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β-carotene and Retinol Reduce Benzo[a]Pyrene Induced Mutagenicity and Oxidative Stress via Transcriptional Modulation of Xenobiotic Metabolizing Enzymes in Human HepG2 Cell Line

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**Abstract:**

Benzo[a]pyrene (B[a]P) is one of the polycyclic aromatic hydrocarbons which is formed due to smoking of foods, incomplete combustion of woods, vehicle exhausts and cigarettes smokes. B[a]P gets entry into human and animal bodies mainly through their diets. Metabolic activation of B[a]P is required to induce mutagenesis and carcinogenesis in animals and humans studies. Carotenoids and retinoids are phytochemicals that if ingested have multiple physiological interferences in the human and animal bodies. In this study, we firstly investigated the protective effects of β-carotene, β-apo-8-carotenal, retinol and retinoic acid against B[a]P induced mutagenicity and oxidative stress in human HepG2 cells. Secondly, we tested the hypothesis of modulating xenobiotic metabolizing enzymes (XMEs) by carotenoids and retinoids as a possible mechanism of protection by these micronutrients against B[a]P adverse effects. The obtained results declared that β-carotene and retinol significantly reduced B[a]P induced mutagenicity and oxidative stress. Tested carotenoids and retinoids reduced B[a]P induced phase I xenobiotic metabolizing enzymes (XMEs) and induced B[a]P reduced phase II and III XMEs. Thus, the protective effects of these micronutrients are probably due to their ability of induction of phase II and III enzymes and interference with the induction of phase I enzymes by the promutagen, B[a]P. It is highly recommended to consume foods rich in these micronutrients in the areas of high PAH pollution.

**Keywords:** carotenoids, retinoids, B[a]P, mutagenicity, oxidative stress, HepG2 cells, diet, pollution
1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are formed in heat-treated meats such as grilled or barbecued, pan-fried and in cured meats or smoked foods (Darwish et al. 2010). Benzo[a]pyrene (B[a]P) is one of the most potent PAH carcinogens in animal studies (Sugimura et al. 1996). B[a]P is considered as human mutagen, carcinogen, and endocrine disruptor, and has been extensively used as a marker of exposure to total carcinogenic PAHs (ATSDR 1995). It has been proved that the carcinogenicity of B[a]P was highly associated with the oxidative stress (Kim and Lee 1997). The oxidative stress could be generated in the biotransformation reaction of B[a]P by cytochrome P450 (CYP) (Hildebrandt et al. 1981; Joseph and Jaiswal 1998).

Diet is an important factor for human exposure to mutagenic and carcinogenic substances such as polycyclic aromatic hydrocarbons (ATSDR 1995). Diet may be directly involved in mutagenicity and carcinogenicity through DNA damage (Phillips 1999). At the same time, diet also provides human with various nutrients, which have counter effects for mutagenesis and carcinogenesis. These nutrients such as vitamin C, vitamin E, flavonoids and carotenoids have been reported to reduce DNA damage related to PAH exposure and could protect against adverse health outcomes related to exposure to such contaminants (Bhuvaneswari et al. 2002; Duarte-Salles et al. 2012).

Cytochrome P450 (CYP) 1A1, 1A2 and epoxide hydrolase 1 (EH1) are major phase I xenobiotic metabolizing enzymes (XMEs) in metabolizing procarcinogenic and environmental pollutants such as polycyclic aromatic hydrocarbons, specifically catalyze the formation of B[a]P reactive metabolites. The formed metabolites pass through conjugation and detoxification reactions via phase II XMEs like UDP-glucuronosyltransferases (UGT) and glutathione S-transferases (GST), and phase III XMEs (xenobiotic transporters) as multidrug resistant protein 1 (MDR1) & multidrug resistance associated protein (MRP2) (Darwish et al. 2014). In addition, NAD(P)H: quinone oxidoreductase-1 (NQO1), phase I/II XME, prevents the redox cycling of B[a]P quinone-semiquinone-quinols, thus reducing ROS generation (Yang et al. 2016).

