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Inflammation Research

The anti-inflammatory and anti-cancer properties of epigallocatechin-3-gallate are mediated by folate cycle disruption, adenosine release and NF-κB suppression

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Abstract. *Objective:* To understand the mechanism by which (-)-epigallocatechin-3-gallate (EGCG), the major polyphenol of green tea, exerts its anti-inflammatory action.
Methods: To check our hypothesis that the anti-inflammatory properties of EGCG could be related to its antifolate action and whether adenosine and its receptors are involved in EGCG action, we investigated the EGCG-induced suppression of NF-κB in Caco-2 cell monolayer, which acted as a model of the human intestinal epithelium.
Results: We demonstrate that the anti-inflammatory properties of EGCG are associated with its antifolate activity. By using a natural stable folate we were able to reverse the EGCG suppression of TNF-α-induced NF-κB activation, the phosphorylation and degradation of IκBα and the phosphorylation of Akt in this human colon carcinoma cell line. These suppressions were mediated by the release of adenosine following disruption of the folate cycle by EGCG. By binding to its specific receptors, adenosine can modulate the Akt and NF-κB pathway. Moreover, EGCG produces a significant increase in a specific adenosine receptor, which could explain the suppression of the constitutive activation of NF-κB in colon cancer cells.
Conclusions: The data suggest that by modulating NF-κB activation, EGCG might not only combat inflammation, but also cancer.

Key words: Green tea – Epigallocatechin-3-gallate – Folic acid – Adenosine – NF-κB pathway

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Abbreviations: AICAR, aminoimidazole carboxamide ribonucleotide; APCP, α,β-methylene adenosine-5'-diphosphate; DHFR, dihydrofolate reductasc; DMPX, 3,7-dimethyl-1-propargylxanthine; EGCG, (-)-epigallocatechin-3-gallate; EMSA, electrophoretic mobility shift assay; FAICAR, formaminoimidazole carboxamide ribonucleotide; FCS, fetal calf serum; FGAR, formylglycinamide ribonucleotide; GAR, glycinamide ribonucleotide; Leucovorin, 5-formyltetrahydrofolate; MTX, methotrexate; NF-κB, nuclear factor-kappa B; THF, tetrahydrofolate; TMP, trimethoprim; TNF-α, tumor necrosis factor-α.

Introduction

The therapeutic uses of tea are confined to alternative medicine. Although the anticarcinogenic, anti-inflammatory and antimicrobial properties of tea have been known for many years, clinical medicine has not included its use in treatments, almost certainly due to the lack of knowledge about its exact mechanisms of action. Recent investigations suggest that green tea and several of its components, including EGCG, might interfere with the cell folic acid metabolism by inhibiting DHFR [1, 2] and/or decreasing the cellular uptake of this vitamin [3]. These results suggest that EGCG could act as an antifolate compound in the same way as MTX. Additional evidence to support this observation can be obtained by examination of the related bibliography. Surprisingly, EGCG has not only found similar applications as antifolate compounds in the treatment of cancer, microbial and fungal infections, Crohn's disease, psoriasis or chronic inflammatory diseases such as rheumatoid arthritis and multiple sclerosis [4, 5] but also shows similar cellular and molecular effects against tumor development and progression to MTX [6].

Inhibitors of the folic acid metabolism, also called antifolates, have provided several important agents for use in cancer chemotherapy and as antibiotics because they inhibit the biosynthesis of nucleic acid precursors [7]. Among the antifolates, MTX, together with the antibacterial drug TMP, is the most widely used DHFR inhibitor in clinical practice. The most common use of MTX is as an anticancer drug, although the drug is also considered to have anti-inflammatory and immunosuppressive properties with accompanying activity against autoimmune disorders [8]. Inflammation is central to our fight against pathogens, but if it is not ordered and timely the resulting chronic inflammation may contribute to diseases such as arthritis, heart attacks and Alzheimer's disease. A functional link between chronic inflammation and cancer has long been suspected [9, 10]. This link is of great interest in the context of this study because green tea has shown remarkable anti-inflammatory activity [4]. Understanding the mechanisms by which EGCG imparts this effect could be of importance for explaining the epidemiological data on the prophylactic effects of diets high in gallate polyphenols for certain forms of cancer. Most solid tumors contain many non-malignant cells, including immune cells and blood-vessel cells, which are important in inflammation, although the crucial molecular pathways that permit communication between abnormally growing cancer cells and these inflammatory cells remain unknown. A mouse model of inflammation-associated cancer now points to the involvement of the gene transcription factor NF-κB and the inflammatory mediator known as TNF-α in cancer progression [11]. Several of the anti-inflammatory effects of MTX and other antifolates can be explained by the suppression of NF-κB activation, a multisubunit factor known to play a role in inflammation, immune modulation and cell proliferation. NF-κB is primarily composed of proteins with molecular masses of 50 kDa (p50) and 65 kDa (p65) and is retained in the cytoplasm by an inhibitory subunit, IκBα. NF-κB is activated by a wide variety of inflammatory stimuli, including TNF-α, which induces the phosphorylation-dependent degradation of IκBα, allowing active NF-κB to translocate to the nucleus and regulate gene expression.

