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(−)-Epicatechin effects in rat liver epithelial cells: stimulation of gap junctional communication and counteraction of its loss due to the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate

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Abstract

Gap junctional intercellular communication (GJIC) is a direct signaling pathway for neighboring cells. Disturbances in GJIC are suggested to play a role in carcinogenesis and may be involved in cardiac arrhythmia. Tumor promoters like 12-*O*-tetradecanoylphorbol-13-acetate (TPA) are capable of inhibiting GJIC, whereas GJIC is stimulated by several micronutrients like genistein, retinoids or carotenoids. (−)-Epicatechin (4–40 μM), a major flavonoid in cocoa and green tea, exhibited stimulatory effects on GJIC in WB-F344 rat liver epithelial cells after 24–72 hr of incubation; no change was observed after 90 min. However, treatment of cells for 90 min with TPA (5 or 10 nM) led to complete loss of GJIC, whereas 40% loss was found with 1 nM. These inhibitory effects of TPA were largely suppressed when (−)-epicatechin or genistein (40 μM) were present during the incubation. In communicating WB-F344 cells, most of the major gap junction protein connexin43 (Cx43) was located in the plasma membrane. When the cells were exposed to TPA, considerably less protein was found in the membrane. Such a delocalization of Cx43 proteins was not observed when TPA was coincubated with the flavonoids, (−)-epicatechin or genistein. It is concluded that TPA affects Cx43 trafficking between cellular compartments, and that this effect is counteracted by (−)-epicatechin or genistein.

Keywords: Gap junctional communication; Phorbol ester; Flavonoids; (−)-Epicatechin; Genistein; Connexin43

1. Introduction

Intercellular signaling is required for the coordination of cellular functions, and one pathway of signal exchange is mediated by GJIC [1]. Gap junctions are specialized plasma membrane structures consisting of cell-to-cell channels that connect the cytosol of neighboring cells and provide a direct pathway for the diffusion of small molecules (<1000 Da). The channels are composed of proteins which belong to the family of connexins. GJIC is involved in the regulation of growth, transmission of developmental signals, coordination of muscle contraction, and maintenance of metabolic homeostasis. There is increasing evidence that intercellular communication *via* gap junctions plays a role in the regulation of tumor cell growth [2,3] and may be involved in the pathogenesis of cardiac diseases predisposing for arrhythmia [4].

Tumor promoting agents such as TPA or the insecticide dichlorodiphenyl-trichloroethane inhibit GJIC [5,6]. Conversely, thyroid hormones [7], vitamin D [8], vitamin A and some of their derivatives [9] stimulate GJIC. The regulation of gap junctional communication is complex; GJIC is sensitive to intracellular pH and calcium levels [1]. It is affected by the transcription rate of connexin genes as well as by stabilization of connexin mRNA [10]. Connexins are also modified posttranslationally, and phosphorylation is one of the most common modifications of these proteins [11]. GJIC is also sensitive to oxidative and nitrosative stress; hydrogen peroxide and peroxynitrite inhibit GJIC [5,12,13]. Some antioxidant micronutrients like flavonoids [14] and carotenoids [15] induce GJIC. Here, we investigated the effects of (−)-epicatechin and genistein on GJIC in the presence of inhibitory TPA.

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Abbreviations: GJIC, gap junctional intercellular communication; Cx43, connexin43; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; NGS, normal goat serum; FCS, fetal calf serum.

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Epicatechin is a major flavonoid in cocoa and green tea, but is also found in various fruits and vegetables [16]; genistein is an important flavonoid of the soy bean [17]. Epidemiological data as well as *in vitro* and *in vivo* studies support the idea that dietary polyphenols like the flavonoids play a role in the prevention of degenerative diseases [17–19].