Recently, micronutrients derived from plants and fruits, that called phytochemicals, have been examined for their protective effects against the adverse effects of endogenous and exogenous toxins (Darwish et al. 2016). These phytochemicals include carotenoids, retinoids and flavonoids. Carotenoids and retinoids like β-carotene and retinol have been reported to have anticarcinogenic, anti-inflammatory, antiproliferative and antiatherogenic properties and used as
chemopreventive agents against cancer in animal studies. In addition, β-carotene and retinol play important roles in immune response, cell differentiation, vision and reproduction (Nishino et al. 2009).

Epidemiological studies indicate that diets rich in fruits and vegetables can be associated with lower risks of numerous diseases and cancers (Bhagavathy and Sumathi 2012). However, the exact mechanisms behind these effects are still unclear.

Thus, the objectives of this study were, firstly, to investigate the protective effects of carotenoids such as β-carotene (BC) & β-apo-8-carotenal (BA8C) and retinoids such as retinol and retinoic acid (RA) against B[a]P induced mutagenicity and oxidative stress. Secondly, to investigate the mechanisms behind these protective effects through studying the modulatory effects of the co-exposure of B[a]P & BC, BA8C, retinol and RA on phase I, II and III XMEs using the human hepatoma (HepG2) cells.
2. Materials and Methods

We followed the guidelines of Hokkaido University, Japan during conducting the experiments of this study.

2.1. Chemicals and reagents

Benzo[a]pyrene, β-carotene, β-apo-8-carotenal, 9-cis retinoic acid and TRI reagent were from Sigma Chemical Co. (St. Louis, MO, USA). Retinol was obtained from Funakoshi Co. (Tokyo, Japan). Co-factor S9, NADPH, glucose-6-phosphate (G-6-P) and G-6-PDH were from Oriental Yeast (Tokyo, Japan). Primer sets were from Invitrogen (Carlsbad, CA). Other reagents were obtained from Wako Pure Chemical Industries (Tokyo, Japan).

2.2. Cell line and culture conditions

The human hepatoma cell line (HepG2) (RIKEN Cell Bank, Tsukuba, Japan), was cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin) at 37°C in a humidified incubator with 5% CO2. Cells were grown to 80%-90% confluence in 60-mm collagen-coated dishes. Cells were exposed to B[a]P (5 or 50 ng/L) in serum-free medium for 24 hours. In protection experiments, HepG2 cells were exposed to either BC, BA8C, retinol or RA with their physiologically relevant concentration (1, 5, 10 and 20 nM) (Darwish et al. 2010b) alone or co-exposed with B[a]P and incubated for 24 hours. Cells were also exposed to DMSO as a negative control. The medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS). Each treatment was represented by five dishes. Each experiment was repeated twice to confirm replication of the obtained results.

2.3. Cell viability assay

Cell viability was examined using the CCK-8 assay (Sigma-Aldrich, St. Louis, MO) according to the manufacturer instructions.

2.4. Ames mutagenicity assay

Ames assay was performed according to the published method (Ames and Gold 1990), with slight modifications. In short, the tester strain was Salmonella typhimurium TA98, which is sensitive to frameshift mutations. The reaction mixture contained 10 mM G-6-P, B[a]P (final concentrations were 1, 5 or 50 nM in dimethyl sulfoxide), 1 mg co-factor S9 mixture. S9 fraction was prepared from human HepG2 cells, according to the method published before (Darwish et al. 2010b). The reaction was started after adding 20 µL of 50 mM NADPH and 200 U/mL of G-6-
PDH mixture. After incubation for 20 min at 37°C, the reaction was terminated by adding of top agar. Histidine-independent mutants were scored after incubation for 48 h at 37°C. Each experiment was done in duplicate and repeated at least five times at different days.