Although the mechanism by which antifolates modulate NF-κB activation has remained unclear for some time, recent investigations have demonstrated that MTX could inhibit the TNF-α-induced NF-κB activation though the release of adenosine [8, 12]. By lowering THF cofactors, MTX inhibits two steps of the purine synthesis pathway: the conversion of GAR to FGAR and the conversion of AICAR to FAICAR. Excess AICAR inhibits the conversion of AMP to IMP by AMP deaminase, while AMP is rapidly converted to adenosine by surface expressed ecto-5' nucleotidase. Adenosine is a potent endogenous regulator of a variety of physiological processes through specific receptors on the cell surface and binds to four different types of G protein-coupled cell surface molecules, termed the A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors [13]. After binding to the cell surface receptors, adenosine alters the immune cell production of soluble mediators such as cytokines, free radicals, and arachidonic acid metabolites [12]. The A_3 adenosine receptor has been found to be highly expressed in inflammatory tissues and peripheral blood mononuclear cells of rats with adjuvant-induced arthritis [14]. To check our hypothesis that the anti-inflammatory properties of EGCG could be related to its antifolate action and whether adenosine and its receptors are involved in EGCG action, we investigated the EGCG-induced suppression of NF-κB in Caco-2 cell monolayer, which acted as a model of the human intestinal epithelium. Colon cancer cells are highly sensitive to antifolate compounds [15] and the results obtained in this model could be of importance for understanding the epidemiological data that correlate the ingestion of green tea with the low risk of suffering gastrointestinal cancer [16].

Materials and methods

Cell culture and treatments

Caco-2 cells were purchased from the American type culture collection (Rockville, USA). Cells were cultured in EMEM (Gibco, Barcelona, Spain) supplemented with 10 % FCS (Gibco), 2 mM L-glutamine (Gibco), 100 µg/ml of penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 1 mM pyruvate and a non-essential aminoacids solution (Gibco) at 37 ºC in a humidified atmosphere of 95 % air-5 % CO_2. Caco-2 cells were plated at a density of 1×10^6 cells per 75 cm^2 flask and treated when they had reached 70 % confluence. Co-treatments of Caco-2 cells with EGCG (Sigma Chemical Co., Madrid, Spain) and other reagents were carried out by supplementing medium cultures with leucovorin (100 µM), APCP (50 µM) or DMPX (50 µM) (all obtained from Sigma). For TNF-α experiments, the cells were exposed to different treatments for the specified time and then 20 ng/ml TNF-α (Sigma) was added for 5 min.

Cell viability

Cell injury was evaluated by a colorimetric assay for mitochondrial function using the MTT test. For this, cells were plated in a 96-well plate at a density of 1000 cells/well and grown until reaching 50–60 % confluence.

NF-κB assays

To study the effect of EGCG on the activation of NF-κB in Caco-2 cells, nuclear fractions were prepared as described elsewhere [17]. EMSAs were performed with a gel shift assay system kit obtained from Promega (Madison, WI, USA), according to the manufacturer's protocol. The NF-κB band was assigned using appropriate negative and positive (Hela nuclear extract) controls, and supershift assays. For the supershift assays, nuclear extracts were incubated with the antibodies against p65 of NF-κB for 30 min at room temperature before the complex was analyzed by EMSA.

