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Transactivation of peroxisome proliferator-activated receptor α by green tea extracts

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Tea is a popular beverage. Recently, green tea was reported to increase the number of peroxisomes in rats. In this study, to find out whether the green tea-induced proliferation of peroxisomes is mediated by PPARα, a transient transfection assay was carried out to investigate the interactions of tea extracts (green tea, black tea, oolong tea and doongule tea) and tea components (epigallocatechin gallate, epigallocatechin, epicatechin gallate, epicatechin and gallic acid), with mouse cloned PPARα. Green tea and black tea extracts, and epigallocatechin gallate, a major component of fresh green tea leaves, increased the activation of PPARα 1.5-2 times compared with the control. It is suggested that the green tea induced-peroxisomal proliferation may be mediated through the transactivation of PPARα and that epigallocatechin gallate may be an effective component of green tea leaves. This would account for the increase in the number of peroxisomes and the activity of peroxisomal enzymes previously reported. However, black tea, a fully fermented product, had a stronger effect than oolong tea extract. These results also suggest, that in addition to epigallocatechin gallate, green tea leaves may possess some active chemicals newly produced as a result of the fermentation process, which act on PPARα like other peroxisome proliferators.

Key words: Peroxisome proliferator-activated receptor, green tea, epigallocatechin gallate

Introduction

Tea is a preparation made from dried leaves of Camellias sinensis, being one of the most widely consumed and popular beverages in the world. Tea was discovered in China, where it has been consumed, due to its medical properties, since BC 3000 [5]. The significance of the daily tea consumption and its cancer prevention in humans is an important issue. Oral administration of tea extract has been demonstrated to inhibit the development of experimental skin tumor of rodents [7], the growth of implanted tumor cells [13], and invasion and metastasis of malignant tumor cells [2]. The aforementioned chemopreventive effects of tea against tumorigenesis and tumor growth have been attributed to the biochemical and pharmacological action of the polyphenols contained in tea.

The most significant properties of tea polyphenols include their antioxidant activity [17], modulation of carcinogen-metabolizing enzymes [9], trapping of ultimate carcinogens [16,18], inhibitory effect in respect of the nitrosation reaction [8], inhibition of cell proliferation-related activity, induction of cell apoptosis and cell cycle arrest [1], blockade of mitotic signal transduction through the modulation of the growth factor receptor binding, and nuclear oncogene expression [10,11].

Recently, green tea as a sole drinking fluid has been found to enhance the hepatic CN^--insensitive palmitoyl CoA oxidase activity and increase the number of hepatic peroxisomes than the control in rats [3]. Hess *et al.* [6] reported that clofibrate, a compound with hypolipidemic properties in man as well as animals, caused an enlargement of the liver in male rats associated with a profound increase of the number of peroxisomes in the liver cells. Later, a number of pharmaceuticals and industrial chemicals were found to induce peroxisome proliferation and liver tumor, first of all in rat and mouse liver [14]. In rodent studies, where the exposure to peroxisome proliferators is associated with hepatocarcinogenicity, the number of peroxisomes in the liver cells has always been found to be 3-fold higher than that in the normal [14]. Accordingly, a response below a 2-fold increase is considered to be of uncertain biological significance. As with many other toxic end points, a 2- to 3-fold increase is considered to be a week response, and a 3-fold and higher response is regarded as a definitely expressed response.

In case with green tea and black tea, the palmitoyl CoA oxidase activity has been found to increase a little, compared with the enzyme activity induced by di(2-ethylhexyl)

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phthalate and Wy-14,643 in other reports [3,12]. Also, considering the world-wide consumption and versatile effects on the tumor cells, it is important that green tea induces changes related to peroxisome proliferation in the liver cells. Therefore, the present study was undertaken to find out whether green tea-induced proliferation of peroxisome is mediated by PPARα, using transient transfection assay.

Materials and Methods

Preparation of tea extracts and chemicals

Green tea (Pacific, Korea), black tea (Harrods, UK), oolong tea (Fortnum & Mason, UK), and doongule tea (Pacific, Korea) were purchased locally and stored at 4°C in a sealed bag. 2.0% tea extracts were prepared by adding the appropriate volume of boiling water to the tea in a pre-warmed thermos flask, leaving to stand for 3 min. with regular inversion every 10 seconds, and then filtering through 40 μm syringe filter and stored at −20°C until use.

Epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC) and gallic acid (GA) were purchased from Sigma (USA) and dissolved in the medium. Wy-14,643 (Tokyo, Japan) and clofibrate (Sigma, USA) were dissolved in 1000-fold stock DMSO, which was 0.1% of the final concentration.

Plasmid

The firefly luciferase reporter plasmid pHD3xLuc, which contains three copies of nts 2956 to 2919 of the rat enoyl-CoA hydratase/3-hydroxylacyl CoA gene cloned into pCPS-Luc, and retinoic acid X receptor α (RXRα) were obtained from Dr. Capone (Mcmaster University, Canada). The mouse PPARα expression plasmid (pCMVmPPARα E272G) was provided by Dr. Johnson (Scripps Research Institute, USA). The renilla luciferase reporter plasmid (pRL-TK) was purchased from Promega (USA).

Cytotoxicity test using MTT assay

The cytotoxic effect of chemicals on COS-1 cells was estimated by measurement of the rate of mitochondrial metabolism of MTT. In short, the control and treated cells were seeded at 5×10^5 cells/well in 100 μl of actinomycin D containing the medium in 96-well plates. After 3 hours, the cells were incubated in the presence of GTE for 24 hrs. 10 μl of a MTT (5 mg/ml in PBS) were added to each well. After 4 hr of incubation of 37°C, 100 μl of a lysing buffer (10% sodium dodecyl sulphate; 45% dimethylformamide; adjusted to pH 4.5 with glacial acetic acid) were added to each well. After overnight incubation at 37°C, the plates were read with a microplate reader, using a test wavelength of 595 nm and a reference wavelength of 655 nm. All the cytotoxicity assays were performed in triplicate.

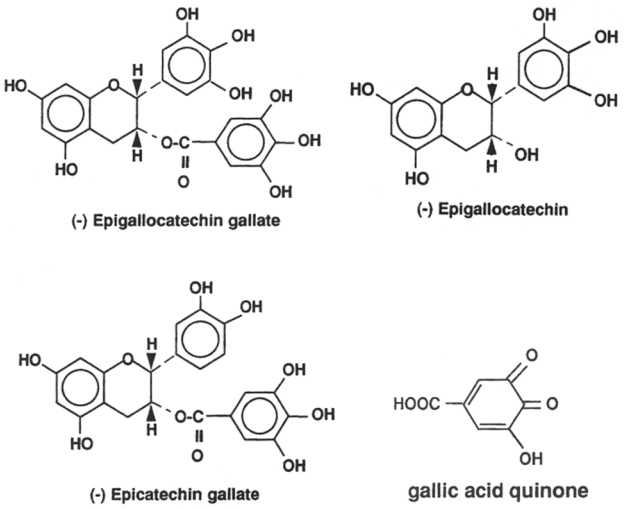

Fig. 1. Chemical structures of major compounds in fresh green tea leaves.

Transient Transfection Assay

COS-1 cells were seeded in 6 well plates at 1×10^5 cells per wells of 6-well culture plate in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum. Then, the cells were cultured for 24 hrs at 37°C and transfected with a mixture of 1 μg of plasmid DNA as described below, using FuGene 6 transfection reagent (Roche, USA). Each well was transfected with 14 ng pCMVmPPARα E272G, 14 ng RXRα, 350 ng pHD3xLuc, and 28 ng pRL-TK, made up to 1 μg with sonicated sperm DNA. After 24 hrs, the medium was replaced by DMEM with serum, containing tea extracts or their major components. Cells were lysed 24 h later, and the firefly and renilla luciferase activity was measured using Dual Luciferase Activity kit (Promega, USA) with luminometer (Berthold, Germany). Firefly luciferase reporter activities were normalized for the level of renilla luciferase activity and data shown are x-fold induction of luciferase activity for cells treated with chemicals compared with the vehicle control. Wy-14,643 (20 μM) was used as a positive control for PPARα.

Statistical analysis

The data shown in each figure are mean values ± SE (for n = 3 triplicates) and are representatives of at least three such independent experiments. Statistical analysis was performed between two groups using two-tailed Student's *t*-test for unpaired values.

