Apigenin and tangeretin enhance gap junctional intercellular communication in rat liver epithelial cells

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Two flavones, apigenin and tangeretin, were studied for their ability to modulate gap junctional intercellular communication (GJIC) in the rat liver epithelial cell line REL. Their cytotoxicity was first determined by cell density and neutral red uptake assays; neither apigenin nor tangeretin are cytotoxic at 10 and 25 μM, the concentrations used in our experiments. We then studied GJIC using the dye transfer assay and observed that both apigenin and tangeretin enhance it, the maximum stimulation (∗1.7–1.8) being achieved at 25 μM for 24 h. When the dye transfer was enhanced, the amount of connexin 43 increased, which was demonstrated by Western blot and immunofluorescence analysis. For apigenin only, Northern blot analysis showed an accumulation of connexin 43 mRNA. In addition, the incubation of REL cells with the two compounds, for 1 or 24 h, prevented the inhibition of dye transfer by 12-O-tetradecanoylphorbol-13-acetate (1 or 10 ng/ml). The enhancement of GJIC by apigenin could be one of the major mechanisms responsible for apigenin’s anti-tumour promoting action in vivo. As for tangeretin, its capacity to enhance GJIC completes its potential protective properties towards the post-initiation process.

Introduction

Gap junctions are membrane channels that permit the direct diffusion of ions, small metabolites and second messengers between adjacent cells (1). They are protein structures constituted of six subunits called connexins (2). Their function can be regulated at the different levels of connexin expression, assembly, gating, internalization and degradation (3,4). Gap junctional intercellular communication (GJIC*) seems to be an important mechanism controlling cellular homeostasis, proliferation and differentiation (5). It can be modulated (inhibited or stimulated) by endogenous and exogenous agents. Many compounds, including tumour promoters, inhibit GJIC (6–9). Conversely, a few compounds, like some retinoids and carotenoids (10–12), dexamethasone (13) and nickel (14), were found to stimulate it. However, opposite effects were also observed with retinoids and nickel in other experimental conditions (10,11,15).

Among the numerous and various flavonoids, plant polyphenolic compounds which can be found in the diet (16,17), only quercetin and green tea extracts have been studied for their ability to modulate GJIC in vitro. Quercetin had no effect by itself on the metabolic cooperation of Chinese hamster V79 lung fibroblasts (18). Green tea extracts did not change the dye transfer in mouse hepatocytes (19,20), WB-F344 rat liver epithelial cells (21) or human keratinocytes (19). Yet these studies showed that they could prevent the inhibition of GJIC by various tumour promoters. These antagonistic effects could be related to in vivo anti-tumour promoting actions reported for some flavonoids. Quercetin (22), as well as kaempferol (23) and apigenin (24), suppressed the tumour promoting effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on mouse skin when applied topically. Nevertheless, when administered per os, it had no significant inhibiting effect towards the tumour promoting actions of teleocidin on mouse skin (25) and DDT in rat liver (26). Recently, it has been observed that green tea catechins reduced small intestinal tumours in rat multi-organ carcinogenesis (27).

So, the only results available on GJIC modulation by flavonoids concern a flavanol and some catechins. To complete these data we studied the action of two flavones, apigenin and tangeretin, alone or in combination with TPA, on GJIC of a rat liver epithelial cell line isolated in our laboratory (named REL). Apigenin was chosen for the reason mentioned above, i.e. it antagonized the promoting effect of TPA in mouse skin (24). Tangeretin deserves attention because (i) it represents a naturally-occurring polymethoxylated class of flavonoids and (ii) it shows marked anti-invasive effects in vitro (28). To study GJIC at the functional level, we used the dye transfer assay, which allows the detection of both stimulating and inhibiting effects of modulators (11). We also investigated their effects on the regulation of connexin 43, the main connexin expressed in this cell system.

Materials and methods

Chemicals

Ham’s F10 medium, fetal calf serum (FCS), antibiotics and L-glutamine, were purchased from Flow Laboratories (Irvine, UK). Apigenin, lucifer yellow CH, dimethyl sulfoxide (DMSO) and TPA were obtained from Sigma Chemical Co (St Louis, MO) and tangeretin from Extrasynthèse (Genay, France).