Western blot analysis

To study the effect of EGCG on adenosine and NF-κB related proteins, cytosolic fractions were obtained as described elsewhere [17]. For immunoblot analysis, 25–50 µg of protein were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated for 1 hour in blocking solution (Tris-buffered saline containing 1 % Tween 20 and 5 % non-fat dry milk) and further incubated overnight at 4 ºC with appropriate primary antibodies. IκBα was detected with primary IκBα antibodies (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA). Total and phosphorylated Akt were detected with primary Akt (1:500) and p-Akt (Ser473) (1:500) (Santa Cruz Biotechnology). The A_1AR (1:500), A_{2A}AR (1:500), A_{2B}AR (1:500) and A_3AR (1:500) antibodies were purchased from Santa Cruz Biotechnology. The membranes were then washed with blocking solution and incu-

bated for two hours with anti-mouse, anti-rabbit or anti-goat secondary antibodies conjugated with horseradish peroxidase. Bound antibodies were detected by chemiluminiscence using an ECL Plus detection kit (Amersham Life Science, Inc., Uppsala, Sweden).

Confocal imaging

Caco-2 cells were cultured over 35 mm glass bottom microwell dishes to 50% confluence and treated with vehicle, EGCG or EGCG plus leucovorin. After washing in PBS, the cells were incubated for 1h with PBS containing primary adenosine receptor antibodies (1:25 for each; Santa Cruz Biotechnology) followed by incubation (1 h) with PBS containing secondary antibodies (Alexa Fluor Dyes, Invitrogen, Barcelona, Spain). Cells were analyzed by confocal laser scanning microscopy (Leica TCS 4D; Leica Microsystems GmbH, Wetzlar, Germany) at 750-fold magnification.

Results

EGCG suppression of TNF-α induced activation of NF-κB in colon cancer cells is reversed by leucovorin

NF-κB activation is suppressed after the treatment of cells with EGCG and TNF-α [17]. To check whether this effect is related with the cellular depletion of folic acid, Caco-2 cells were grown in the presence of 20 μM EGCG alone or in combination with leucovorin for 24 h and then induced with TNF-α. Then nuclear extracts were prepared and assayed for NF-κB by EMSA. TNF-α produced a 9-fold activation of NF-κB (Figure 1A). As shown in this figure, EGCG inhibited TNF-α-mediated NF-κB activation, but the effect was reversed by co-treatment with leucovorin. Control experiments showed that leucovorin alone had not any detectable effect on NF-κB activation both in the absence or the presence of TNF-α (data not shown). Leucovorin, also known as 5-formyl-THF, is the most stable natural folate and it is used together with MTX to rescue healthy cells from the toxic effects of MTX. Leucovorin restores the cells' reduced folate pools and, therefore, is an efficient agent to reverse antifolate effects in the cell.

EGCG inhibits TNF-α-dependent phosphorylation and the degradation of IκBα, while both are reversed by leucovorin

The translocation of NF-κB to the nucleus is preceded by the phosphorylation, ubiquitination and proteolytic degradation of IκBα [18]. To determine whether inhibition of TNF-α-induced NF-κB activation was due to reduced IκBα degradation, we pretreated cells with EGCG for 24 h, before exposing them to 20 ng/ml TNF-α for 5 min. The content of IκBα in the cytosolic fraction of treated cells was checked by Western blot analysis (Figure 1B). EGCG was seen to inhibit IκBα degradation in a dose-dependent manner, with maximum inhibition occurring at 20 μM. The effect of EGCG was mitigated by co-treating cells with leucovorin (Figure 1B).

EGCG inhibits TNF-α-dependent phosphorylation of Akt

We next investigated the pathway that EGCG uses to suppress NF-κB activation in the presence of TNF-α by studying the levels of Akt activation in treated cells. It has been proposed that TNF-α activates the PI3K/PDK-1/Akt signalling pathway [19]. This pathway culminates in the phosphorylation of IKK by Akt, which is necessary for IκBα degradation and NF-κB activation. Figure 2 shows that EGCG was able to inhibit Akt phosphorylation, while co-treatment of cells with EGCG and leucovorin restored cell p-Akt levels.