2. Materials and methods

2.1. Cell culture

WB-F344 rat liver epithelial cells (kindly provided by Dr. Trosko, East Lansing, MI) were grown in Dulbecco's modification of Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and penicillin/streptomycin in 35-mm plastic dishes. Cells were incubated at 37° in a humidified atmosphere containing 5% CO_2. The confluent cells were incubated with DMEM (without FCS) for 24 hr to decrease the basal level of cell-to-cell communication. (−)-Epicatechin, genistein (Sigma) or TPA were dissolved in DMSO and added to the medium; controls received DMSO only. The final concentration of DMSO in the medium was 0.2%. The epicatechin dimer was provided by Mars, Inc.

2.2. Gap junctional communication assay

Cells were treated with (−)-epicatechin (4–40 μM) and genistein (40 μM) for 24 and 72 hr and GJIC was determined. For the experiments with TPA, incubation was for 90 min with TPA (1, 5, and 10 nM), TPA (1, 5, and 10 nM) plus epicatechin (40 μM), and TPA (1, 5, and 10 nM) plus genistein (40 μM); DMSO (0.2%) was used as a control. GJIC was measured by microinjection of the fluorescent dye Lucifer Yellow CH (10% in 0.33 M LiCl) into selected cells by means of a micromanipulator and a microinjector system (Eppendorf) One minute after injection, the number of fluorescent cells around a single cell, which was loaded with the dye, were counted. Ten individual cells were injected per dish and means of the number of fluorescent neighboring cells were calculated. Images were taken with a Zeiss axiovert fluorescent microscope coupled to a CCD camera (ORCA II, Hamamatsu).

2.3. Western blot analysis

To obtain cell lysate, WB-F344 rat liver epithelial cells were washed with PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 10 mM Na_2HPO_4; pH 7.4) and treated two times with SDS–PAGE lysis buffer (125 mM Tris, 4% SDS, 20% glycerol, 100 mM dithiothreitol, 0.2% bromophenol blue; pH 6.8). After sonication, the lysates were heated for 5 min at 95° and applied to SDS–PAGE (10% (w/v) acrylamide). The gels were blotted onto polyvinylidene difluoride membranes. Immunodetection was performed with a polyclonal rabbit anti-Cx43 antibody (Zymed) as primary antibody and goat anti-rabbit IgG (Dianova) as secondary antibody.

2.4. Immunohistochemistry

For immunohistochemistry, WB-F344 cells were grown in complete medium with FCS on coverslips in 35-mm plastic dishes until they reached 90% confluence. The cells were incubated for 90 min with TPA (1, 5, or 10 nM), (−)-epicatechin (40 μM) or genistein (40 μM) or a combination of (−)-epicatechin (40 μM) and (1, 5, and 10 nM) TPA or genistein (40 μM) and (1, 5, and 10 nM) TPA; DMSO (0.2%) was used as a control.

After treatment, cells were washed with PBS and fixed with methanol for 10 min at −20°. After further washing with PBS, non-specific binding sites were blocked with 3% NGS in PBS containing 0.3% (v/v) Triton X-100 for 45 min at room temperature. Cells were incubated with a polyclonal anti-Cx43 antibody (Zymed); diluted 1:1500 in PBS with 1% (v/v) NGS overnight at 4°. Cells were washed with PBS, and incubated with an Alexa 546-coupled goat anti-rabbit IgG (H + L) secondary antibody (Molecular Probes; diluted to 1:800 in PBS) for 45 min at 37°. After washing and embedding, images were taken with a Zeiss axiovert fluorescent microscope coupled to a CCD camera (ORCA II, Hamamatsu).

3. Results and discussion

Genistein affects GJIC in cell culture at levels above 10 μM [20]. In the present study, the exposure of WB-F344 rat liver epithelial cells to genistein or (−)-epicatechin (40 μM) led to a stimulation of GJIC which was dependent on the time of incubation. After 24 hr of exposure, the stimulation of GJIC with genistein and (−)-epicatechin was 2.1-fold and 1.5-fold of control, respectively. Upon incubation for 72 hr, the increase was 2.9-fold above controls for genistein and 2.1-fold for (−)-epicatechin. The controls were treated with DMSO (0.2%) only; baseline communication was 13.4 ± 1.0 cells at 24 hr and 12.1 ± 1.3 cells at 72 hr, respectively. Fig. 1 shows typical examples of communication measurements with the dye transfer assay; control (Fig. 1A), inhibition with TPA (10 nM) after 90 min (Fig. 1B), stimulation with genistein (Fig. 1C) or (−)-epicatechin (Fig. 1D) at 40 μM after 72 hr of treatment.

