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Note

Artificial Food Colorants Inhibit Superoxide Production in Differentiated HL-60 Cells

Hang QI,[1,2,*] Machiko KAWAGISHI,[1,*] Motoko YOSHIMOTO,[1] Hiroshi TAKANO,[1] Beiwei ZHU,[2] Yasuaki SHIMOISHI,[1] Mikiro TADA,[3] Yoshiyuki MURATA,[1] and Yoshimasa NAKAMURA[1,†]

[1]*Graduate School of Natural Science and Technology, Okayama University, Okayama 700-8530, Japan*
[2]*College of Food Technology, Dalian Polytechnic University, Dalian 116034, China*
[3]*Faculty of Contemporary Life Science, Chugoku Gakuen University, Okayama 701-0197, Japan*

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We tested synthetic food colorants for their antioxidative potential by the *in vitro* superoxide generation assay in differentiated HL-60 cells in response to phorbol ester. Among the 12 colorants tested, such fluorescein-type red colorants as rose bengal showed potent inhibitory activity without any cytotoxicity under dark conditions. The intracellular accumulation and superoxide anion scavenging effect of rose bengal were at least partly involved in the inhibitory activity.

Key words: food colorant; rose bengal; superoxide; HL-60 cell; xanthine oxidase

An acute or chronic inflammatory status has been implicated as the mediator of a number of pathological disorders, including epithelial tumorigenesis in the lung, bowel, bladder, colon and skin. Inflammatory cells produce a highly complicated mixture of growth and differentiation cytokines, as well as biologically active arachidonic acid metabolites. In addition, they possess the capacity to generate and release a spectrum of reactive oxygen species (ROS) and free radicals during an oxidative burst. Among the inflammatory cells, polymorphonuclear leukocytes are particularly adept at generating and releasing ROS.[1,2] ROS production is closely associated with the metabolic activation of proximate carcinogens[3] and the increased level of oxidized DNA bases.[4] We have demonstrated that the potent inhibitors of leukocyte-derived superoxide generation[5,6] effectively suppressed inflammation-related carcinogenesis.[6,7] Inhibitors of excessive superoxide generation have also been accepted as more effective antioxidants than radical scavengers, because the superoxide anion is one of the precursors of several types of ROS.

Synthetic colorants are a very important class of food additive. They are widely used to compensate for the loss of natural colors in food, which can be destroyed during processing and storage, and to provide the desired colored appearance. The synthetic colorants that are approved by the Food and Drug Administration (FDA) for use in foods, pharmaceuticals and cosmetic preparations have undergone rigorous scrutiny for their toxicity. However, surprisingly little effort has been devoted to studying these FDA-approved colorants as potentially useful medicinal agents, except for the anti-tumor promoting property.[8]

We examined in the present study the effect of twelve kinds of artificial food colorant that have been approved by the Food Sanitation Law of Japan (some of these colorants have been approved by FDA as indicated), including tartrazine (Japanese name, Yellow no. 4; FDA name, FD&C yellow no. 5), sunset yellow FCF (Yellow no. 5; FD&C yellow no. 6), amaranth (Red no. 2; D&C red no. 2), erythrosine B (Red no. 3; FD&C red no. 3), allura red AC (Red no. 40; FD&C red no. 40), new coccin (Red no. 102), phloxine B (Red no. 104; D&C red no. 28), rose bengal (Red no. 105), acid red (Red no. 106), brilliant blue FCF (Blue no. 1; FD&C blue no. 1), indigo carmine (Blue no. 2; FD&C blue no. 2), and fast green FCF (Green no. 3; FD&C green no. 3), on the superoxide generation in differentiated HL-60 cells that is used as a model for activated neutrophils in inflammatory processes.

