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

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ARTICLE INFO

Keywords:
β-Carotene
High glucose condition
Oxidative stress
Inflammation-related genes
Histone modification

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 IL3RA, 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...
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 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 Pena 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 CO2 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 conducted to identify genes whose expressions were upregulated by β-carotene in culture medium for 15 min at 37°C. The results were 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 the 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 FGR, PLCB2, GRN, SULF2, SCUBE1, OR2A1, SLCA342A, TBC1D2, PRAM1, SPDYE2B, HIPK2, MERTK, PSAP, SPDYE2B, TOLL4, LRG1, OGRF, 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 FBH1 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 ILSRA, NCFB1, 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, SLCA342A, PRAM1, PSAP, LRG1, and CSF3R were higher in the BC group than in the HG 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 NCFB1 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; NF-kB p65. The average of normal IgG for each region 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

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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 (IL31RA, 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

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

### 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-bisphosphatase 3 | NM_001145443 | 1.5    | 0.016  | 1.63   | 0.007  |
| **Immune response** | IL31RA     | interleukin 31 receptor A | NM_00124636 | 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_00114380      | 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_00116841      | 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  |
|                   | SLCO4A2     | 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       | FML-RARA regulated adaptor molecule 1 | NM_032152 | 1.7    | <0.001 | 1.77   | 0.000  |
|                   | SFDYE2B     | 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_00142465      | 1.65   | <0.001 | 1.8    | 0.000  |
|                   | SFDYE2B     | 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  |
|                   | TTL14       | 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  |
|                   | OGFGR       | opioid growth factor receptor  | NM_007346        | 1.58   | 0.010  | 1.74   | 0.012  |
|                   | PPP2R2B     | protein phosphatase 2, regulatory subunit B, beta | ENST0800381625 | 1.54   | 0.005  | 1.65   | 0.011  |
|                   | RCD51       | RCD domain containing 1        | NM_05206        | 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).
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 IL31RA, 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-acetylgalactosamine 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 transcriptional elongation in the body region in active chromatin by recruiting a DNA methyl-transferase, DMNT3b [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 DNA, 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,
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/L-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/L-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. 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-κB, 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).
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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. 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-κB, 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).

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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 downregulated 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.

**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-κB, 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).

**Funding**

This work was supported by the KAKENHI program of the Japan Society for the Promotion of Science, Japan, [grant numbers JP20H04103, JP17H01964, JP20K21750] from the Ministry of Education, Culture, Sports, Science, and Technology; the Takeda Science Foundation; and the Uehara Memorial Foundation, Japan.

**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.
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