Cell culture

REL cells were cloned from a rat liver epithelial cell line which had been isolated as previously described (29). They were grown in Ham’s F10 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 μl/ml penicillin, 100 μg/ml streptomycin and 10 μg/ml gentamycin at 37°C in a humidified atmosphere containing 5% CO2.

We dissolved apigenin and tangeretin in DMSO and added them to pure FCS to prevent their adsorption on flasks. The complete media were sonicated and stored in silicone-coated flasks (Ventron, Germany). TPA dissolved in DMSO was directly added to the medium to reach 1 or 10 ng/ml concentrations 1 h before microinjection. All studies, cytotoxicity, microinjection, Northern blot, Western blot and immunofluorescence, were determined at cell sub-confluency after the same time of culture. Cells (4X10^4) for each treatment

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were plated in duplicate in a 35 mm Petri dish. Twenty-four hours after
plating the medium was replaced by the complete medium containing apigenin or
tangeretin or DMSO alone (0.1%) for an additional 24 h incubation.

Only for the first kinetic study of dye transfer were 1.5×10³ cells plated
for each treatment and the molecules added for 48, 24 or 4 h before microinjection.
As 0.1% DMSO had no effect on GJIC and all plates were microinjected at
the same time (3 days after plating), a common control could be taken for
the different treatments, i.e. 0.1% DMSO, 48 h.

Cytotoxicity assays

For GJIC studies, both flavonoids had to be tested at concentrations inducing
no cytotoxic effects nor reduction in cell density, which influences the ability
to cells to communicate. A concentration reducing by at least 25% (inhibiting
concentration, IC₂₅) either cell density or neutral red uptake was considered
as cytotoxic. Cytotoxicity was determined in two separate experiments.

Cell density determination

For each treatment, cells from three separate Petri dishes were counted with
a Coulter counter-channelizer (Coultronics) 48 h after plating.

Neutral red uptake assay (30)

For each treatment, incorporation of neutral red was measured in eight wells
from two different 96-well microplates 48 h after plating 9×10³ cells/well.
The cells were treated for 24 h with a control or a flavonoid-containing
medium and then maintained for 3 h at 37°C with the neutral red solution
(50 μg/ml) (Sigma, St Louis, MO) in the culture medium. Then
they were washed 3 times with warm phosphate-buffered saline (PBS) (37°C) and fixed
with destain solution (1% glacial acetic acid, 50% ethanol, 49% distilled water).
The neutral red uptake was finally quantified by spectrophotometry at
540 nm.

Dye transfer

We used the protocol described by Enomoto et al. (31). Confluent cells from
two separate dishes were microinjected with a 5% (w/v) solution of lucifer
yellow CH in 0.33 M lithium chloride by means of a glass capillary
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Table 1. Cytotoxicity of apigenin and tangeretin

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>0</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
<th>25</th>
<th>50</th>
<th>100</th>
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<tr>
<td>Apigenin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell density</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell no. (×10⁶)b</td>
<td>551±9</td>
<td>528±32</td>
<td>543±9</td>
<td>537±41</td>
<td>493±18</td>
<td>342±11</td>
<td>66±2</td>
</tr>
<tr>
<td>Per cent controlb</td>
<td>100</td>
<td>96</td>
<td>99</td>
<td>97</td>
<td>89</td>
<td>62*</td>
<td>12*</td>
</tr>
<tr>
<td>Neutral red uptake</td>
<td>Optical density (×10⁻³)c</td>
<td>129±11</td>
<td>135±8</td>
<td>133±13</td>
<td>120±16</td>
<td>110±14</td>
<td>68±12</td>
</tr>
<tr>
<td>Per cent controlb</td>
<td>100</td>
<td>105</td>
<td>103</td>
<td>93</td>
<td>85</td>
<td>53*</td>
<td>26*</td>
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<tr>
<td>Tangeretin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell density</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell no. (×10⁶)b</td>
<td>457±41</td>
<td>429±21</td>
<td>471±13</td>
<td>520±5</td>
<td>510±12</td>
<td>539±9</td>
<td>378±3</td>
</tr>
<tr>
<td>Per cent controlb</td>
<td>100</td>
<td>94</td>
<td>103</td>
<td>114</td>
<td>112</td>
<td>118</td>
<td>83</td>
</tr>
<tr>
<td>Neutral red uptake</td>
<td>Optical density (×10⁻³)c</td>
<td>431±18</td>
<td>421±41</td>
<td>387±39</td>
<td>506±28</td>
<td>422±32</td>
<td>333±47</td>
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<tr>
<td>Per cent controlb</td>
<td>100</td>
<td>98</td>
<td>90</td>
<td>117</td>
<td>98</td>
<td>77</td>
<td>95</td>
</tr>
</tbody>
</table>

