and histone modification around these genes in THP-1 cells treated with a high-glucose medium. Indeed, all-trans retinoic acid enhanced phagocytosis but reduced the expression of CD14/TLR4 in macrophages [49]. Therefore, vitamin A, which is converted from β-carotene, may affect that expression levels of genes related to the induction of ROS and inflammation in THP-1 cells treated with a high-glucose medium. However, these issues should be examined in further studies. In addition, we should examine whether β-carotene is metabolized to oxidative β-carotene, vitamin A, and/or other metabolites in THP-1 cells with a high-glucose medium.

The plasma concentrations of β-carotene in human subjects consuming 7.2 ± 1.5 mg/day supplements was 2.5 μM after 4 weeks [50]. The β-carotene supplements given to patients with solar urticaria from 30 to 180 mg/day over 4 months was 7.5 μM on day 35, 11.2 μM on day 55, and 14.2 μM at 4 months [51]. In addition, we previously demonstrated 10 μM β-carotene in 3T3-L1 adipocytes enhanced the expression of genes related to insulin sensitivity [47]. These studies suggest that 5 μM β-carotene concentration is adequate. It should be examined whether expression of genes related to the ROS production, inflammation, metabolism, and histone modifications around these genes are altered at lower or higher doses of β-carotene compared with a 5 μM dose in THP-1 cells treated with a high-glucose medium.

In this study, we did not describe whether DNA damage was induced

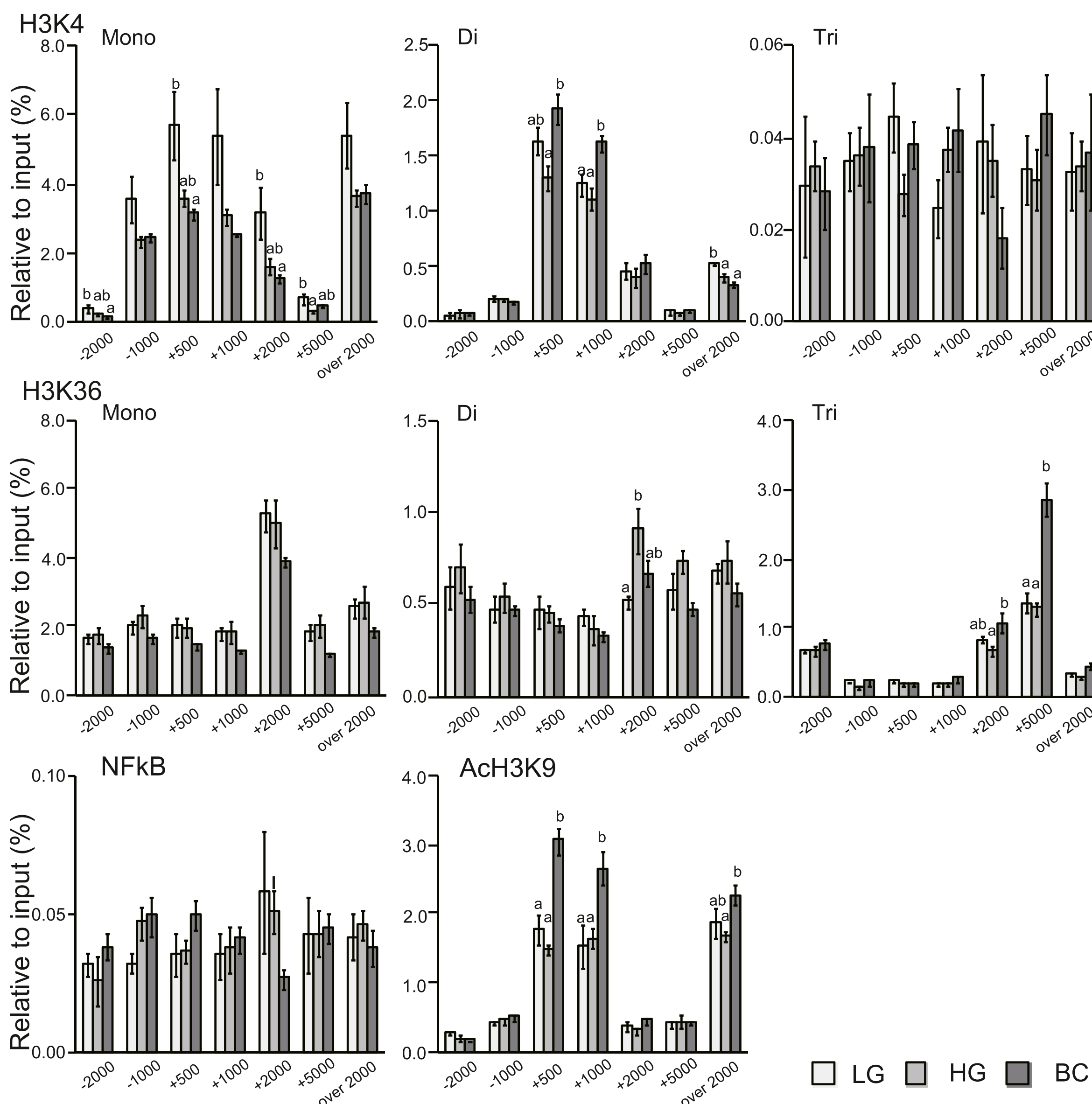

Fig. 6. Methylated histones H3K4 and K36, and acetylated H3K9 around the *NCF1B* gene in THP-1 cells treated with low glucose or high glucose with/without β-carotene.

ChIP signals for anti-acetyl-histone H3K4, anti-acetyl-histone H3K36, anti–NF–κ B, and anti-AcH3K9 were quantified using qRT-PCR in THP-1 cells treated with low glucose (LG), high glucose (HG), and/or β-carotene (BC). LG, low glucose; HG, high glucose; BC, high glucose with β-carotene. Data are expressed as mean ± SEM for six plates per group. Significant differences are indicated by different alphabets ($p < 0.05$).

by high glucose and/or β-carotene in THP-1 cells. A previous study demonstrated that culturing THP-1 cells in a high-glucose medium induced DNA damage [52]. In further studies, we must examine whether β-carotene induces DNA damage and/or oxidation in THP-1 cells cultured in a high-glucose medium. Another limitation of our study is that we did not focus on genes whose expression levels were down-regulated by β-carotene in THP-1 cells. Further studies should examine these genes.

In conclusion, our study suggests that treatment of THP-1 cells with β-carotene under high-glucose conditions enhanced the expression of oxidative stress- and inflammation-related genes and the histone H3 acetylation, histone H3K4 dimethylation, and K3K36 trimethylation around genes. These results suggest that β-carotene may induce adverse effects by inducing the expression of oxidative stress- and inflammation-related genes via histone H3 acetylation and histone H3K4 dimethylation in patients with diabetes.

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Author contributions

Shinnosuke Kondo: Investigation, Writing – Original draft. **Rina Suzuki**: Investigation. **Yuki Nakashima:** Investigation. **Kazuki Mochizuki**: Project administration, Writing – Original draft. All authors have approved the final manuscript.

Data statement

Not available.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101325.

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