Results

To confirm the transient transfection assay, we examined the effect of Wy-14,643 and clofibrate on the transactivation

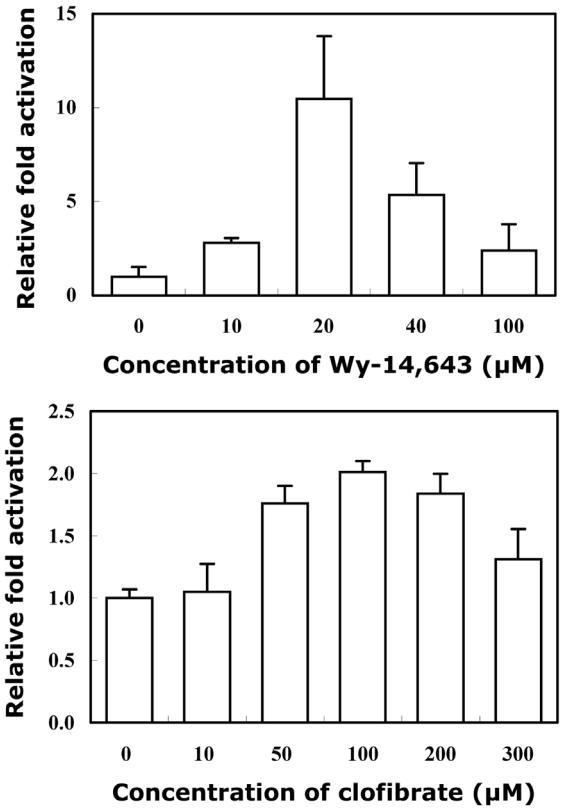

Fig. 2. Mouse PPARα activation is stimulated by a potent peroxisome proliferator, Wy-14,643 and a hypolipidemic drug, clofibrate. This transfection assay system is appropriate to test PPARα activation by other chemicals. Wy-14,643 and clofibrate stimulate PPARα activation at a concentration of 20 μM and 100 μM, respectively.

of PPARα. A characteristic activation of PPARα of Wy-14,643 at 20-40 μM and clofibrate at 100 μM was noted. After several runs of the experiment, the concentration of the positive control was determined to be 20 μM (Fig. 2).

Whether green tea extract (GTE) was cytotoxic or proliferative in respect to COS-1 cells was determined using the MTT assay, because the cytotoxicity or proliferation can affect the interpretation of the results due to the non-specific changes of renilla luciferase. GTE showed a dose dependent cytotoxicity in COS-1 cells (Fig. 3). GTE began to stimulate PPARα activation at a concentration of 0.001% GTE, and a level of above 0.01% GTE induced a stable transactivation (Fig. 4). Although 0.4% GTE showed little cytotoxic effect in Fig. 2, it sometimes caused severe cytotoxicity (data not shown), depending upon the preparation of the tea extracts. In these results, 0.02% GTE generally maintains the maximum transactivation.

Like GTE, the black tea extract (BTE) is also derived from leaves of green tea, manufactured with further processing. After the treatment for 24 hrs, BTE induced 1.5-2 times activation of PPARα (Fig. 5a). The most effective transactivation was observed at 0.02% BTE, similar to GTE.

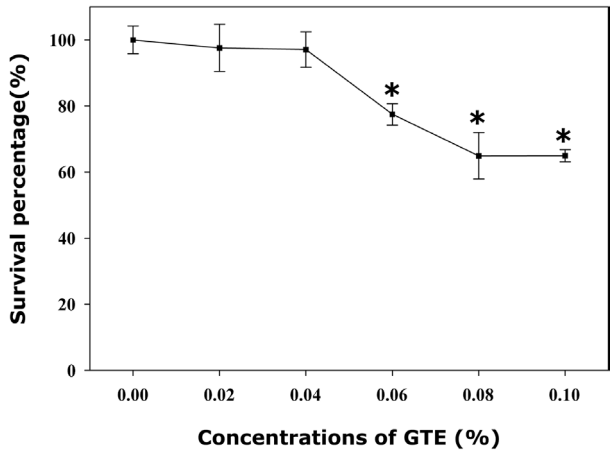

Fig. 3. Dose dependent effect of GTE on the cytotoxicity in COS-1 cells. Cells were incubated in the presence of GTE for 24 hrs. Then the cytotoxic effect was detected by MTT assay method as described in materials and methods. The cytotoxicity is expressed as the percentage of mitochondrial MTT reduction activity and the data are expressed as mean SD of three determinations (each in triplicate). *$p < 0.001$ compared with control.