*a Mean ± SD of three dishes.
*Ratio of cell number in treated dishes×10⁶/cell number in control dishes.
*c Mean ± SD of 16 wells.
*d Ratio of optical density in treated dishes×10⁶/optical density in control dishes.
*Cytoxic effect.

2326
Apigenin and tangeretin enhance intercellular communication in 1× SSPE, 0.5× SSPE and 0.1× SSPE at 65°C for 30 min. Blots were exposed to Kodak Omat AR film at -70°C using double intensifying screens. Northern blot analysis was repeated on extracts from four independent cultures.

Results

Cytotoxicity of apigenin and tangeretin

Before studying GJIC, we evaluated the cytotoxicity of apigenin and tangeretin by two complementary assays: cell density and neutral red uptake. For each molecule, similar results were obtained with the two assays (Table I): up to 25 μM apigenin is neutral, between 25 and 50 μM it has a cytostatic effect and beyond 50 μM it is cytotoxic. Up to 100 μM tangeretin is not cytotoxic.

As the cell density of a monolayer influences the capacity

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Number of dye-coupled cellsa</th>
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</thead>
<tbody>
<tr>
<td>Control (DMSO 0.1%)</td>
<td>94 ± 18</td>
</tr>
<tr>
<td>Apigenin 10 μM</td>
<td>117 ± 26</td>
</tr>
<tr>
<td>Apigenin 25 μM</td>
<td>136 ± 33</td>
</tr>
<tr>
<td>DMSO 0.1%b</td>
<td>110 ± 19</td>
</tr>
<tr>
<td>Tangeretin 10 μM</td>
<td>127 ± 25</td>
</tr>
<tr>
<td>Tangeretin 25 μM</td>
<td>142 ± 28</td>
</tr>
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</table>

Results are mean ± SD of 20–40 microinjections in two separate dishes.

Table II. Kinetic study of apigenin and tangeretin effects on dye transfer

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Number of dye-coupled cellsa</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>103 ± 10</td>
</tr>
<tr>
<td>Apigenin 10 μM</td>
<td>152 ± 17b</td>
</tr>
<tr>
<td>Apigenin 25 μM</td>
<td>187 ± 24b</td>
</tr>
<tr>
<td>Tangeretin 10 μM</td>
<td>149 ± 20b</td>
</tr>
<tr>
<td>Tangeretin 25 μM</td>
<td>175 ± 24b</td>
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</table>

Table III. Estimation of the stimulation of junctional transfer by apigenin and tangeretin

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. of independent experiments</th>
<th>Average number of fluorescent cells</th>
<th>Stimulation factor</th>
<th>Total number of microinjections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (DMSO 0.1%)</td>
<td>9</td>
<td>103 ± 10</td>
<td>1</td>
<td>233</td>
</tr>
<tr>
<td>Apigenin 10 μM</td>
<td>9</td>
<td>152 ± 17b</td>
<td>1.5 ± 0.2b</td>
<td>208</td>
</tr>
<tr>
<td>Apigenin 25 μM</td>
<td>9</td>
<td>187 ± 24b</td>
<td>1.8 ± 0.1b</td>
<td>185</td>
</tr>
<tr>
<td>Tangeretin 10 μM</td>
<td>8</td>
<td>149 ± 20b</td>
<td>1.4 ± 0.1b</td>
<td>210</td>
</tr>
<tr>
<td>Tangeretin 25 μM</td>
<td>8</td>
<td>175 ± 24b</td>
<td>1.7 ± 0.2b</td>
<td>170</td>
</tr>
</tbody>
</table>

*Inter-experiment SD of the mean.
Significantly higher than the control value (P < 0.001).
Statistical analysis is described in Materials and methods.