All artificial colorants were supplied by San-Ei Gen F.F.I. (Osaka, Japan). The RPMI-1640 medium and fetal bovine serum were purchased from Gibco-Invitrogen (Carlsbad, CA, USA). 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was obtained from Research Biochemicals International (Natick, MA, USA), and cytochrome *c* was obtained from Sigma (St. Louis, MO, USA). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Acute promyelotic leukemia HL-60 cells (Health Science Research Resources Bank, Osaka, Japan) were maintained in the RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Trace Scientific, Melbourne, Australia). The inhibitory tests on the TPA-induced superoxide generation in dimethylsulfoxide (DMSO)-differentiated HL-60 cells were done as previously reported.[5,6] Briefly, a test compound dissolved in 5 µl of DMSO was added to a DMSO-induced differentiated HL-60 cell suspension (1×10^6/ml) and incubated at 37 °C for 15 min. TPA (100 nM)

† To whom correspondence should be addressed. Tel/Fax: +81-86-251-8300; E-mail: yossan@cc.okayama-u.ac.jp
* Equally contributed to this work.
Abbreviations: ROS, reactive oxygen species; FDA, Food and Drug Administration; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DMSO, dimethylsulfoxide; BSA, bovine serum albumin; ESR, electron spin resonance; HXA, hypoxanthine; XOD, xanthine oxidase; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; NBT, nitroblue tetrazolium

and a cytochrome *c* solution (1 μg/ml) with or without superoxide dismutase (150 unit/ml) were added to the reaction mixture which was incubated for another 15 min. The visible absorption at 550 nm was then measured after centrifuging at 250 × *g*. The inhibitory effect is expressed by the decrease in ratio of the absorbance of a test compound to the that in the control experiment. We performed a trypan blue dye (Gibco-Invitrogen) exclusion assay to determine the cell viability. The differentiated HL-60 cells (1×10^6) were treated with each compound in a 6-well plate, and the plate incubated for 1 h. Each cell suspension was mixed with 0.4% trypan blue stain. The viable cells (those that excluded the blue dye) were counted with a hemocytometer under an optical microscope (Nikon, Tokyo, Japan). A flow cytometric analysis[9] was performed with EPICS XL System II (Beckman Coulter, Tokyo, Japan) to determine the intracellular uptake of a colorant. The cells were treated with rose bengal at the indicated concentrations for 30 min at 37 °C. After washing twice with PBS, a flow cytometric analysis was performed to detect the intracellularly incorporated rose bengal. The inhibition experiments were performed with FBS or bovine serum alubumin (BSA) which was co-incubated with rose bengal (50 μM) for 30 min.

Electron spin resonance (ESR) spin trapping experiments were performed as previously reported.[10] A typical incubation mixture was composed of hypoxanthine (HXA, 500 μM), xanthine oxidase (XOD, 0.1 unit/ml), and 150 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO) in a phosphate buffer (50 mM at pH 7.4) containing DTPA (1 mM) with or without rose bengal (25 μM). Each sample was subsequently transferred to a 100-μl capillary tube, and the ESR spectrum was recorded within 30 s after starting the reaction. ESR spectra were recorded at room temperature by a Jeol JES-TE200 spectrometer operated at 9.8 GHz. Typical spectrometer parameters were as follow: receiver gain, 5×10^4; sweep width, 100 G; field set, 3560 G; time constant, 0.01 s; scan time, 30 s; modulation amplitude, 1.0 G; modulation frequency, 100 kHz; and microwave power, 10 milliwatts. The spectra shown were the average of 10 scans.

Each value is expressed as the mean ± SE. A statistical analysis was performed by using Student's *t*-test, a level of $p < 0.05$ being considered significant in all statistical tests.

We examined the inhibitory effect of twelve artificial food colorants on the TPA-induced superoxide generation under dark conditions which was detected by the cytochrome *c* reduction method. As shown in Fig. 1A, among the colorants tested, the three fluorescein-type red colorants, erythrosine, phloxine B, and rose bengal (Fig. 1B), significantly inhibited superoxide generation without any severe cytotoxicity at 100 μM. Figure 1C shows that the inhibitory activity of the most potent inhibitor, rose bengal, was dose-dependent with an IC_{50} value of approximately 15 μM. This activity is comparable with that of curcumin[11] and much higher than that of carotenoids,[12] both of which are well known naturally occurring antioxidative pigments.