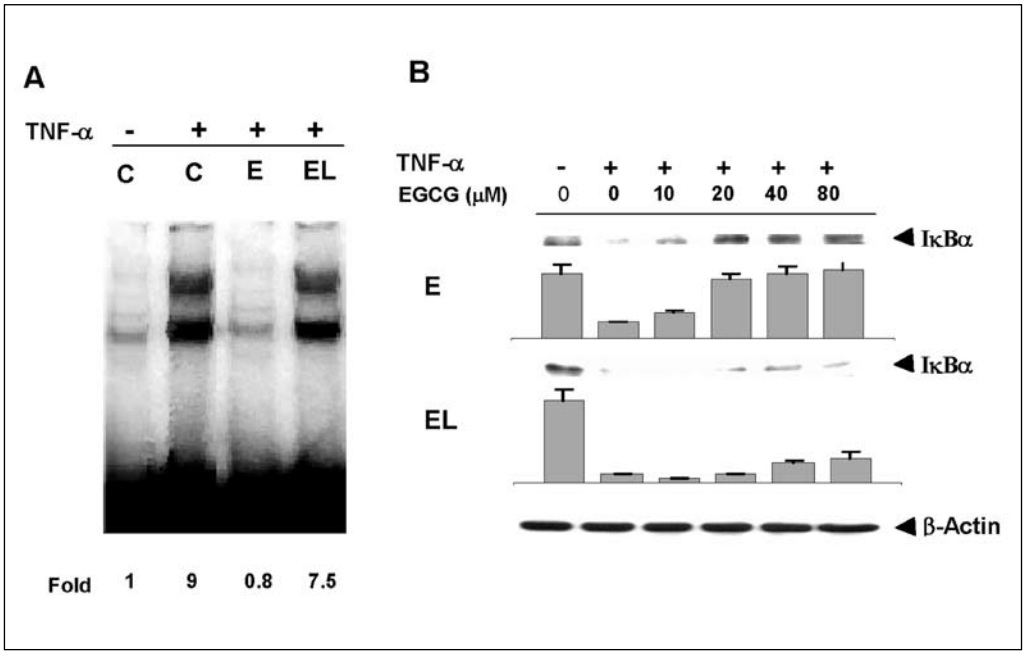

Fig. 1. (A) Effect of TNF-α alone (control) or combined with EGCG (E; 20 μM) in the absence or the presence of leucovorin (EL; 100 μM) on NF-κB activation assayed by EMSA. The data shown here are from a representative experiment repeated five times with similar results and the induction fold was calculated with respect to an untreated control without TNF-α. (B) Effect of EGCG on TNF-α-mediated degradation of IκBα. Caco-2 cells were treated with vehicle only or the specified concentration of EGCG for 24 h. When specified, cells were treated with TNF-α. Treatments included EGCG alone, E, or combined with 100 μM leucovorin, EL. Band intensity was determined with densitometry and the values presented are the means of three independent experiments; bars, ± SD.

EGCG suppression of TNF-α-induced activation of NF-κB in colon cancer cells is mediated by adenosine

Although the mechanism by which antifolates modulate NF-κB activation has not been totally elucidated, recent investigations have suggested that MTX could inhibit TNF-α-induced NF-κB activation though the release of adenosine [8, 12]. Whether EGCG inhibits NF-κB activation through the release of adenosine by disturbing folate metabolism was investigated in our laboratory using two independent approaches. The first approach involved suppression of TNF-α mediated NF-κB activation by treatment of cells with adenosine and the second involved the use of inhibitors to block the production or action of adenosine. To determine whether adenosine blocks TNF-α-mediated NF-κB activation, Caco-2 cells were pretreated with 10 μM adenosine and then examined for NF-κB activation by EMSA (Figure 3A). Our results indicate that preincubation of Caco-2 cells with adenosine for 24 h inhibited TNF-α-mediated NF-κB activation.

Adenosine is produced in the cell from adenosine monophosphate in a reaction catalyzed by the enzyme ecto-5' nucleotidase. This enzyme is inhibited in a competitive manner by APCP. Figure 3B shows that APCP reversed the EGCG-induced suppression of NF-κB activation by activating IκBα degradation. This indicated that adenosine plays an important role in the EGCG-mediated inhibition of NF-κB activation by TNF-α. To further understand the role of adenosine in the EGCG-mediated suppression of NF-κB activation, we examined the effect of DMPX, an antagonist of the A_2AR. As shown in Figure 3B, DMPX reversed the EGCG-mediated suppression of NF-κB activation in the presence of TNF-α. These results also suggest that the inhibitory effect of EGCG on NF-κB activation is mediated though adenosine and that the A_{2A}AR signaling pathway is the predominant event in the presence of TNF-α.

