Free Radical Research, October 2006; 40(10): 1086–1094

informa
healthcare

Modulation of transcription factor NF-κB in Hodgkin's lymphoma cell lines: Effect of (−)-epicatechin

GERARDO G. MACKENZIE[1] & PATRICIA I. OTEIZA[1,2]

[1]*Departments of Nutrition, and Environmental Toxicology, University of California Davis, Davis, CA 95616, USA, and* [2]*Department of Biological Chemistry, IQUIFIB (UBA-CONICET), School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, Buenos Aires C1113AAD, Argentina*

Accepted by Professor J. Vina

(Received 27 February 2006; accepted 26 April 2006)

Abstract
Transcription factor NF-κB plays a central role in tumorogenesis and in different types of cancer, including Hodgkin's lymphoma. Previously, we described that (−)-epicatechin (EC) inhibits PMA-induced NF-κB activation in Jurkat T cells. Therefore, we investigated the capacity of EC to inhibit NF-κB activation, the underlying mechanisms and the effects of EC on cell viability in Hodgkin's lymphoma cells. EC inhibited NF-κB–DNA binding activity in L-428 and KM-H2 cells. This inhibition was not associated with EC antioxidant activity, with changes in p65 phosphorylation or NF-κB nuclear translocation. Results suggest that EC acted inhibiting the binding of NF-κB to DNA. The combined treatment with EC and an inhibitor of NF-κB nuclear translocation (SN-50) caused an additive inhibitory effect on NF-κB activation. The partial cell viability decrease, under conditions that EC and SN-50 completely prevented NF-κB–DNA binding, indicates that the inhibition of other signaling pathways should be also targeted in the treatment of Hodgkin's lymphoma.

Keywords: *NF-κB, Hodgkin's lymphoma, (−)-epicatechin, SN-50, flavonoids*

Abbreviations: *NF-κB, nuclear factor-κB; HD, Hodgkin's disease; H–RS cells, Hodgkin and multi nucleated Reed-Sternberg cells; TNF-α, tumor necrosis factor-α; EBV, Epstein-Barr virus; PMA, phorbol 12-myristate 13-acetate; EC, (−)-epicatechin; RANTES, regulated on activation normal T cell expressed and secreted; LA, (±)-α lipoic acid; CAT, catalase; FBS, fetal bovine serum; EDTA, ethylenediamine tetraacetate; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; DCDHF, 5(or 6)-carboxy-2′7′-dichlorodihydrofluorescein diacetate; DCF, 2′7′-dichlorofluorescein; EMSA, electrophoretic mobility shift assay; EGCG, (−)-epigallocatechin gallate; EBV, Epstein-Barr virus; LMP-1, latent membrane protein-1; TRAF, tumor necrosis factor receptor-associated factor; NIK, NF-κB inducing kinase; IKK, IκB kinase; NF-IL-6, nuclear factor of IL-6; IRF-3, interferon regulatory factor-3; RFLAT-1, RANTES factor of late activated T lymphocytes-1*

Introduction

The activation of nuclear factor-κB (NF-κB) has been described in solid tumors (breast, gastric and colonic cancers) as well as in leukemia and lymphoma (reviewed in [1]), including Hodgkin's disease (HD) [2,3]. Due to the fact that the deregulation of NF-κB occurs in different types of cancer, the research interest in this pathway has intensified in search for molecules that could inhibit NF-κB, as a therapeutic strategy against cancer. Up to date, known members of the Rel/NF-κB family of proteins in eukaryotic cells include c-Rel, RelB, p65 (RelA), p50/p105 and p52/p100. In the classical pathway, the activity of the Rel/NF-κB homo- and hetero-dimers (mainly p50–p65 heterodimer) is regulated by their interaction with inhibitory IκB proteins which anchor the transcription factor to the cytosol [4]. In general, the phosphorylation of two serines present in IκB mediates the activation of NF-κB by a specific IκB kinase complex [5]. After IκB phosphorylation,

Correspondence: P. I. Oteiza, Department of Nutrition, University of California, Davis One Shields Avenue, Davis, CA 95616, USA. Tel: 1 530 754 6074. Fax: 1 530 752 8966. E-mail: poteiza@ucdavis.edu

ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Informa UK Ltd.
DOI: 10.1080/10715760600788396

ubiquitination at two lysine residues targets the protein for its subsequent degradation by the proteasome leading to the complete activation of NF-κB [6]. In the nucleus, NF-κB binds to its consensus sequence and modulates the transcription of numerous genes involved in inflammation, cell survival, proliferation, tumor promotion and angiogenesis [7–11]. NF-κB has been proposed to link inflammation with tumor promotion in part through the upregulation of tumor necrosis factor-α (TNF-α) [12]. Based on the role of NF-κB in cancer and inflammatory disorders, the field of the development of new therapeutic agents that could inhibit the NF-κB activation cascade has gained particular interest. The numerous and diverse natural or chemically-designed inhibitors described so far, include: antioxidants, proteasome and proteases inhibitors, IκBα phosphorylation and/or degradation inhibitors and other agents with still not completely understood mechanisms of action [13].

HD is a lymphoma characterized by the presence of mononuclear Hodgkin (H) and multi-nucleated Reed-Sternberg (RS) cells. HD has been recently defined [14], as being determined by malignant H–RS cells with self-growth-promoting potential in a hyperplastic environment constituted by normal reactive lymphocytes, neutrophils, histiocytes, plasma cells, eosinophils and stromal cells, and present a significant and constitutive activation of NF-κB. H–RS cells are characterized by a particular phenotype and by the expression of a particular set of lymphoid activation markers that lead to the histopathological presentation and clinical characteristics of HD. Several cytokines as well as proteins involved in cell proliferation and resistance to apoptosis produced by H–RS cells are regulated by NF-κB. The constitutive activation is a common characteristic of H–RS cells in culture as well as in cells isolated from Hodgkin's patients. However, the underlying mechanisms are heterogeneous (Figure 1) including: a, a constitutive high activity of IκB kinase leading to an increased IκBα degradation [15]; b, mutations in the IκBα [16–18] and *IκBε* genes [19], c, amplifications of the *c-Rel* gene locus [20], d, overexpression of CD30 leading to a CD-30-ligand-independent NF-κB activation [21] and, e, in Epstein-Barr virus (EBV)-positive cases, NF-κB activation generally occurs secondary to the expression of the EBV-encoded latent membrane protein-1 (LMP-1) which activates NF-κB [22].

Several epidemiological studies suggest a role for different flavonoids in the prevention of cancer (reviewed in [23]). Despite the inverse relationship between flavonoid intake and cancer risk, the underlying mechanisms are not completely elucidated. Select flavan-3-ols and procyanidins inhibit cell proliferation and induce apoptosis in cancer cells [24–27]. These effects could be, in part, mediated by their capacity to modulate transcription factor NF-κB [26,28–31].

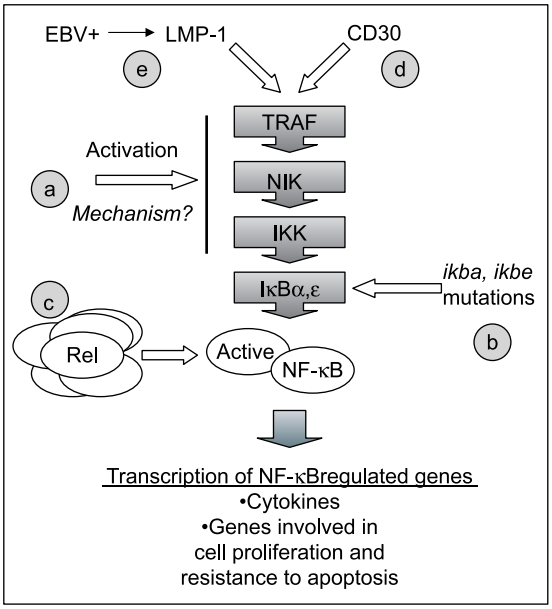

Figure 1. Molecular alterations leading to NF-κB constitutive activation in H–RS cells. The current known mechanisms (a–e) are described in the text. Abbreviations: EBV, Epstein-Barr virus; LMP-1, latent membrane protein-1; TRAF, tumor necrosis factor receptor-associated factor; NIK, NF-κB inducing kinase; IKK, IκB kinase.