Both flavonoids showed no significant effect on GJIC after 90-min incubation. With increasing levels of (−)-epicatechin in cell culture (4–40 μM), the number of communicating cells was increased (Table 1). In cells treated with the lowest concentration (4 μM), the communication was still 1.6-fold above solvent control. The dimeric form of the epicatechin tested at the 20 μM level stimulated GJIC to about the same level as 40 μM of the monomer.

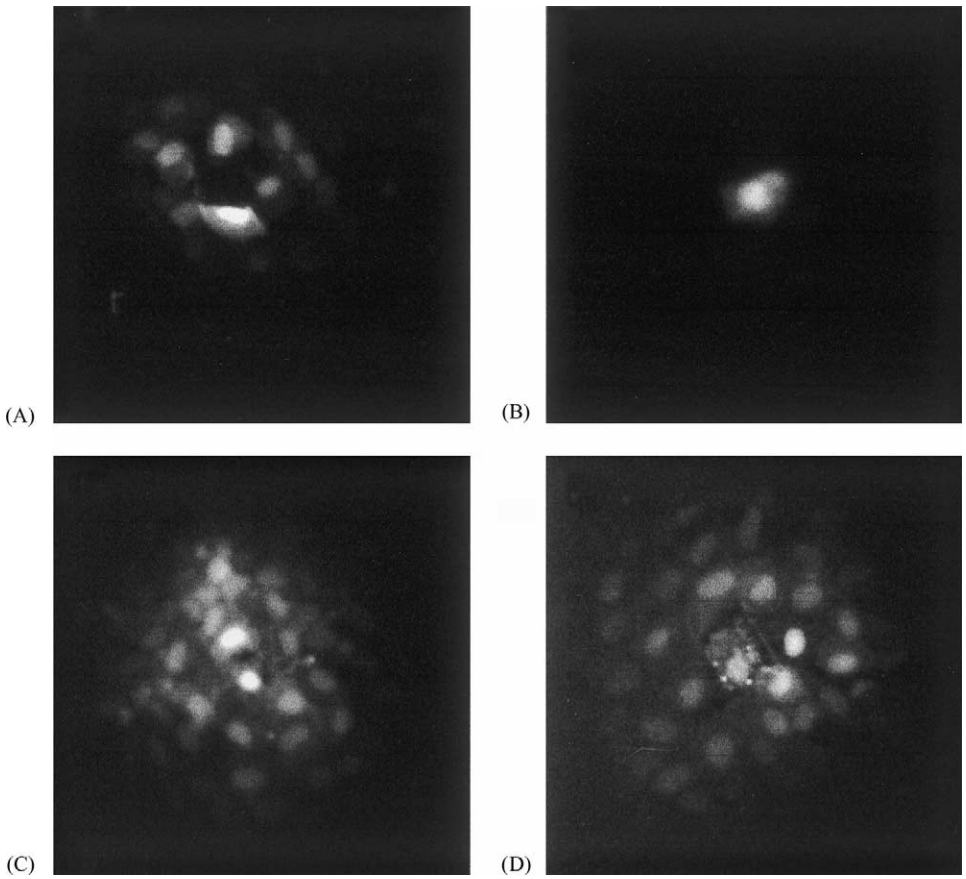

Fig. 1. Gap junctional intercellular communication in WB-F344 cells measured by the Lucifer Yellow dye transfer assay. (A) Control (0.2% DMSO); (B) TPA (10 nM, 90 min); (C) genistein (40 μM, 72 hr); (D) (−)-epicatechin (40 μM, 72 hr).