2.5. Determination of reactive oxygen species (ROS)

ROS production was measured using the fluorogenic probe 2',7'-dichlorofluorescein diacetate (DCF-DA) (Sigma) as described previously (Korashy and El-Kadi 2012). Briefly, HepG2 cells were seeded in dark 96 well plate and incubated with the B[a]P (0-50 nM) for 24 hours. In protection experiment, cells were exposed to B[a]P 50 nM or co-exposed to either BC, BA8C, retinol or RA (1, 5, 10 or 20 nM). The cells were then stained with DCF-DA (5 µM) for 1 hour at 37°C. The fluorescence intensity was measured at excitation and emission wavelengths of 485 and 535 nm, respectively, using a 96-well plate reader (Baxter, Deerfield, IL). Each treatment was represented by five wells. Each experiment was repeated twice to confirm replication of the obtained results.

2.6. RNA extraction & cDNA synthesis

Total RNA was extracted using TRI reagent (Sigma-Aldrich) according to the manufacturer’s instructions. The cDNA was synthesized as described previously (Amara et al. 2010).

2.7. Quantitative real-time polymerase chain reaction

Quantitative real-time PCR for human XMES mRNA levels was performed using StepOne™ Real-Time PCR System (Applied Biosystems). The investigated genes included human CYP1A1, CYP1A2, EH1, NQO1, UGT1A6, GSTa, MDR1 and MRP2. The primer sequences for the investigated genes are described in Table 1 (Amara et al. 2010; Ohno and Nakajin 2009).

PCR was performed in a volume of 10 µL according to the protocol described previously (Mureithi et al. 2012). Briefly, the PCR mixture was prepared with SYBR® qPCR Mix (Toyobo), 10 µM of each primer, 600 ng of cDNA, and 50× ROX reference dye, and then made up to a final volume of 10 µL with RNase-free water. The reaction cycle consisted of an initial holding stage at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 minute, and extension at 72°C for 30 s. We confirmed amplification of a single amplicon by melting curve analysis. Agarose gel electrophoresis confirmed the absence of
primer dimers and genomic DNA amplification. β-actin was used for normalization in the comparative Ct method. Each experiment was repeated at least three times at different times.

2.8. Statistical analysis

Statistical significances were evaluated by either Tukey's Kramer HSD difference test or Dennett’s using JMP (SAS Institute, Cary, NC, USA). P<0.05 was considered to be significant.
Results and Discussion

B[a]P is one of the polycyclic aromatic hydrocarbons which is formed during barbequing of meat and smoking of fish and additionally released in tobacco smoke, car exhausts and during incomplete combustion of wood. B[a]P is categorized as Group I carcinogen as established before (IARC 2010). B[a]P induced carcinogenicity events starts with mutagenesis. One possible mechanism of B[a]P induced DNA damage includes production of reactive oxygen species (ROS) leading to oxidative stress and DNA adducts formation. B[a]P can induce tumors at various sites of the body including liver, lung, kidney, skin, oral cavity, gastrointestinal tract and brain depending on the route of entry to body (Kim and Lee 1997).

In this study, we, firstly, confirmed the mutagenicity of B[a]P using Ames mutagenicity assay as well as B[a]P ability of ROS formation in the human liver HepG2 cell line after exposure to environmentally relevant concentrations of B[a]P. It notes worthy that all tested B[a]P or phytochemicals’ concentrations did not affect HepG2 cells viability (data are not shown). The obtained results of the mutagenicity assay reflected the bio-activation and the high mutagenic ability of B[a]P in HepG2 cells as indicated by the high production of histidine-positive revertants in a concentration dependent manner in Salmonella typhimurium mutagenicity assay (Fig 1A). Furthermore, B[a]P strongly produced ROS in the human HepG2 cells in a concentration dependent fashion as clear in figure 1B. These results go in line with our previous report, as we had detected high mutagenic ability of B[a]P in-vitro using cattle, horse and deer liver microsomes (Darwish et al. 2010a). The high ability of B[a]P to produce ROS in HepG2 cells, goes in line with Kim and Lee (1997), who recorded high oxidative DNA damage in the different organs of the female Sprague-Dawley rats orally treated with B[a]P. Moreover, high induction of ROS in normal human epidermal keratinocytes exposed to elevated concentrations of B[a]P ranged from 20 nM to 10 µM was recorded (Tsujii et al. 2011).