Given that NF-κB constitutive activation is a consistent finding in Hodgkin's lymphoma cells and that EC has the ability to inhibit phorbol 12-myristate 13-acetate (PMA)-induced NF-κB activation in Jurkat T cells [32], in the present work, we studied the capacity of (−)-epicatechin EC to inhibit NF-κB in H–RS cells, the underlying mechanisms and the functional consequences. The possible combined action of EC with SN-50, an agent that affects the nuclear transport of the active NF-κB, was investigated.

Materials and methods

Materials

KM-H2 and L-428 cells were obtained from DSMZ (Braunschweig, Germany). Cell culture media and reagents were obtained from Invitrogen Life Technologies (Carlbad, CA, USA). The CellTiter-Glo Luminescent Cell Viability Assay, the oligonucleotides containing the consensus sequence for NF-κB, SP-1, and the reagents for the EMSA assay were from Promega (Madison, WI, USA). TNF-α ELISA kit was obtained from BD Biosciences (San Diego, CA, USA). Regulated on activation normal T cell expressed and secreted (RANTES) ELISA kit was obtained from Biosource (Camarillo, CA, USA). The protease inhibitor cocktail was obtained from Roche Applied Science (Mannheim, Germany). The NF-κB nuclear translocation inhibitor (SN-50), its mutated control (SN-50M), and the antibodies for p65 and p50 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for p-p65 (S276)

was purchased from Rockland (Gilbertsville, PA, USA). PVDF membranes were obtained from BIO-RAD (Hercules, CA, USA) and Chroma Spin-10 columns were obtained from Clontech (Palo Alto, CA, USA). The ECL Western blotting system was from GE Healthcare (formerly Amersham Pharmacia Biotech Inc.) (Piscataway, NJ, USA). Propidium iodide (PI) and 5(or 6)-carboxy-2′7′-dichlorodihydro-fluorescein diacetate (DCDHF) probes were obtained from Molecular Probes (Eugene, OR, USA). EC, (+)-α-lipoic acid (LA), catalase and all other reagents were from the highest quality available and were purchased from Sigma (St Louis, MO, USA).

Cell culture and incubations

KM-H2 and L-428 cells were cultured in RPMI 1640 media supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (10 U/ml penicillin and 10 μg/ml streptomycin). Cells were incubated in the absence or the presence of (5–50 μM) EC and/or 20 μM SN-50 or SN-50M, and/or catalase (100 U/ml) or with 0.5 mM LA for variable periods of time (2–48 h). Cell viability, after 24 and 48 h incubation under the different treatments, was measured evaluating the cell ATP content (CellTiter-Glo Luminiscent Cell Viability assay), following the manufacturer's protocol.

Determination of cell oxidants

Cell oxidants were evaluated as previously described [32] using the probe DCDHF. This probe can cross the membrane, and after oxidation, is converted into a fluorescent compound. Cells (1.5×10^6) were incubated in the absence or presence of EC, LA and/or SN-50 for 24 h. Subsequently, cells were centrifuged at 800*g* for 10 min, rinsed with warm PBS and suspended in 200 μl of RPMI 1640 medium containing 10 μM DCDHF. After 30 min of incubation at 37°C, the media were removed, cells rinsed with PBS, and then incubated in 200 μl of PBS containing 0.1% (v/v) Igepal. After a brief sonication and 30 min incubation with regular shaking, the fluorescence at 525 nm (λ_{exc}: 475 nm) was measured. To evaluate DNA content, samples were added with 50 μM PI. After incubating for 20 min at room temperature, the fluorescence (λ_{exc}: 538, λ_{em}: 590) was measured. Results are expressed as the ratio 2′,7′-dichlorofluorescein (DCF) fluorescence/PI fluorescence.