Fig. 1. Average increase of dye transfer by apigenin in a REL cell monolayer. (A) control cells; (B) cells treated with 25 μM apigenin for 24 h (×400).

Fig. 2. Effects of apigenin and tangeretin on the distribution of dye-coupled cells per microinjection. Numerical data are provided by the independent experiments presented in Table III. (A) Apigenin; (B) tangeretin. White bars: without flavonoid; hatched bars: 10 μM apigenin or tangeretin; black bars: 25 μM apigenin or tangeretin.

Fig. 3. Effects of apigenin and tangeretin on the amount of connexin 43, demonstrated by Western blot analysis. Total proteins were extracted from rat liver (L), rat heart (H) or REL cells treated or not (C, control) with 10 μM (T10) or 25 μM (T25) tangeretin or with 10 μM (A10) or 25 μM (A25) apigenin.
of cells to come into contact and to communicate, it must be prevented from decreasing too much. The concentration causing 25% inhibition (IC25) of cell density is 40 μM for apigenin and 100 μM for tangeretin. Therefore, the concentrations 10 μM and 25 μM for both apigenin and tangeretin can be used in further GJIC studies.

Stimulation of intercellular communication by apigenin and tangeretin

The effects of apigenin and tangeretin on GJIC of REL cells were studied at 4, 24 and 48 h treatment (Table II). Both increase the dye transfer in a dose–response manner. The maximal stimulation is obtained after 24 h. Figure 1 illustrates the stimulation of dye transfer in a cell monolayer treated with 25 μM apigenin for 24 h (Figure 1B) as compared to control cells (Figure 1A).

Furthermore, numerous independent experiments, focusing on 24 h incubations, were performed to estimate the mean stimulation factor and to analyse the distribution of the number of dye-coupled cells. The average number of dye-coupled cells per microinjection is significantly higher for flavonoid-treated cells as compared to control cells (P < 0.001). The mean stimulation factors are 1.8 ± 0.1 for apigenin and 1.7 ± 0.2 for tangeretin, at a concentration of 25 μM (Table III).

The control population is distributed between classes 60 and 200 (Figure 2A) and the treated population between 100 and 300 (Figure 2B).

Effects of apigenin and tangeretin on the expression of connexin 43 and corresponding mRNA

The effects of apigenin and tangeretin on the amount of connexin 43 were determined by Western blot analysis after 24 h treatment (Figure 3). Compared to untreated cells, the treated cells present a higher amount of connexin 43 detected with a specific antibody, especially with 25 μM apigenin. As shown by immunofluorescence micrographs (Figure 4A), connexin 43 is located in cell–cell contact regions as expected.

Then, connexin 43 mRNA was compared in treated and untreated cells by Northern blot analysis (Figure 5). It clearly increased only with total RNA extracted from 25 μM apigenin-treated cells.

Table IV. Decrease in TPA-induced inhibition of dye transfer by apigenin and tangeretin.

<table>
<thead>
<tr>
<th>Percentage of inhibitiona</th>
<th>TPA (ng/ml)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Control (without flavonoids)</td>
<td>41±13</td>
</tr>
<tr>
<td>Apigenin 25 μM, 1 h</td>
<td>15±12</td>
</tr>
<tr>
<td>Apigenin 25 μM, 24 h</td>
<td>12±7</td>
</tr>
<tr>
<td>Tangeretin 25 μM, 1 h</td>
<td>1±2</td>
</tr>
<tr>
<td>Tangeretin 25 μM, 24 h</td>
<td>5±8</td>
</tr>
</tbody>
</table>

Results are means ± SD of three independent experiments.
aFor each treatment, the percentage of inhibition of dye transfer is calculated using the corresponding control (without TPA).
bSub-confluent REL cells were treated with TPA for 1 h before microinjection.
Apigenin and tangeretin prevent the inhibition of dye transfer by TPA

The effects of treatment with apigenin or tangeretin, for 1 h or 24 h, on the inhibition of intercellular communication by TPA were studied using the dye transfer assay (Table IV).