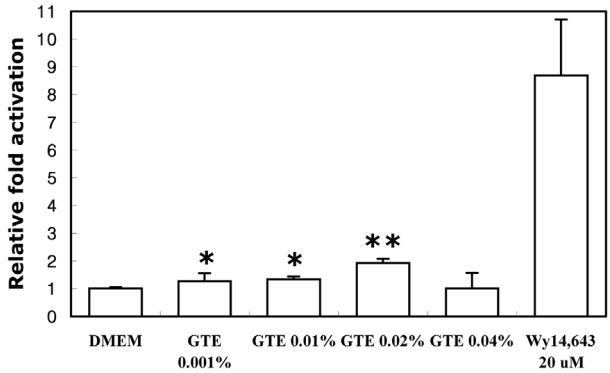

Fig. 4. Mouse PPARα activation is stimulated by the green tea extracts. This transfection assay system is appropriate to test PPARα activation by other chemicals. The data are expressed as mean SEM of three determinations (each in triplicate). *$p < 0.05$, **$p < 0.01$ compared with control.

In contrast to GTE and BTE, although the oolong tea extract (OTE) and the doongule tea extract (DTE) showed significant increase of transactivation, it failed to reach the level induced by GTE (Fig. 5). Actually, when all of 0.02% extracts were compared, only GTE and BTE induced the activation of PPARα (Fig. 6). In spite of the fact that OTE is also derived from green tea leaves, its action is not as effective as that of GTE and BTE. To find out the components that could explain the effect of GTE, we examined the action of EGCG, EGC, ECG, EC, and gallic acid. EGCG proved to increase the activation of PPARα in a dose dependent manner, but not EGC, ECG, EC, and GA (Fig. 7). When the action of the chemicals at their maximum effective concentration was compared to that of clofibrate,

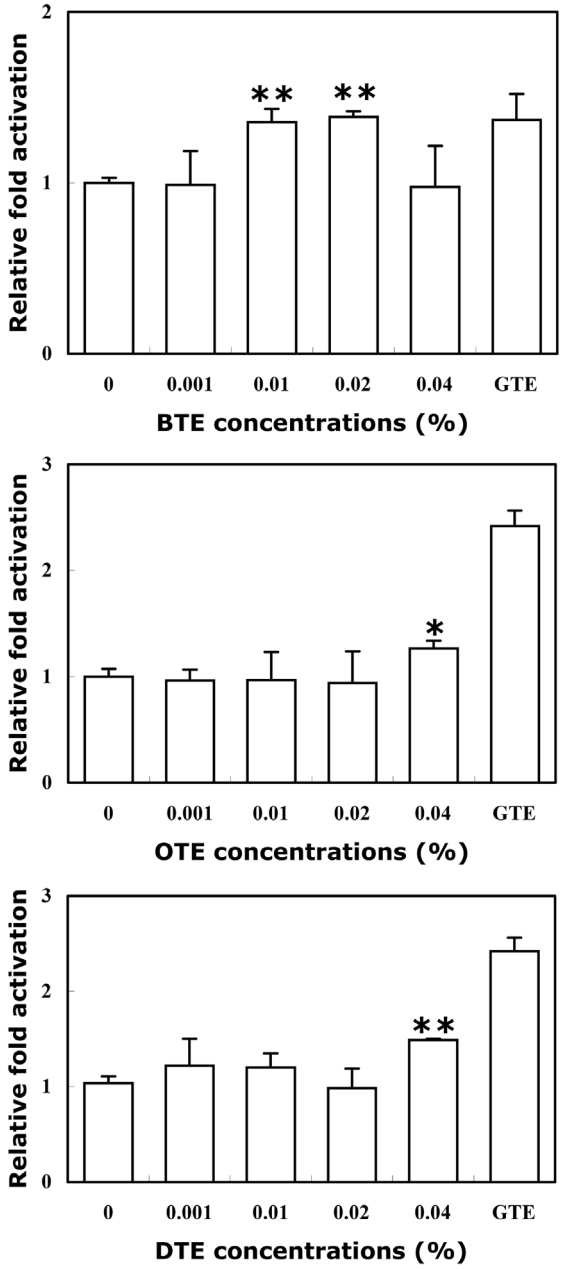

Fig. 5. Mouse PPARα activation is stimulated by BTE. Although OTE and DTE significantly increase the activation of PPARα, their activations are very weak compared to that of GTE. BTE begins to stimulate PPARα activation at a concentration of 0.01% and has activity similar to GTE. The data are expressed as mean SEM of three determinations (each in triplicate). *$p < 0.05$, **$p < 0.01$ compared with control.