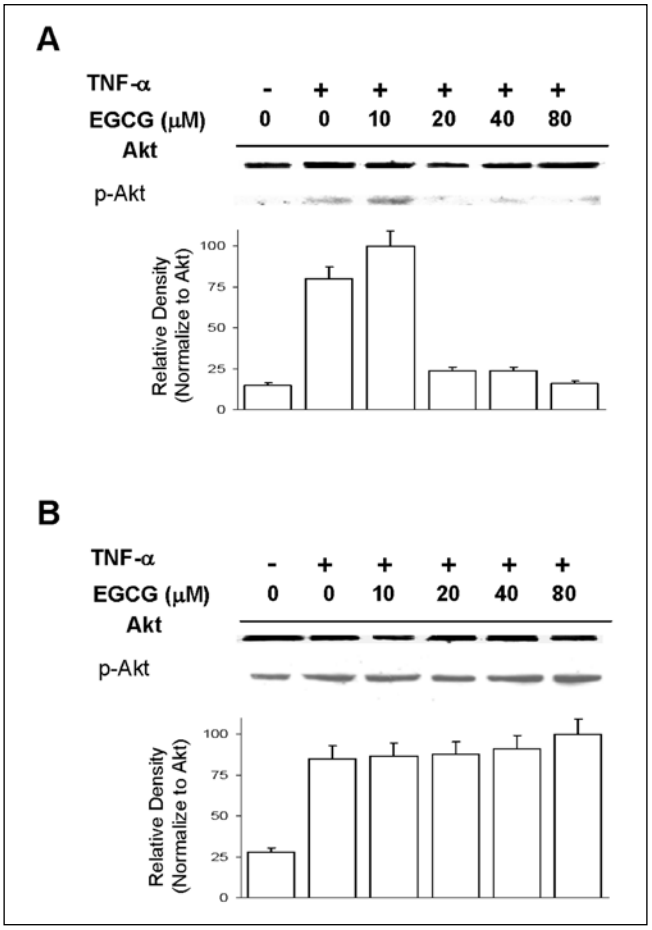

Fig. 2. (A) Akt and p-Akt levels in the cytoplasm of Caco-2 treated for 24 h with different concentrations of EGCG, and then with or not TNF-α. (B) Effect of leucovorin (100 μM) on Akt and p-Akt levels in the cytoplasm of Caco-2 treated for 24 h with different concentrations of EGCG, and then with or not TNF-α. The data shown here are from a representative experiment repeated three times with similar results. The columns represent the means of the experiments; bars, ± SD.