It is interesting to note that, even though all the artificial colorants had polar substituted groups such as carboxylates or sulfonates to give them water-soluble properties, only the fluorescein-type ones showed significant activity. To evaluate the difference in hydrophobicity among the colorants tested, we examined the relationship between the superoxide inhibitory activity and the octanol/water partition coefficient (log P) evaluated by CS Chem Draw 3D Ultra version 7.0 software (Cambridge Soft, USA). An analysis by simple linear regression showed a tendency toward positive correlation ($r = 0.83$, Fig. 1D), suggesting that the active fluorescein-type colorants, having only a carboxylate group, had higher hydrophobicity than the other non-active compounds, all of which had more than two sulfonates. This idea was also supported by the previous reports.[13,14] To obtain further evidence, the cellular accumulation of the dye was determined by a flow cytometric analysis, because the fluorescence of fluorescein-type dyes can be collected through a 610 nm band-pass filter for propidium iodide, a well characterized fluorescence probe for cellular DNA labeling. As shown in Fig. 2A, HL-60 cells treated with rose bengal at the indicated concentrations for 1 h resulted in significant and dose-dependent incorporation into the cells. However, a negative control group without the dye showed no fluorescence peak. Since rose bengal has been reported to readily bind to human serum albumin,[15] the effect of FBS or BSA on rose bengal incorporation was examined. As expected, the cellular incorporation was significantly decreased by pretreating with FBS as well as BSA (Fig. 2B). This indicated that not only preferential cellular incorporation but also protein-binding action might contribute to the inhibition of superoxide production by rose bengal in differentiated HL-60 cells. During this activation, $p47^{phox}$, an essential component of the NADPH oxidase complex, was phosphorylated on several serine residues, and protein kinase Cβ (PKCβ) was involved, particularly in the differentiated HL-60 cells, in the activation of NADPH oxidase as well as the $p47^{phox}$ phosphorylation.[16] However, rose bengal did not affect the PKCβ translocation to the membrane (data not shown).

The possible involvement of a protein-binding action led us to address the inhibitory efficacy against superoxide generation in an HXA/XOD cell-free system by measuring the nitroblue tetrazolium (NBT) reduction and uric acid formation as previously reported.[7] The data are not shown, but rose bengal had the expected inhibitory effect on superoxide-dependent NBT reduction with an IC_{50} value of about 10 μM. On the other hand, it did not inhibit any uric acid formation as determined by an HPLC analysis. These observations strongly suggested that the inhibition might not be merely due to non-specific binding to XOD, but that a superoxide scavenging effect is also attributable. To confirm this possibility, the ESR spin trapping method was employed by using the HXA/XOD system. As shown in Fig. 3A, signals for DMPO-OOH (a spin adduct of DMPO with superoxide) as well as for DMPO-OH (a spin adduct of DMPO with hydroxyl radical), which were assigned according to the previous report,[17] were observed. The signal intensity of DMPO-OOH was reduced by rose bengal at 50 μM (Fig. 3B), but the reduced intensity of DMPO-OH was not as significant. This result strongly suggested that rose bengal might scavenge the superoxide rather than hydroxyl