It has been assumed that TPA affects GJIC by modification of the Cx43 protein [11,21,22]. Since Cx43 is a major gap junction protein in WB-F344 cells [23], we investigated the effects of TPA on GJIC in the presence of (−)-epicatechin and genistein (Table 2). When the cells were exposed for 90 min to TPA alone, intercellular communication was completely disrupted at levels of 5 and 10 nM (Fig. 1B); GJIC was diminished by about 40% when TPA (1 nM) was used. No effect was found with TPA (0.1 nM). In all experiments, cell viability was not affected (data not shown). The incubation of cells with TPA for 72 hr had no effect on GJIC; the number of communicating cells was

Table 1
Effects of (−)-epicatechin and genistein on GJIC in WB-F344 rat liver epithelial cells

Compound	Number of communicating cells	Control (%)
Control		
DMSO (0.2%)	12.1 ± 1.3	100 ± 11
(−)-Epicatechin		
4 μM	18.1 ± 2.2*	156 ± 18*
10 μM	20.1 ± 6.8*	173 ± 56*
40 μM	24.2 ± 3.1*	209 ± 26*
Epicatechin dimer		
20 μM	23.4 ± 3.1*	202 ± 26*
Genistein		
40 μM	35.4 ± 2.4*	293 ± 20*

Cells were exposed to different concentration of (−)-epicatechin (4–40 μM). Genistein was measured at 40 μM. Communication was determined after 72 hr of incubation. GJIC is given as numbers of communicating cells measured by the dye transfer assay and as percent of control. Controls were treated with DMSO (0.2%); basal communication was 12.1 ± 1.3 cells which was set to 100%. The data represent means ± SD of three independent experiments.

* Significantly different from control ($P < 0.001$).

Table 2
Inhibitory effects of TPA on GJIC; counteraction by (−)-epicatechin and genistein

TPA (nM)	TPA (% control)	TPA plus 40 μM (−)-epicatechin (% control)	TPA plus 40 μM genistein (% control)
0.1	94 ± 14	95 ± 14	n.m.
1	55 ± 19	95 ± 19*	90 ± 23*
5	0	61 ± 15*	83 ± 18*
10	0	50 ± 16*	72 ± 17*

Cells were exposed to different levels of TPA and combinations of different amounts of TPA with either (−)-epicatechin (40 μM) or genistein (40 μM). GJIC is presented as percent of control. Controls were treated with DMSO (0.2%); basal communication was 18.6 ± 2.9 cells, which was set to 100%. Communication was measured after 90 min of incubation. The data represent means ± SD of three independent experiments. n.m.: Not measured.

* Significantly different from treatment with TPA ($P < 0.001$).