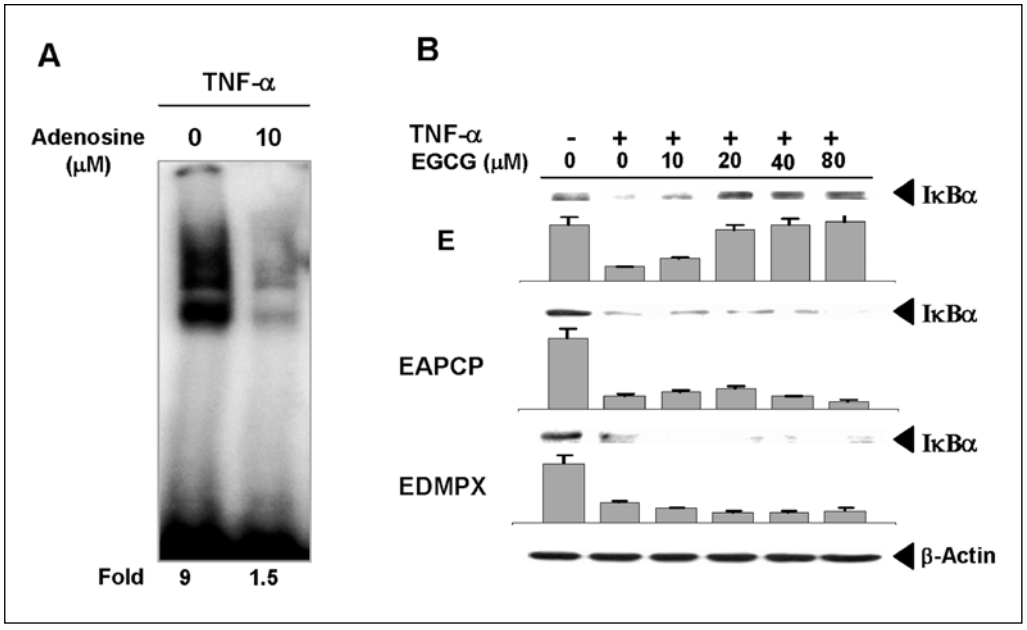

Fig. 3. (A) Effect of adenosine on TNF-α-mediated activation of NF-κB assayed by EMSA. The data shown here are from a representative experiment repeated five times with similar results and the induction fold was calculated with respect to an untreated control without TNF-α. (B) Effect of APCP and DMPX on the suppression of NF-κB induced by EGCG in the presence of TNF-α. Caco-2 cells were treated with vehicle only or the specified concentration of EGCG for 24 h. When specified, cells were treated with TNF-α. Treatments included EGCG alone, E, combined with 50 μM APCP, EAPCP or 50 μM DMPX, EDMPX. Band intensity was determined with densitometry and the values presented are the means of three independent experiments; bars, ± SD.

EGCG modulates the constitutive activation of NF-κB in colon cancer cells through adenosine receptors in a time-dependent manner

To further understand the role of adenosine in this process we investigated the effect of EGCG on the constitutive activation of NF-κB in Caco-2 cells since it has been shown that NF-κB is constitutively activated in human colorectal carcinoma tissue [20]. Here we show that EGCG differentially modulates NF-κB in a time-dependent manner. After treatment of Caco-2 cells with 20 μM EGCG for one day, NF-κB was highly activated compared with an untreated control (Figure 4A). This activation was effectively reversed by co-treatment with leucovorin (Figure 4A). After one day adenosine alone or in combination with EGCG showed a similar effect, enhancing NF-κB activation (data not shown). It is well-known that by binding to its A_1AR, adenosine activates NF-κB through a pathway that involves the decrease of cAMP, the liberation of calcium from endoplasmic reticulum and activation of the PKC pathway [21]. Although adenosine had the same effect after longer treatments (three days), the lack of NF-κB activation by EGCG (Figure 4B) cannot be explained by the participation of this adenosine receptor.

To understand the involvement of different types of adenosine receptors in this process, their levels in the plasmatic membranes of Caco-2 cells and their response to EGCG treatment were analyzed by both Western blot analysis and confocal microscopy. EGCG differentially modulated the expression of adenosine subtype receptors. While A_1AR and A_2AR expression was not significantly increased after 3 days' treatment with 20 μM EGCG, A_3AR did show a significant increase after this time (Figure 4C). A comparison of the expression of A_{2A}AR and A_3AR using confocal microscopy is presented in Figure 4C. The data clearly indicate that the A_3AR signaling pathway was the predominant event in the more prolonged treatments with EGCG, when its action was able to suppress the constitutive activation of NF-κB in colon cancer cells.

Discussion

Antifolates, which have been used for decades as anticancer agents, play a major therapeutic role in non-neoplastic diseases, acting as anti-inflammatory and immunosuppressive drugs [22]. Currently, MTX is commonly used to treat rheumatoid arthritis, psoriasis, primary biliary cirrhosis, Crohn's disease and intrinsic asthma [12]. The mechanism by which MTX exerts its anti-inflammatory action was unknown for many years, although several of its effects were explained through the suppression of activation of NF-κB. Recently, it has been clearly demonstrated that MTX suppresses NF-κB activation through the release of adenosine, which may contribute to the anti-inflammatory, immunomodulatory and antiproliferative effects of MTX [12]. In a recent study, we