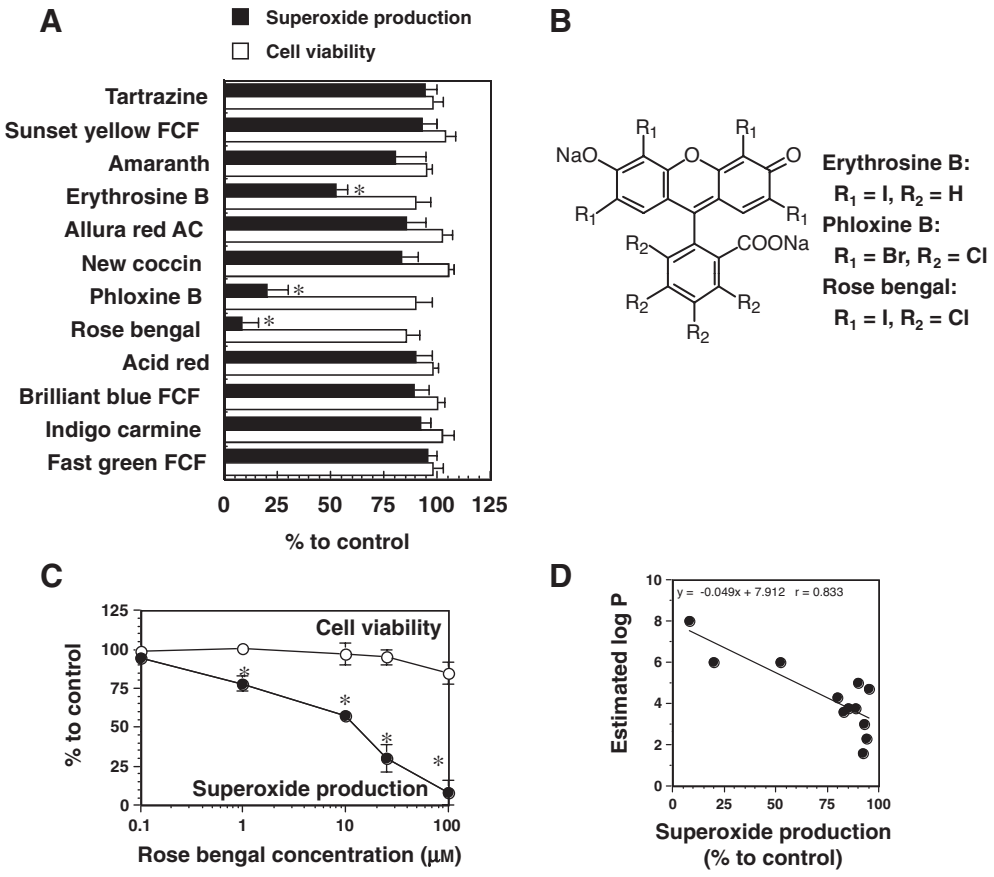

Fig. 1. Inhibitory Effect of Artificial Food Colorants on Superoxide Generation in Differentiated HL-60 Cells.

A, Effects of 12 artificial food colorants approved by the Food Sanitation Law of Japan on TPA-induced superoxide production (filled bars) and cell viability (unfilled bars) in differentiated HL-60 cells in the dark condition. The final concentration was 100 μM. B, Chemical structure of the active fluorescein-type food colorants. C, Dose-dependent effects by rose bengal on superoxide production (filled circles) and cell viability (unfilled circles) in differentiated HL-60 cells in the dark condition. Each result is expressed as the mean ± SE of 3 independent experiments. Bars with asterisks are statistically different ($p < 0.05$). D, Relationship for the 12 colorants between superoxide production inhibition and the octanol/water partition coefficient (log P) evaluated by CS Chem Draw 3D Ultra version 7.0 software. A simple linear regression analysis was performed on all data. Filled circles represent the mean value for each group.