Electrophoretic mobility shift assay (EMSA)

Nuclear fractions were isolated as previously described in Ref. [32]. After the corresponding treatments, cells (6×10^6 cells) were collected by centrifugation at 800*g* for 10 min. Cells were resuspended in 150 μl of buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% (v/v) Igepal), incubated for 10 min at 4°C, and centrifuged for 1 min at 12,000*g*. The supernatant fraction was discarded and the nuclear pellets resuspended in 60 μl of buffer B (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM PMSF). Samples were incubated for 20 min at 4°C and centrifuged at 10,000*g* for 15 min at 4°C. The supernatant was transferred to a new tube, protein concentration was determined by the method of Bradford [33] and samples were stored at −80°C.

For the EMSA, the oligonucleotide containing the consensus sequence for NF-κB was end labeled with [γ^{-32}P] ATP using T4 polynucleotide kinase, and purified using Chroma Spin-10 columns. Samples were incubated with the labeled oligonucleotide (20,000–30,000 cpm) for 20 min at room temperature in binding buffer [5X binding buffer: 50 mM Tris–HCl buffer, pH 7.5, containing 20% (v/v) glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl and 0.25 mg/ml poly(dI–dC)]. The products were separated by electrophoresis in a 6% (w/v) non-denaturing polyacrylamide gel using 0.5 × TBE (45 mM Tris/borate and 1 mM EDTA) as the running buffer. The gels were dried and the radioactivity was quantitated in a Phosphoimager 840 (GE Healthcare, Piscataway, NJ, USA).

Western blot analysis

Nuclear fractions were obtained as described above. Aliquots of nuclear fractions containing 25–40 μg protein were separated by reducing 10% (w/v) polyacrylamide gel electrophoresis and electroblotted to PVDF membranes. Molecular weight standards (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were run simultaneously. For p-p65, p65 and p50 evaluation, membranes were blocked overnight in 5% non-fat milk and subsequently incubated in the presence of the corresponding antibodies (1:1000 dilution) for 90 min at 37°C. After incubation, for 90 min at room temperature, in the presence of the secondary antibody (HRP-conjugated) (1:10,000 dilution) the conjugates were visualized by chemiluminescence detection in a Phosphoimager 840.

Measurement of TNF-α and RANTES

TNF-α and RANTES were measured by ELISA. Briefly, cells (1.5×10^6) were incubated for 24 h in the different experimental conditions. TNF-α and RANTES were measured in the cell culture media, after separating the cells by centrifugation at 800*g* for 10 min. TNF-α (BD Biosciences, San Diego, CA, USA) and RANTES (Biosource, Camarillo, CA, USA) were measured following the manufacturer's protocols.

Statistical analysis

One way analysis of variance (ANOVA) with subsequent *post hoc* comparisons by Scheffe was performed using Statview 512 + (Brainpower Inc., Calabazas CA, USA). A $p < 0.05$ was considered statistically significant. Values are given as means ± SEM. Three independent experiments with at least duplicates for each experimental condition were run for the different assays.

Results and discussion

EC inhibits NF-κB constitutive activation in H–RS cells

Different flavonoids have the capability of inhibiting transcription factor NF-κB and the expression of NF-κB-regulated genes [29,30]. With regard to flavan-3-ols and procyanidins, (−)-epigallocatechin gallate (EGCG), the major polyphenol in tea, inhibited *in vitro* and *in vivo*, lipopolysaccharide-induced NF-κB nuclear binding activity and TNF-α secretion [31]. Furthermore, EGCG also inhibited 12-*O*-tetradecanoylphorbol-13-acetate-induced IκBα phosphorylation and NF-κB–DNA binding activity in JB6 mouse epidermal cells [34]. EC, catechin and dimers B1 and B2 inhibited the interferon γ-induced expression of NF-κB-dependent genes in RAW 264.7 macrophages [28]. Procyanidins present in grape seeds inhibit the expression of matrix metalloproteinases in human prostate carcinoma cells, in association with the inhibition of the activation of NF-κB [26].