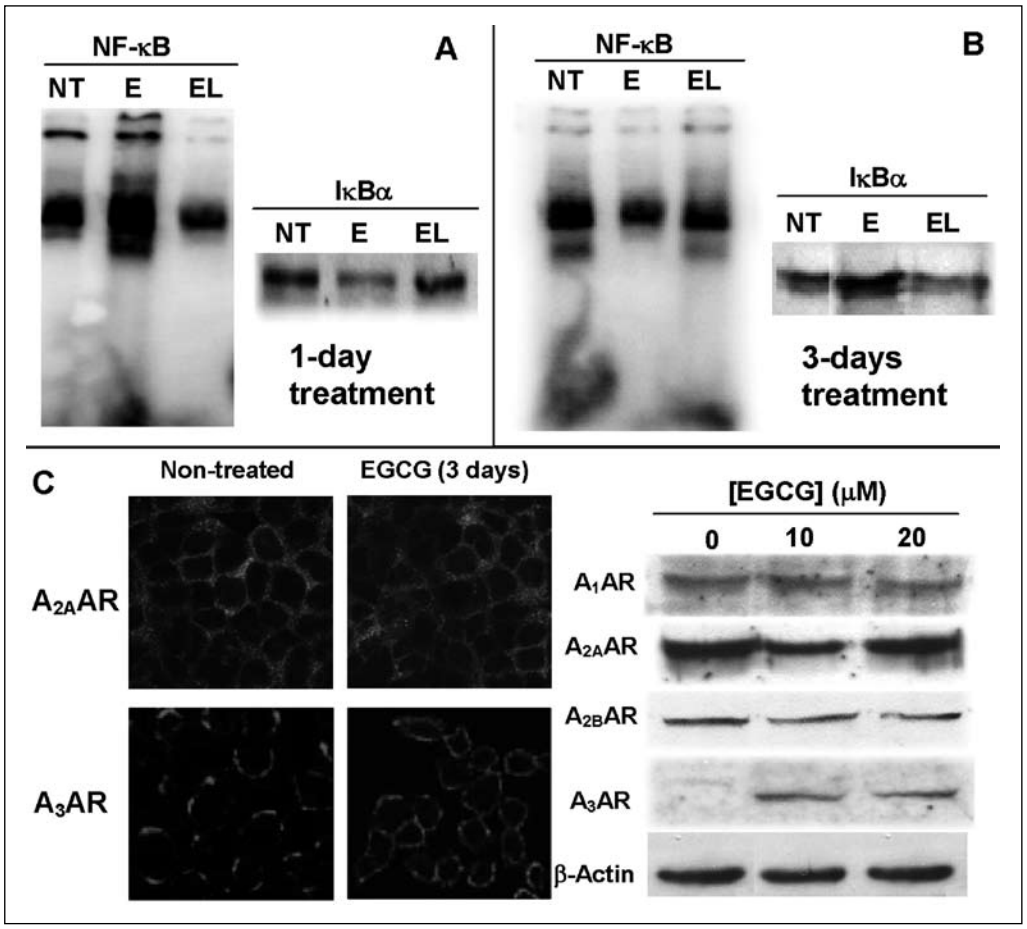

Fig. 4. Nuclear NF-κB and cytosolic IκBα level in Caco-2 cells subject to one day (A) or three days (B) of treatment with EGCG (20 μM), alone or combined with leucovorin (L; 100 μM). The data shown here are from a representative experiment repeated three times with similar results. (C) Florescence confocal microscopy of Caco-2 after 3 days' treatment with vehicle only or 20 μM EGCG and revealed with A_{2A}AR and A_3AR antibodies. Adenosine receptor levels assayed by Western blot analysis. The data shown here are from a representative experiment repeated three times with similar results.

demonstrated that EGCG was an efficient inhibitor of DHFR [1], and encouraged by numerous studies that demonstrated that EGCG showed remarkable anti-inflammatory effects in many animal tumor and cell culture systems [4, 5, 17], we decided to investigate the mechanism by which EGCG suppressed TNF-α-induced NF-κB activation. The data indicate that EGCG action could also be mediated by adenosine. Our results demonstrate that adenosine blocks TNF-α-stimulated NF-κB activation in Caco-2 cells and that the inhibitor of 5'-ectonucleotidase, APCP, completely reverses the effect of EGCG on TNF-α-induced NF-κB activation. In addition, the A_{2A}AR antagonist, DMPX, reversed the EGCG-mediated suppression of TNF-α-induced NF-κB activation, indicating the involvement of this adenosine receptor in this process. Although adenosine, at low concentrations, binds preferentially to the high affinity A_1AR, it seems that in the conditions mentioned its signaling pathway is switched off [8]. The activation of A_{2A}AR produces a constellation of effects that can attenuate inflammation. An increase in intracellular cAMP would have an inhibitory effect on the PI3K/PDK1/Akt signaling pathway by blocking the coupling between Akt and its upstream regulator, PDK1, in the plasma membrane [19]. Inactivation of the PI3K/PDK1/Akt signaling pathway would affect multiple components of the apoptotic cascade such as caspases, GSK-3β, ceramide, BAD/Bcl-2, CREB, and NF-κB [23]. Thus, EGCG may induce apoptosis by regulating multiple molecules in the Akt and NF-κB pathway. The suppression of TNF-α-induced NF-κB-mediated gene transcription may also downregulate several genes involved in inflammation, angiogenesis and metastasis, including COX-2, iNOS, MMP-9, cell surface adhesion molecules (e. g. ICAM-1, E-selectin, and VCAM-1), urokinase-type plasminogen activator, TNF-α, IL-1, IL-2, IL-6, and GM-CSF. Although we can not discard the possibility that the effects described in this study may correspond to a Caco-2 specific response, the downregulation of genes mediated by Akt and NF-κB pathways has been described in several cell models treated with EGCG or MTX [24–30].