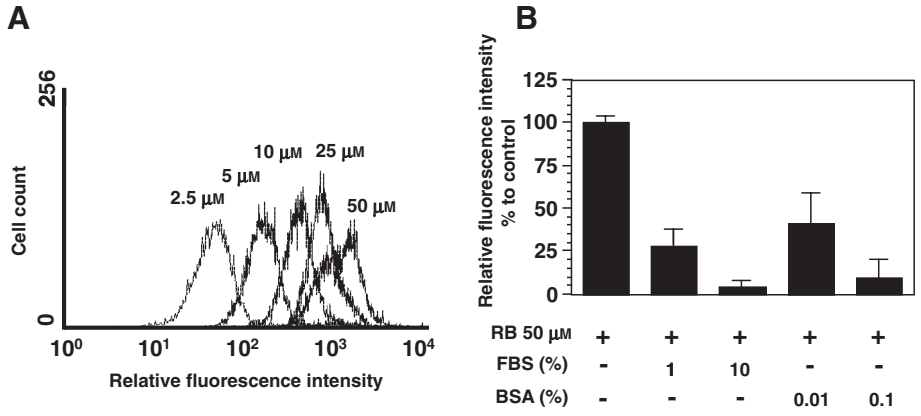

Fig. 2. Cellular Incorporation of Rose Bengal in HL-60 Cells.

A, Representative cytograms of the cells treated with rose bengal. After incubating with rose bengal at the indicated concentrations for 30 min, HL-60 cells were washed and analyzed by a flow cytometer. B, Inhibitory effects of FBS and bovine serum albumin on the intracellular incorporation of rose bengal. FBS or bovine serum albumin at the indicated concentrations was co-incubated with rose bengal (50 μM) for 30 min, and then the cells were washed and analyzed.

radical, which might be partly responsible for inhibiting superoxide generation in the differentiated HL-60 system.

In conclusion, we have demonstrated that fluorescein-type food red colorants showed potent inhibitory activity against superoxide production under dark conditions. Intracellular accumulation and a superoxide anion scavenging effect were suggested to be involved in one of the potential mechanisms. Artificial colorants have attracted a poor public image, because some of them pose a potential risk to human health, especially if they are excessively consumed. Furthermore, certain

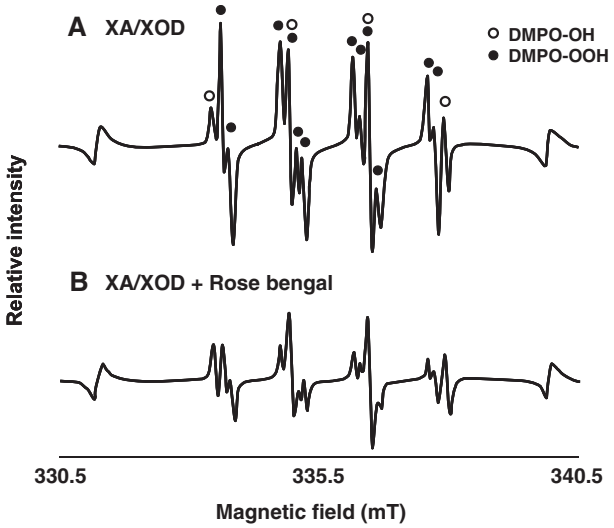

Fig. 3. Effect of Rose Bengal on Spin Adduct Formation of DMPO with Superoxide in the Hypoxanthine/Xanthine Oxidase System.

Incubated mixtures contained the following components as indicated: hypoxanthine (HXA, 500 μM), xanthine oxidase (XOD, 0.1 unit/ml), and 150 mM DMPO in a phosphate buffer (50 mM at pH 7.4) containing DTPA (1 mM) with (A) or without (B) rose bengal (25 μM). Immediately after adding all of the components, the reaction mixture was transferred to an ESR capillary tube (100 μl), and spectra were recorded at room temperature. Results are representative of 3 independent.

food colorants, including rose bengal, serve as effective singlet oxygen generators or pro-oxidants of lipids when the peroxide value is measured as an oxidation index.[15,18] However, the fluorescein-type colorants indeed showed an antioxidative effect on differentiated HL-60 cells in the dark condition with comparable activity to that of the well-known naturally occurring pigments already discussed. Such antioxidants as (−)-epigallocatechin-3-gallate are known to have a pro-oxidative effect depending on the conditions[19] and biological activity through the oxidative reaction.[20] Future studies will be concerned with the *in vivo* biological significance and elucidating the molecular mechanism to control the beneficial/harmful balance of antioxidative food colorants, including direct modification of the electron transfer system of cytochrome b558 gp91^{phox} in NADPH oxidase.

Acknowledgments

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