Based on our previous findings that EC-inhibited PMA-induced NF-κB activation in Jurkat cells, we initially investigated the capacity of EC to inhibit NF-κB in the H–RS cell lines KM-H2 and L-428. Dietary EC undergoes extensive metabolization with the appearance in plasma of methylated and glucuronidated derivatives [35,36]. Flavonoids conjugates have differential antioxidant and biological activities [37,38]. However, in the present study, we investigated the effects of the parent compound EC on H–RS cells, considering EC as a potential therapeutic agent that could be administered intravenously, rather than as a dietary constituent. Both, KM-H2 and L-428 cells, are characterized by a constitutive activation of NF-κB secondary to the absence of functional IκBα or IκBα and IκBε, respectively [16–19]. The active form of NF-κB in these cells is composed basically by p50 and p65 as demonstrated by EMSA supershift assays (Figure 2A). The specificity of the NF-κB–DNA complex in the EMSA assays was assessed by competition with a 100-fold molar excess of unlabeled oligonucleotide containing the consensus sequence for either NF-κB or SP-1 (Figure 2A). After 24 h of incubation, EC (5–50 μM) caused a dose-dependent inhibition of NF-κB–DNA nuclear binding activity in KM-H2 and L-428 cells (Figure 2B). At 25 μM, EC caused a significant inhibition (40 and 42%) of NF-κB–DNA binding activity in L-428 and KM-H2 cells, respectively.

H–RS cells are characterized by overexpressing numerous NF-κB-driven genes. They include cytokines, chemokines and their modulators (RANTES, TNF-α), genes encoding for proteins involved in the regulation of the cell cycle (cyclin D2), proteins that prevent apoptosis (Bfl-1/A1, c-IAP2, TRAF1 and Bcl-X_L) and cell surface receptors (CD86 and CD40) [39,40]. We next tested the capacity of EC to inhibit the transactivation of the NF-κB-dependent genes RANTES and TNF-α (Figure 3). At 25 μM concentration, EC did not affect TNF-α secretion (Figure 3A). EC treatment significantly inhibited RANTES secretion in L-428 and KM-H2 cells (16 and 7%, respectively) (Figure 3B). Although in Jurkat cells, EC markedly decreased PMA-induced IL-2 secretion [32], only a modest EC-mediated decrease in RANTES and TNF-α secretion was observed in H–RS cells. These findings suggest that the extent of EC-mediated inhibition of NF-κB–DNA binding is not sufficient to downregulate TNF-α and RANTES. Furthermore, the promoter of both chemokines could be under the control of other transcription factors.

Combined action of EC and SN-50 in the inhibition of NF-κB

We hypothesized that compounds that could affect the NF-κB signaling cascade at different steps could act synergistically in the inhibition of NF-κB in H–RS cells. Based on previous results in Jurkat cells, EC could act inhibiting NF-κB at the initial steps by acting as an antioxidant. Furthermore, we have presented evidence that EC could directly interact with NF-κB proteins preventing the binding of the active transcription factor to DNA [32]. Since the main NF-κB deregulation in L-428 and KM-H2 cells is the lack of the inhibitory peptide IκB, we considered that inhibiting NF-κB nuclear translocation (a downstream event) would be the logic step to target in these H–RS cells. For this purpose, we used a cell permeable peptide (SN-50) containing the nuclear localization signal of p50 that inhibits NF-κB nuclear transport [41]. Thus, the possible combined effects of EC with SN-50 were next evaluated in L-428 and KM-H2 cells. Cells treated with 20 μM SN-50 showed a lower NF-κB–DNA binding activity in nuclear fractions while no effect was observed when the mutated control peptide (SN-50M) was used. This indicates a specific inhibitory effect of SN-50 on NF-κB nuclear translocation in H–RS cells (Figure 2C). The simultaneous incubation of cells with 50 μM EC and 20 μM SN-50 showed an almost complete inhibition of NF-κB binding activity in both L-428 and KM-H2 cells (Figure 2C).

The effects of LA, EC and SN-50 on two cytokines partially regulated by NF-κB was next evaluated.