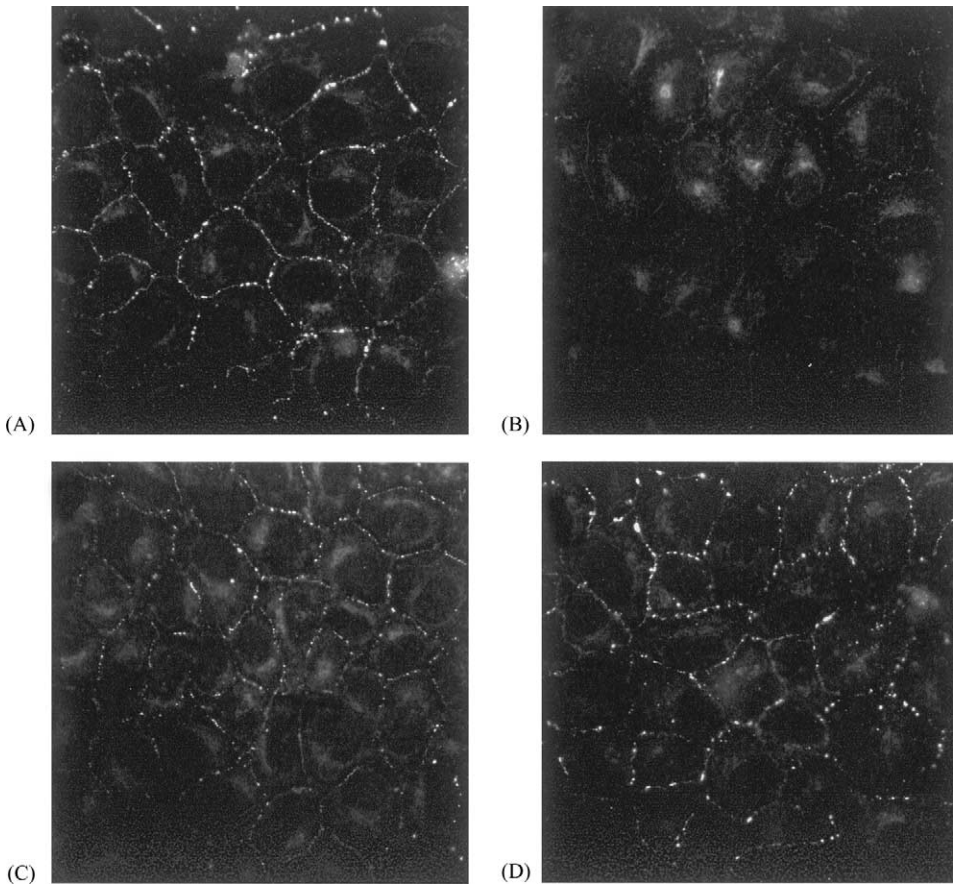

Fig. 2. Immunofluorescence analysis of the subcellular distribution of Cx43. (A) Control (0.2% DMSO); (B) TPA (10 nM); (C) TPA (10 nM) plus (−)-epicatechin (40 μM); (D) TPA (10 nM) plus genistein (40 μM). Incubation time for all treatments was 90 min.

comparable to the control, i.e. there was a complete recovery from the loss observed at 90 min.

The complete loss of GJIC by TPA after 90 min (5 and 10 nM) was partially counteracted when TPA was coincubated with (−)-epicatechin (40 μM) or genistein. Genistein was slightly more effective than (−)-epicatechin. Both compounds restored GJIC to basal levels when applied together with TPA (1 nM). This is in accordance with data from literature where similar effects of epicatechin on TPA-treated cells were described [24]. Other flavonoids such as apigenein and tangeritin also counteract tumor promoter-induced inhibition of intercellular communication [14].

In parallel, the distribution of Cx43 protein within the cells was investigated by immunostaining (Fig. 2). A typical control is shown in Fig. 2A. The cells are confluent, and connexin proteins are localized at the plasma membrane. Some protein signal is also detectable within the cytoplasm. Upon treatment with TPA for 90 min (10 nM), the signal of membrane-associated Cx43 decreased dramatically (Fig. 2B), no more areas of accumulated Cx43 protein being detectable. When (−)-epicatechin (Fig. 2C) or genistein (Fig. 2D) was present in cell culture, the TPA effect was attenuated. Cx43 protein was detectable in the membrane organized in clustered structures comparable to the control (Fig. 2A). Similar counteracting effects of (−)-epicatechin and genistein were found when TPA was used at 1 and 5 nM, respectively (data not shown). It has been speculated that the phosphorylation of gap junction proteins causes an alteration in GJIC due to structural changes of the protein which results in a translocation from the cell membrane into the cytoplasm [25]. The tumor promoter TPA is known to activate protein kinase C. TPA-mediated phosphorylation of Cx43 may be responsible for the inhibitory effects of this phorbol ester on GJIC, probably due to disturbances in intercellular trafficking as shown here. However, Western blot analyses did not exhibit significant changes in the phosphorylation pattern of Cx43 in WB-F344 in the presence or absence of TPA (data not shown). Thus, it is not yet known whether flavonoids affect protein kinases or phosphoprotein phosphorylases with impact on the GJIC. Several mechanisms may overlap and lead to the functional changes observed in cellular communication.

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