β-carotene is a micronutrient, that found in vegetables and fruits with variable concentrations. However, retinoids like retinol and its primary active metabolite, retinoic acid, are mainly found in dairy products, liver and eggs. These micronutrients have well-documented antioxidant activities through their radical scavenging effects. Epidemiological studies in humans have suggested that BC intake had a reverse correlation with the incidence of gastric cancer (Larsson et al. 2007). However, earlier studies reported that BC treatment resulted in an
increased risk in the incidence of lung cancer among male smokers (De Luca and Ross 1996). Thus, we tested the protective effects of BC, BA8C, retinol and RA against B[a]P induced mutagenicity and oxidative stress in the human liver cells. Clear antimutagenic effects for the tested carotenoids and retinoids were observed (Figure 2A). Especially, BC, retinol and its metabolite RA had the highest antimutagenic effects. Retinol could achieve more than 80% reduction at both 10 and 20 nM followed by BC and RA as clear in figure 2A. In agreement with these protective effects of the tested phytochemicals, clear antimutagenic and anti-genotoxic effects for the extracts of *Randia echinocarpa* fruit, which is rich in carotenoids and polyphenols were recorded (Santos-Cervantes et al. 2007).

The results obtained in figure 2B showed a clear reduction of B[a]P-induced ROS by carotenoids and retinoids. Particularly, retinol and BC could protect HepG2 cells from oxidative stress in a concentration dependent mode achieving the end-point at 10-20 nM. The protective effects of retinol against B[a]P-induced ROS correspond with (Alsharif and Hassoun 2004), who recorded clear protective roles for retinol against 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced body wasting, hepatomegaly, thymic atrophy, production of ROS and DNA damage in C57BL/6J mice. Reduction of B[a]P-induced ROS by BC is going in agreement with Kasperczyk et al. (2014), who observed a clear reduction of ROS in lead exposed-workers after BC dietary supplementation. Additionally, Armentano et al. (2015) recorded strong *in-vitro* antioxidant activities for *Sclerocarya birrea* methanolic root extract rich in carotenoids in HepG2 cells. Thus, consumption of dietary substances rich in BC, retinol and RA in highly polluted areas with PAHs, HCAs, and heavy metals may help in reducing cancer risk and oxidative stress among consumers. Using of phytochemicals in reducing oxidative stress and the resultant mutagenic and carcinogenic risks is a well-documented strategy in several studies. For instance, several natural antioxidants such as vitamin E, vitamin C, garlic extract, glabridin (flavonoid), the rosmarinic acid and carnosic acid (polyphenols) had strong antioxidant and antimutagenic activities (Fuhrman et al. 2000). In addition, it was reported that green tea and oak fruit extracts, rich in carotenoids and flavonoids, could be effectively used as a substituent of synthetic antioxidant BHT (Ranjbar et al. 2015). Furthermore, it was found that wild raspberry (*Rubus hirsutus Thunb.*) extract rich in flavonoids and carotenoids reduced ROS production in rats having acrylamide-induced oxidative damage (Chen et al. 2016).
Metabolism has a major contribution in conversion of chemical carcinogens into reactive species that damages cellular macromolecules, interferes with signaling pathways and causes cancer. Among the XMEs is the aryl hydrocarbon receptor (AhR)-regulated gene battery, which includes phase I enzymes such as CYP 1A1 and 1A2; phase II enzymes such as UGT 1A6, and GSTa and phase III enzymes such as MDR1 and MRP2. The AhR gene battery is responsible for metabolism and detoxification of promutagenic environmental pollutants, such as B[a]P (Nebert et al. 2000). Thus, modulation of transcriptional regulation of AhR gene battery may contribute to reducing the ROS production and mutagenic activity of B[a]P. Therefore, we investigated the modulatory effects of carotenoids and retinoids on the gene expression of AhR gene battery using the quantitative RT-PCR. Furthermore, EH1 is a key enzyme involved in the bioactivation of B[a]P to its ultimate mutagenic metabolite B[a]P-7,8-dihydrodiol-9,10-epoxide. Additionally, NQO1, which is classified as phase I/II metabolizing enzyme, bypasses the formation of semiquinones and prevents quinone-semiquinone-quinol redox cycles, thus reducing ROS generation (Yang et al. 2016). B[a]P induced XMEs such as CYP1A1, 1A2, NQO1, MDR1 and MRP2 but reduced phase II enzymes compared with the non-treated cells (Figures 3-6). Consistent with this finding, treatment of the human Caco-2 cell line with PAHs, such as B[a]P, chrysene, phenanthrene, benzo[a]fluoranthene, dibenzo[a,b]pyrene, and pyrene, induced mRNA expression of various XMEs, including CYP1A1, CYP1B1, epoxide hydrolase, and ABC-transport MBR1 (Lampen et al. 2004; Yang et al., 2016). Interestingly, retinol, RA, BC and BA8C reduced B[a]P-induced phase I enzymes examined as declared (Fig. 3 & 4). Surprisingly, tested carotenoids and retinoids induced phase II XMEs, UGT1A6 and GSTa, which were reduced following B[a]P exposure (Figure 5 A & B). Retinol had the highest ability to induce phase III drug transporters, MDR1 and MRP2 mRNA expressions (Fig. 6 A & B). To support our new hypothesis, HepG2 cells were exposed to the tested carotenoid and retinoids in the absence of B[a]P. The obtained results showed clear modulatory effects for the tested phytochemicals towards AhR gene battery. As retinol and RA significantly reduced CYP1A1, CYP1A2 and NQO1 (Figs. 3 & 4). In addition, BC, BA8C, retinol and RA significantly induced phase II detoxifying enzymes and drug transporters (Fig. 5 & 6).