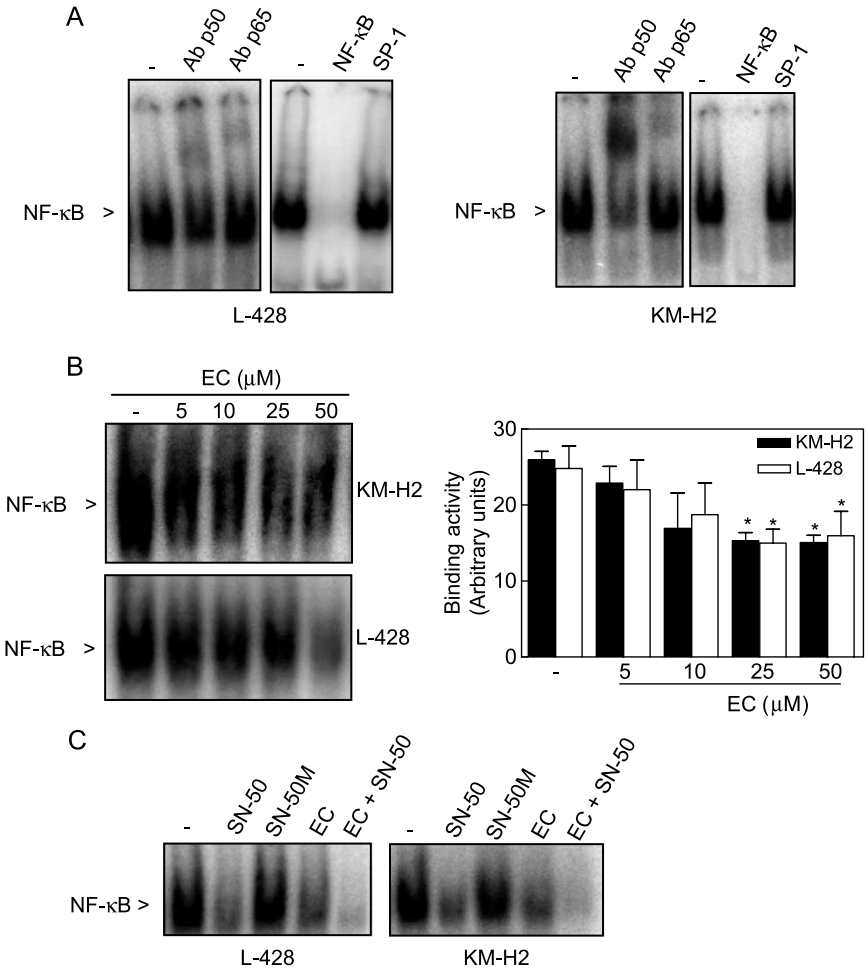

Figure 2. EC inhibits NF-κB binding activity in L-428 and KM-H2 cells. (A) To determine the protein components of the active NF-κB in L-428 and KM-H2 cells, a nuclear fraction isolated from each cell line was incubated in the absence (−) or presence of antibodies against p50 or p65 (Ab p50 and Ab p65, respectively) for 30 min prior to the binding assay. To determine the specificity of the NF-κB–DNA complex, a nuclear fraction (−) was incubated in the presence of 100-fold molar excess of unlabeled oligonucleotide containing the consensus sequence for either NF-κB (NF-κB) or SP-1 (SP-1) before the binding assay. (B) NF-κB nuclear binding activity was measured by EMSA in KM-H2 and L-428 cells incubated for 24 h without (−) or with 5-50 μM EC. After the EMSA, bands were quantitated and results are shown as means ± SEM of 3 independent experiments. *Significantly different compared to untreated cells ($p < 0.01$, one-way ANOVA test). (C) EMSA analysis of nuclear extracts isolated from cells after 2 h incubation without (−) or with 50 μM EC (EC), 20 μM SN-50 (SN-50), its mutated control (SN-50M) or with the simultaneous incubation of 50 μM EC and 20 μM SN-50 (EC + SN-50). One representative experiment out of three independent experiments is shown.