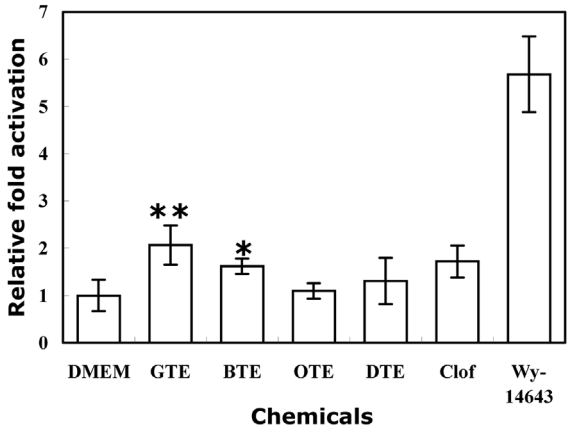

Fig. 6. Mouse PPARα activation is stimulated by tea extracts. GTE and BTE were shown to activate PPARα. Cells were incubated with 0.2% of GTE, BTE, OTE, and DTE, 100 μM of clofibrate, and 20 μM of Wy-14,643 24 hrs before preparation of cell extracts and measurement of luciferase activity. The data are expressed as mean SEM of three determinations (each in triplicate). *$p < 0.05$, **$p < 0.01$ compared with control.

interestingly, EGCG turned out to induce the transactivation as much as clofibrate (Fig. 8). EGC, EC and ECG did not cause apparent transactivation. It suggests that EGCG may be an effective component of green tea leaves which is accountable for an increase in the peroxisomal enzyme activity in other reports, and its effect may be mediated through the transactivation of PPARα. However, the black tea, a fully fermented product, had a stronger effect than the oolong tea extract. These results also suggest that in addition to EGCG, the leaves of green tea may possess some active chemicals that may have been newly produced in the result of the fermentation process and act on PPARα like other peroxisome proliferators.

Discussion

Bu-Abbas reported that an extract of either green tea or black tea increased the activity of peroxisomal enzymes and the number of peroxisomes in rat liver cells [3]. This suggests that green tea acted as a peroxisomal proliferator, believed to activate PPARα and induce the transcription of its target genes. It is not known whether green tea induces the transactivation of genes through the activation of PPARα. In this study, green tea induced the activation of PPARα, and its two components, EGCG and EGC, were shown to be effective. In this transient transfection assay, transactivation is dependent on the activation of PPARα and the binding of PPARα to PPRE, which is its corresponding response element. Although these results cannot verify the identity of the effective materials, peroxisomal proliferation by green tea extract is considered to be mediated through the activation of PPARα.

Generally, black tea is derived as a result of full fermentation of the leaves of green tea. The concentration of its ingredients is different from that of green tea [3]. For example, EGCG, the best known ingredient, is largely degraded by fermentation. In addition, the composition of green tea leaves varies, depending upon the climate, the