As mentioned in the introductory section, a functional link between chronic inflammation and cancer is suspected and, therefore, it is easy to speculate that the use of anti-inflammatory drugs could represent a strategy to prevent cancer formation. Although MTX is widely used for the treatment of inflammatory and autoimmune diseases, its use as a chemopreventive agent is precluded, even at low doses, due to its adverse side effects. However, there is no evidence for such side effects as a result of the regular consumption of tea. The finding that EGCG shares mechanisms of action with MTX could be of interest, and suggests that the regulation of chronic inflammation by EGCG could represents a strong possibility to explain the epidemiological data concerning the prophylactic effects of diets high in gallate polyphenols for certain forms of cancer [6]. In addition, the data presented here indicate that EGCG may well be beneficial, not only in the prevention but also in the treatment of cancer. EGCG was able to modulate the constitutive activation of NF-κB in colon cancer cells by disturbing its folate metabolism, which could be of importance for cancer treatment. Constitutively activated NF-κB is common in a wide variety of tumors. Thus, several investigators have reported the constitutive activation of NF-κB in various types of human tumor cell line, including those of lymphoid origin, such as T-cell lymphoma Hut 78 cells, and multiple myeloma cells [20]. Our results demonstrate that adenosine is required for EGCG to affect NF-κB activation/deactivation. However, adenosine alone could not suppress the constitutive activation of NF-κB in the absence of TNF-α; in fact, adenosine produced the activation of NF-κB by binding to A_1AR. This explains the activation of NF-κB by EGCG at short treatment times (Figure 4A). At longer times the induction of A_3AR in EGCG-treated cells permits the binding of adenosine to A_3AR and the inhibition of NF-κB activation through a pathway that involves a decrease in cAMP, the inhibition of PKA and downregulation of the PKB/Akt arm [31, 32]. PKA and PKB/Akt utilize GSK-3β as a substrate and, upon phosphorylation, its activity is inhibited. GSK-3β has been widely implicated in cell homeostasis, for its ability to phosphorylate a broad range of substrates, including β-catenin, a key component of the Wnt pathway. In normal cells, GSK-3β phosphorylates β-catenin, thereby inducing its ubiquitination and degradation by the proteosome system. However, in tumor cells, GSK-3β fails to phosphorylate β-catenin, leading to its accumulation in the cytoplasm. It then translocates to the nucleus, where it acts in concert with LEF-1 to induce the transcription of the cell cycle progression genes, such as cyclin D1 and c-Myc [31]. Downregulation of these two oncogenes by EGCG has been widely described [33, 34]. Activation of A_3AR by EGCG treatment would suppress the activation of cyclin D1 and c-Myc, leading to cell cycle arrest and the induction of apoptosis [31]. Moreover, the PKA signaling cascade is also connected with several pathways that have been shown to be affected by EGCG, such as the MAPKs, including extracellular regulated kinases 1/2 (ERK1/2) or p38 [35]. By modulating the PKA cascade, EGCG may interfere with the pathways involved in glucose utilization and lipolysis [36], which would justify its traditional inclusion in weight loss diets.

In conclusion, we demonstrate that EGCG, by inhibiting DHFR and/or folic acid uptake [3], can disturb the metabolism of this vitamin in Caco-2 cells, producing the release of adenosine and the suppression of NF-κB. The data suggest that by modulating NF-κB activation, EGCG might not only combat inflammation, but also cancer. Since by reducing chronic inflammation there is a strong possibility of modulating tumorogenesis, these results could be of importance for explaining the prophylactic effects of diets high in gallate polyphenols for certain forms of cancer.

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