In agreement with our results, retinol had been shown to inhibit phase I enzymes as CYP1A1 and CYP1A2 in 3-methylcholanthrene treated rats (Huang et al. 1999). Likely, it was reported that retinol and RA had strong inhibitory effects on xenobiotic oxidations catalyzed by
recombinant CYP1A1 and CYP1A2 through a competitive inhibition fashion (Yamazaki and Shimada 1999). The mechanism of reduction or inhibition of CYP1A expression and dependent metabolism by retinol and RA is still unclear. Our earlier studies reported modulation of XMEs in the meat-producing animals (cattle, deer and horses) and in rat hepatoma H4IIE cells by BC and retinol (Darwish et al. 2010b). Furthermore, it was reported that astaxanthin could alter CYP1A dependent activities via induction of protein expression and inhibition of NADPH P450 reductase dependent-electron transfer in the male Wistar rats (Ohno et al. 2011). Additionally, we confirmed that astaxanthin rich crude extract of *Haematococcus pluvialis* could induce cytochrome P450 1A1 mRNA by activating AhR in rat hepatoma H4IIE cells (Ohno et al. 2012). We here declare the proposed mechanism of protective effects of BC and retinol against B[a]P induced mutagenicity and oxidative stress in figure 7.

In brief, B[a]P induces phase I enzymes and reduces Phase II metabolizing enzymes leading to production of highly reactive metabolites which subsequently induce mutagenesis and ROS giving rise to initiation of carcinogenesis. Unlikely, BC and retinol can reversely react leading to reduction of the induced phase I metabolizing enzymes and induction of phase II and III metabolizing enzymes producing a state of balance between the bio-activation and detoxification pathways leading to protection.

**Conclusions**

This study declared that B[a]P had strong mutagenic activities and could produce reactive oxygen species in the human HepG2 cells. BC and retinol had clear protective effects against B[a]P induced mutagenicity and oxidative stress. Modulation of the transcriptional regulation of xenobiotics metabolizing enzymes is a possible mechanism for these protective effects. Thus, consumption of foods rich in these micronutrients may help to reduce the adverse effects of B[a]P in areas with high levels of PAH pollution or during barbeque parties.
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Author Contributions

W. S. Darwish designed the study, conducted the experiments, drafted the manuscript and interpreted the results. Y. Ikenaka designed the study and interpreted the results. S. M. Nakayama and L. Thompson drafted the manuscript, interpreted the results and performed statistical analysis. H. Mizukawa collected the test data. M. Ishizuka designed the study, supervised the work and interpreted the results.