In agreement with the lack of effect of LA on NF-κB binding activity, LA did not affect RANTES and TNF-α secretion in both cell lines. SN-50 partially inhibited TNF-α (56 and 20%) (Figure 3A) and RANTES (20 and 11%) (Figure 3B) secretion in L-428 and KM-H2 cells, respectively. The combined treatment of cells with EC and SN-50 lead to additive effects on TNF-α and RANTES secretion in both cell lines compared to the individual treatments, reaching significance ($p < 0.05$) only for RANTES secretion. The mild effect on RANTES expression could be due to the complexity of the RANTES promoter. In T lymphocytes, RANTES promoter contain two sites for NF-κB, and sites for nuclear factor of IL-6 (NF-IL-6), interferon regulatory factor-3 (IRF-3) and RANTES factor of late activated T lymphocytes-1 (RFLAT-1) [42]. Similarly, several motifs have been identified in the TNF-α promoter, including binding sites for NFAT, NF-κB, ATF-2, cyclic AMP response element (CRE), AP-1 and AP-2 [43]. However, NF-κB inhibition by SN-50 led to a marked inhibition of TNF-α secretion in L-428 cells.

Comparative action of EC and LA on cell oxidant levels and NF-κB activation

Oxygen and nitrogen active species and changes in cell thiol redox state are recognized signals in NF-κB activation (See [44–49] for reviews). Experimental evidence has shown that different flavanols inhibit NF-κB activation through their antioxidant activity. EGCG diminished the IL-1beta-induced production of NO and inhibited the expression of NF-κB-dependent

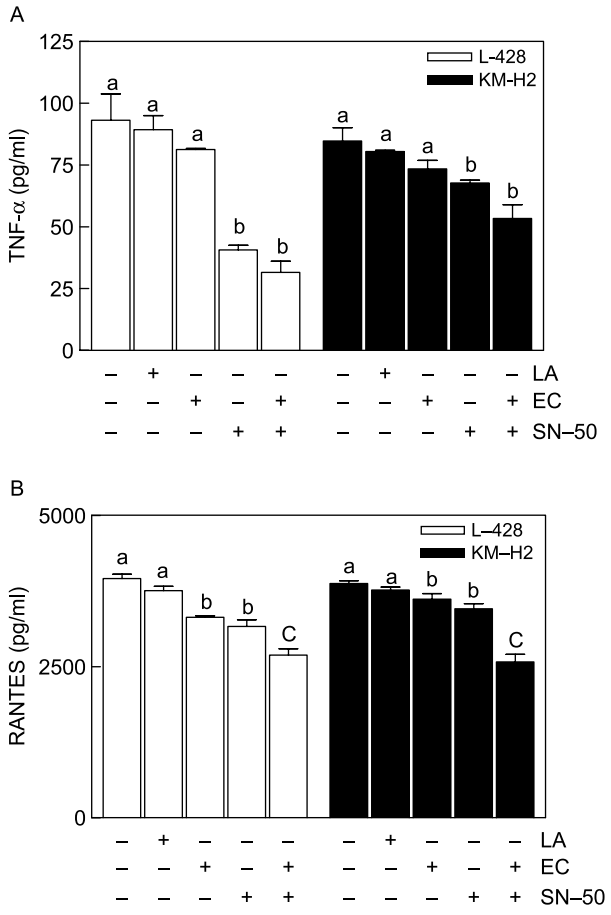

Figure 3. Effect of EC and SN-50 on TNF-α and RANTES secretion in L-428 and KM-H2 cells. L-428 and KM-H2 cells were incubated for 24 h without (−) or with 25 μM EC, 20 μM SN-50 (SN-50), 25 μM EC and 20 μM SN-50 (EC + SN-50) or 0.5 mM LA for 24 h. The concentration of TNF-α (A) and RANTES (B) released to the media was measured by ELISA. Results are means ± SEM of three independent experiments. a is significantly different from b and c; b is significantly different from c ($p < 0.05$, one way ANOVA test).

