Mutation Research 671 (2009) 40–44

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Differential regulation of the hydrogen-peroxide-induced inhibition of gap-junction intercellular communication by resveratrol and butylated hydroxyanisole

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ARTICLE INFO

Article history:
Received 23 July 2009
Received in revised form 22 August 2009
Accepted 24 August 2009
Available online 29 August 2009

Keywords:
BHA
Cx43
ERK
GJIC
H_2O_2
Resveratrol

ABSTRACT

The present study was performed to evaluate the effects of two different phenolic antioxidants, resveratrol (3,5,4′-trihydroxystilbene) and butylated hydroxyanisole (BHA), on the hydrogen peroxide (H_2O_2)-induced inhibition of gap-junction intercellular communication (GJIC) in WB-F344 rat liver epithelial cells (WB-F344). Resveratrol is a naturally occurring polyphenolic antioxidant; on the other hand, BHA is a synthetic phenolic compound. We found that only resveratrol protects WB-F344 cells from H_2O_2-induced inhibition of GJIC, and BHA has no effect. The extracellular-signal-regulated protein kinase 1/2 (ERK1/2)–connexin 43 (Cx43) signaling pathway is crucial for the regulation of GJIC in rat liver epithelial cells, and resveratrol, but not BHA, blocked the H_2O_2-induced phosphorylation of Cx43, a critical regulator of GJIC, and ERK1/2 in WB-F344 cells. Resveratrol appears to attenuate the H_2O_2-mediated ERK1/2–Cx43 signaling pathway and consequently reverses H_2O_2-mediated inhibition of GJIC. DPPH and ABTS radical-scavenging assays revealed that the protective effect of resveratrol on the H_2O_2-mediated inhibition of GJIC was not mediated through its free radical-scavenging activity.

1. Introduction

Gap-junction intercellular communication (GJIC) is an important mode of cell–cell communication and maintains the homeostatic balance in multi-cellular organisms [1]. Gap junctions are composed of transmembrane proteins called connexins (Cxs) [2] and play an important role in the exchange of ions and molecules up to a molecular mass of 1 kDa between neighboring cells [2]. GJIC is crucial for tissue differentiation and development [3]. Modulation of the function of gap junctions via a number of different mechanisms has been shown to cause abnormal cell proliferation [4–6]. Multiple lines of evidence indicate that GJIC is dysfunctional in most cancer cells and that its inhibition is strongly related to carcinogenesis, and to tumor promotion in particular [7,8]. Most tumor promoters, such as pesticides, peroxisome proliferators, organic peroxides, and dietary additives, inhibit GJIC; however, anti-tumor drugs can reverse GJIC disruption [4,9–11]. The molecular mechanism responsible for the inhibition of GJIC correlates with reduced gap-junction number and size, as well as hyper-phosphorylation of connexin 43 (Cx43), a critical regulator of GJIC [3,4,12]. The inhibition of GJIC has been shown to be related to the activation of mitogen-activated protein kinases (MAPKs) [11,13]. In particular, phosphorylation of extracellular-signal-regulated kinase (ERK) was reported to play a key role in the inhibition of GJIC *in vitro* [14,15].

Recent reports suggest that the carcinogenicity of oxidative stress is attributable to the inhibition of GJIC [11,13,16]. Hydrogen-peroxide (H_2O_2) is a well-known cancer promoter that inhibits GJIC [13,16,17]. H_2O_2-mediated inhibition of GJIC correlates with the up-regulation of hyper-phosphorylated Cx43 [17,18]. Chemopreventive natural antioxidants such as vitamin C [19], germanium dioxide [4], honeybee propolis [12], green tea and components of Korean ginseng [20] have been shown to prevent or abolish the inhibition of GJIC.

The present study was performed to compare the effect of two different phenolic antioxidants, resveratrol (3,5,4′-

Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; DPPH, 1,1-diphenyl-2-picrylhydrazyl; BHA, butylated hydroxyanisole; Cx, connexin; ERK 1/2, extracellular-signal-regulated protein kinase 1/2; GJIC, gap-junction intercellular communication; H_2O_2, hydrogen-peroxide; MAPKs, mitogen-activated protein kinases; MEK, MAPK/ERK kinase.

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doi:10.1016/j.mrfmmm.2009.08.011

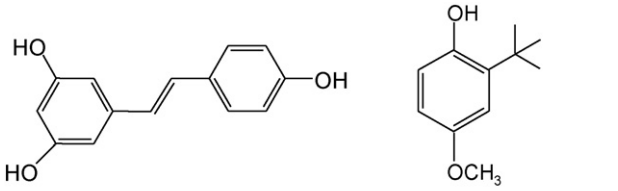

Fig. 1. Chemical structures of 3,5,4′-trihydroxystilbene (resveratrol) and butylated hydroxyanisole (BHA).

trihydroxystilbene) and butylated hydroxyanisole (BHA) (Fig. 1), on the H_2O_2-mediated inhibition of GJIC in WB-F344 rat liver epithelial cells. Resveratrol is a naturally occurring polyphenolic antioxidant present in grape skins and seeds, peanuts, and mulberries [21]. On the other hand, BHA is a synthetic phenolic antioxidant that is widely used as a food additive. We found that resveratrol, but not BHA, attenuates the H_2O_2-mediated ERK1/2–Cx43 signaling pathway and consequently reverses H_2O_2-mediated inhibition of GJIC in WB-F344 cells.