Conflict of interest

The authors declare no conflicts of interest
References


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**Fig 1: B[a]P induced mutagenicity and oxidative stress**

A) Mutagenicity of B[a]P: the number of histidine+revertant colonies reflects the mutagenic activity of B[a]P (0-50 nM) in *Salmonella typhimurium* TA98 mutagenicity assay. The data represent the mean ± SD (n=5). Identical letters are not significantly different from each other (P< 0.05). B) B[a]P induced oxidative stress: ROS production in HepG2 cells exposed to B[a]P (0-50 nM). Cells were incubated with DCF-DA (5 µM) for 1 hour at 37°C. The fluorescence intensity was measured at excitation and emission wavelengths of 485 and 535 nm, respectively, using a Baxter 96-well plate reader. Each treatment was replicated in five wells (n=5) (P< 0.05).

**Fig 2: Protective effects of carotenoids and retinoids against B[a]P induced mutagenicity and oxidative stress**

The reduction percentage due to adding 1, 5, 10 and 20 nM of BC, BA8C, retinol and RA to HepG2 cells on the same time with B[a]P (50 nM) on A) B[a]P induced mutagenicity B) B[a]P induced oxidative stress. Data are presented as the means ± SD (n=5). Star marks indicate significant differences with non-protected ones (0 treatment) (P < 0.05).

**Fig 3: Expression of CYP1A mRNA in HepG2 human cells exposed to B[a]P and different carotenoids and retinoids**

The effects of co-exposure of HepG2 cells to B[a]P (5 or 50 nM) and different carotenoids and retinoids (10 nM) on A) CYP1A1, B) CYP1A2 mRNA expressions as determined by real-time RT-PCR. Data are presented as the mean ± SD (n=5). Columns with same color carrying different superscript letters either (A, B, C, D), (a, b, c, d) or star mark represent expression levels that are significantly different from each other (P<0.05).

**Fig 4: Expression of epoxide hydrolase 1 and NQO1 mRNA in HepG2 human cells exposed to B[a]P and different carotenoids and retinoids**

The effects of co-exposure of HepG2 cells to B[a]P (5 or 50 nM) and different carotenoids and retinoids (10 nM) on A) EH1, B) NQO1 mRNA expressions as determined by real-time RT-PCR. Data are presented as the mean ± SD (n=5). Columns with same color carrying different
superscript letters either (A, B, C, D), (a, b, c, d) or star mark represent expression levels that are significantly different from each other ($P<0.05$).

**Fig 5: Expression of phase II enzyme mRNA in HepG2 human cells exposed to B[a]P and different carotenoids and retinoids**

The effects of HepG2 cells co-exposure to B[a]P (5 or 50 nM) and different carotenoids and retinoids (10 nM) on A) UGT1A6, B) GSTa mRNA expressions as determined by real-time RT-PCR. Data are presented as the mean ± SD (n=5). Columns with same color carrying different superscript letters either (A, B, C, D), (a, b, c, d) or star mark represent expression levels that are significantly different from each other ($P<0.05$).

**Fig 6: Expression of phase III transporter mRNA in HepG2 human cells exposed to B[a]P and different carotenoids and retinoids**

The effects of HepG2 cells co-exposure to B[a]P (5 or 50 nM) and different carotenoids and retinoids (10 nM) on A) MDR1, B) MRP2 mRNA expressions as determined by real-time RT-PCR. Data are presented as the mean ± SD (n=5). Columns with same color carrying different superscript letters either (A, B, C, D), (a, b, c, d) or star mark represent expression levels that are significantly different from each other ($P<0.05$).

**Fig 7: The proposed mechanism for the protective effects of carotenoids and retinoids against B[a]P induced mutagenicity and oxidative stress**

...
A) CYP1A1

B) CYP1A2

Fig. 3
A) Epoxide hydrolase

B) NQO1

Fig. 4
A) UGT1A6

Expression relative to control

B) GSTα

Expression relative to control

Fig. 5
A) MDR1

B) MRP2

Fig. 6