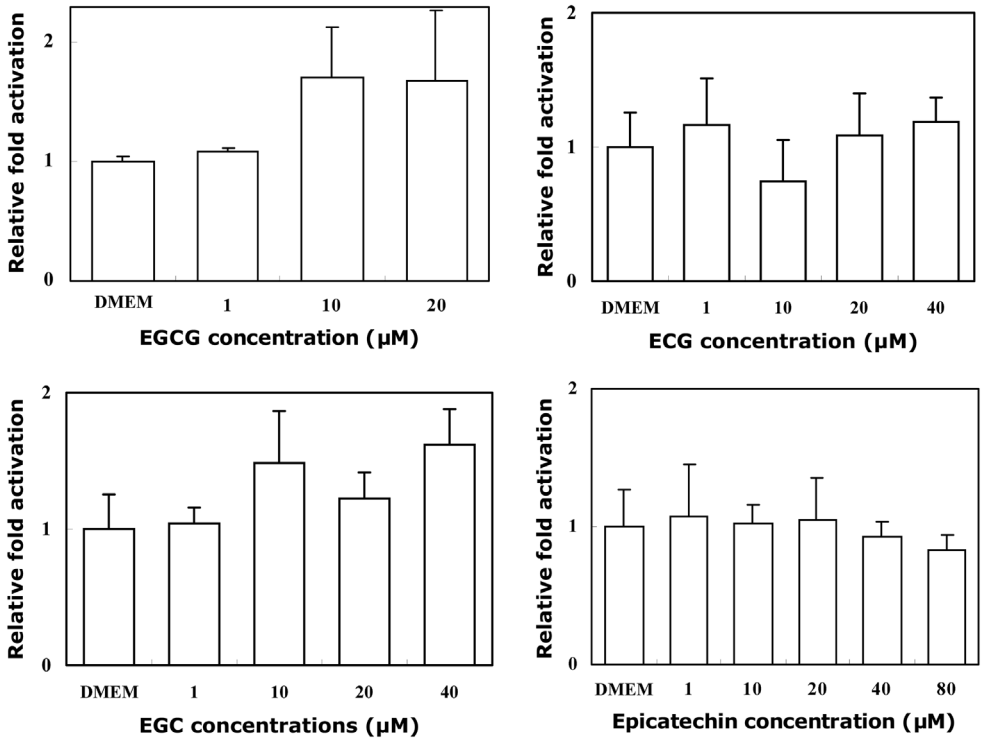

Fig. 7. Mouse PPARα activation is stimulated by EGCG. EGCG begins to stimulate PPARα activation at a concentration of 10 μM. The data are expressed as mean SEM of three determinations (each in triplicate).

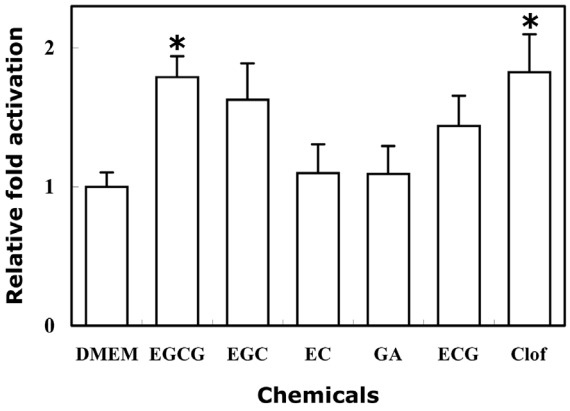

Fig. 8. Mouse PPARα activation is stimulated by EGCG. Cells were incubated with 10 μM EGCG, 40 μM EGC, 20 μM EC, 40 μM GA, and 10 μM ECG 24 hrs before preparation of cell extracts and measurement of luciferase activity. The data are expressed as mean SEM of three determinations (each in triplicate). *$p < 0.05$ compared with control.

season and the processing [5]. Although the concentrations of EGCG and EGC in green tea and black tea are apparently different, these two tea extracts increase the activation of peroxisomal enzymes to a similar extent [3]. In the case of oolong tea, the leaves are dried for a short time, scorched and then fermented. The concentration of EGCG in oolong tea falls between that of green tea and black tea [19]. However, the transactivation expressed by oolong tea was less than the transactivation expressed by black tea. This signifies that, in addition to EGCG, other effective ingredients could be contained in green tea, or some new chemicals may have been produced during the manufacturing process.

Peroxisome proliferators, including hyperlipidemics, plasticizers and pesticides, have been known to induce hepatocarcinogenesis in rat liver. However, whether this carcinogenic effect also works in human beings has not yet been elucidated. Green tea has been used since the year 3000 B.C. and is now consumed worldwide. Recently, the chemopreventive effect of green tea on chemically induced tumors and its inhibitory action on tumor metastasis, was reported [15]. These reports support the speculation, that the overall beneficial effect of green tea by far outweighs its possible negative effect. Eventually, the activation of PPARα and peroxisome proliferation by green tea could be suggested to have some regulatory role in physiologic and pharmacological mechanisms, e.g. lipid metabolism and PPARα-dependent gene expression.

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