2. Materials and methods

2.1. Chemicals

BHA, H_2O_2, lucifer yellow, sodium dodecyl sulfate (SDS), acrylamide, and Tris–HCl were purchased from Sigma (St. Louis, MO). Triton X-100 was obtained from AMRESCO (Solon, OH). All other chemicals were obtained from Fisher (Springfield, NJ).

2.2. Cell culture

WB-F344 rat liver epithelial cells (WB-F344 cells) were kindly provided by Dr. J.E. Trosko (Michigan State University, MI, USA). WB-F344 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Gaithersburg, MD), supplemented with 10% fetal bovine serum (GIBCO) and penicillin/streptomycin (GIBCO) at 37 °C in a 5% CO_2 humidified incubator (Forma Scientific, Marietta, OH).

2.3. Bioassay of GJIC

GJIC was measured by the scrape-loading/dye-transfer technique as described previously [22]. Briefly, WB-F344 cells were preincubated with noncytotoxic doses of resveratrol or BHA for 30 min and then stimulated with 300 μM H_2O_2 for 30 min. After H_2O_2 treatment, cells were washed twice with PBS. Lucifer yellow was added to the washed cells, and three scrapes were made using a scalpel with a surgical-steel-blade under low light intensity. Each scrape traversed a large group of confluent cells. After 3 min of incubation, the cells were washed four times with 2 ml of PBS and then fixed with 2 ml of 4% formalin solution. The number of communicating cells was counted under an inverted fluorescence microscope (I × 70, Olympus, Okaya, Japan).

2.4. Western blot analysis

Western blot analysis was performed to measure the protein level of Cx43, ERK1/2, phosphorylated ERK1/2 (p-ERK1/2), p38 and phosphorylated p38 (p-p38). Antibodies specific for ERK1/2, p-ERK1/2, p38 and p-p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to Cx43 was obtained from Zymed Laboratories (South San Francisco, CA). Briefly, total cell lysates were suspended in 4× sample buffer (8% SDS, 20% glycerin, 250 mM Tris–HCl [pH 7.5], 0.2% bromophenol blue, and 40 mM DTT), heated for 5 min, and separated on a 12.5% SDS-polyacrylamide electrophoresis gel. The separated proteins from the gel were then transferred to a 0.45-μm polyvinylidene fluoride transfer membrane (Gelman Laboratories, Ann Arbor, MI). Membranes were incubated in blocking buffer (25 mM

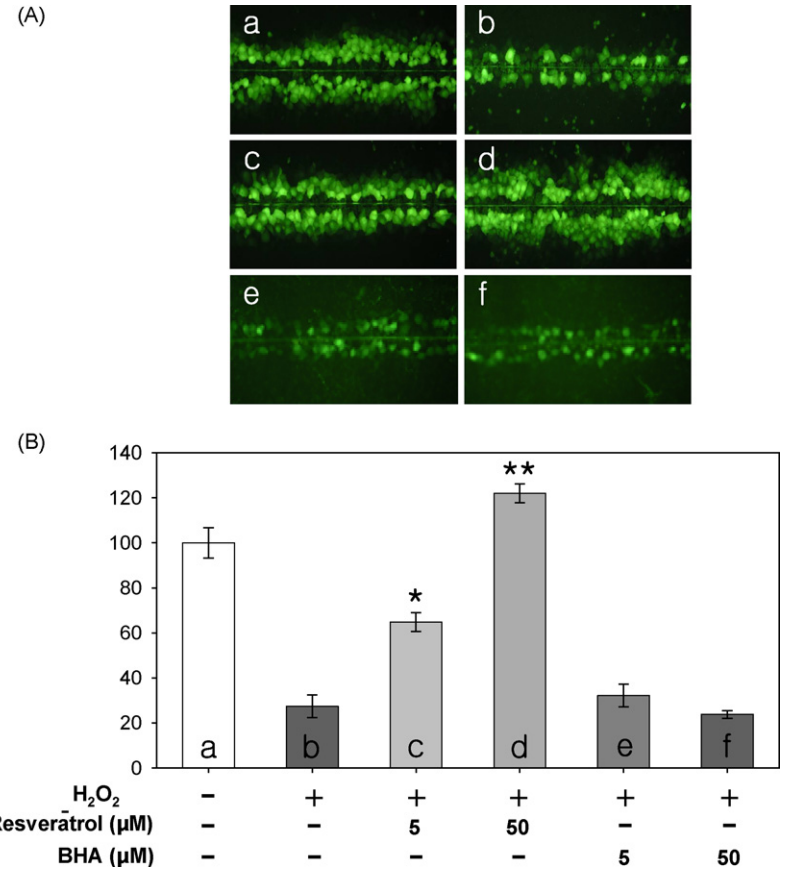

Fig. 2. Effect of resveratrol and BHA on H_2O_2-induced inhibition of GJIC in WB-F344 cells. GJIC was assessed using the scrape-loading/dye-transfer method under an inverted fluorescence microscope (100×). (A) Representative photographs of the following treatments: (a) untreated control, (b) H_2O_2 (300 μM), only, for 30 min, (c) resveratrol (5 μM) for 30 min and then H_2O_2 (300 μM) for 30 min, (d) resveratrol (50 μM) for 30 min and then H_2O_2 (300 μM) for 30 min, (e) BHA (5 μM) for 30 min and then H_2O_2 (300 μM) for 30 min, and (f) BHA (50 μM) for 30 min and then H_2O_2 (300 μM) for 30 min. (B) The number of communicating cells was counted. Values are expressed as means ± SD ($n = 3$). The asterisks indicate a significant increase ($^*p < 0.05$; $^{**}p < 0.01$) in the number of communicating cells, compared to H_2O_2 only treatment.