As expected, 1 ng/ml TPA for 1 h reduces the number of dye-coupled cells by half and the inhibition is almost complete with 10 ng/ml TPA.

Both flavonoids at 25 μM, particularly tangeretin, markedly antagonize TPA-induced inhibition of dye transfer. They almost completely abolish the inhibitory action of 1 ng/ml TPA. As for 10 ng/ml TPA, apigenin and tangeretin also have antagonistic effects, especially when co-administered with TPA for 1 h.

Discussion

To our knowledge, our results are the first to demonstrate enhancement of intercellular communication by flavonoids alone. They were obtained with REL cells, which, among the different rat liver epithelial cells tested, are particularly responsive to stimulation of GJIC.

Our data clearly show that both apigenin and tangeretin enhance dye transfer. The maximal stimulation is seen after 24 h treatment at 25 μM and the enhancement of dye transfer is associated with an increase in the amount of connexin 43, determined by Western blot analysis and in situ immunofluorescence. Northern blot analysis reveals that, while tangeretin has no effect on the amount of connexin 43 mRNA, apigenin caused it to increase, either by a stimulation of connexin 43 transcription or by mRNA stabilization. The increase in connexin 43 caused by tangeretin could be due to translational or post-translational mechanisms. It has been demonstrated that, in different cell types, connexin 43 is synthesized as a single species and then converted to two species (connexin 43-P1 and connexin 43-P3) by serine phosphorylation (35). Although phosphorylation does not seem to affect the connexin 43 half-life, it could be involved in establishment and/or maintenance of gap junctional plaques (4). In further experiments it will be interesting to study whether the stimulation of dye transfer by apigenin and tangeretin is related to a change of the phosphorylation state of connexin 43.

Secondly, apigenin and tangeretin enhance dye transfer in TPA-treated REL cells. It has been previously shown that quer cetin counteracts TPA- and DDT-induced inhibition of metabolic cooperation (18). A catechin gallate-containing green tea extract also prevents inhibition of dye transfer by various tumour promoters in liver cells (19–21). Thus, flavonoids having different chemical structures antagonize the inhibitory action of different tumour promoters.

Hence, multiple mechanisms could explain the flavonoid-induced prevention of inhibition of GJIC by tumour promoters. In our study, the flavonoid-induced enhancement of GJIC without TPA takes 24 h, whereas the antagonistic effect against 10 ng/ml TPA is quicker (1 h), indicating that apigenin and tangeretin can act at different levels of gap junction regulation. Further studies are being carried out in our laboratory to elucidate the mechanisms by which the two compounds antagonize TPA action. In addition, the modulation of GJIC by flavonoids of different classes is being studied to analyse the structure-activity relationships in REL cells and other cell systems. In particular, we have modified REL cells by transfection of human c-fos cDNA (36). These c-fos over-expressing transfectants, which are partly transformed, will be very helpful to study the mechanisms of action of potential anti-promoters.

Finally, we have identified two new GJIC stimulators. As shown for some retinoids and carotenoids (37–39), apigenin and tangeretin stimulate GJIC by enhancing the amount of connexin 43. It remains to be determined whether they can up-regulate connexin 43 expression in a relevant whole animal model. It is now hypothesized that stimulation of GJIC at the early stages of tumour promotion could prevent the development of transformed cell foci (40). Associated with the anti-proliferative (41) and anti-invasive effects (42,28) of tangeretin, the stimulation of GJIC could confer on this flavonoid wide protective properties during the post-initiation process. Also, enhancement of GJIC by apigenin in TPA-treated cells may be an important mechanism for the inhibition of tumour promotion (24).

Acknowledgements

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References