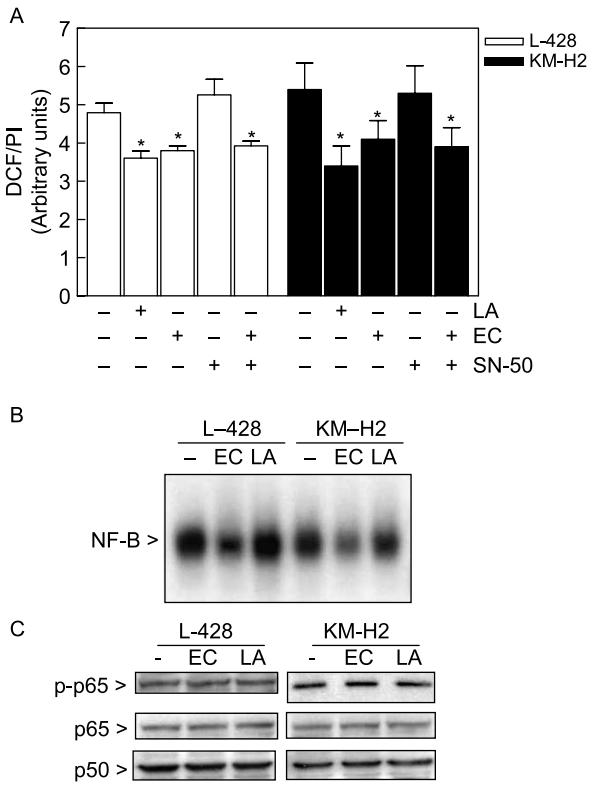

Figure 4. EC decreases cell oxidant levels in L-428 and KM-H2 cells. (A) Global oxidant levels were evaluated with the probe DCDHF in L-428 and KM-H2 cells after incubation without (−) or with 25 μM EC, 20 μM SN-50 (SN-50), 25 μM EC +20 μM SN-50 (EC + SN-50) or 0.5 mM LA for 24 h. Global oxidant levels were determined as described under Materials and Methods. DCF fluorescence was normalized to the PI fluorescence. Results are shown as means ± SEM of three independent experiments. *Significantly different from the untreated group ($p < 0.05$, one way ANOVA test). (B) NF-κB nuclear binding activity was measured by EMSA in nuclear fractions isolated from L-428 and KM-H2 cells incubated for 2 h without (−) or with 50 μM EC (EC) or 0.5 mM LA (LA). (C) Western blot analysis for p65 phosphorylation (p-p65), p65 and p50 in nuclear extracts isolated from L-428 and KM-H2 cells incubated for 2 h without (−) or with 50 μM EC (EC) or 0.5 mM LA (LA). One representative experiment out of three independent experiments is shown.

gene *iNOS* in human chondrocytes by interfering with NF-κB activation [50]. EGCG also inhibited NF-κB activation induced by repetitive exposure to hydrogen peroxide in fibroblasts [51]. Similarly, numerous flavonoids inhibited NO production, TNF-α secretion and NF-κB-dependent gene expression in RAW 264.7 macrophages stimulated with interferon-γ [28]. Thus, the inhibition of early steps in NF-κB activation could be partially mediated by the well described antioxidant action of EC [52]. In this regard, we have previously observed that EC inhibits PMA-induced increase in cellular oxidants in Jurkat cells [32].

To assess whether EC could inhibit NF-κB activation in H–RS cells by acting as an antioxidant, we compared the effects of EC with those of the antioxidant LA in their capacity to decrease cell oxidants and inhibit NF-κB–DNA binding activity in L-428 and KM-H2 cells. LA can act as antioxidant through different mechanisms including its capacity to directly scavenge oxidant species, chelate redox-active metals and reduce glutathione [53–55]. L-428 and KM-H2 showed high levels of cell oxidants, measured with the probe DCDHF. Thus, basal cell oxidant levels were higher in L-428 and KM-H2 cells (DCF fluorescence/PI fluorescence: 4.5 ± 0.4 and 5.4 ± 0.7, respectively) when compared to Jurkat cells (2.0 ± 0.2). Treatment of L-428 and KM-H2 cells with 25 μM EC or 0.5 mM LA for 24 h decreased cell oxidant levels at a similar extent in both cell lines (Figure 4A). As a control, the presence of SN-50 did not affect cell oxidant levels and, in combination with EC, did not cause an additional decrease of cell oxidants (Figure 4A).

While in L-428 and KMH-2 cells EC inhibited nuclear NF-κB–DNA binding activity, LA did not, indicating that the antioxidant status of the cell does not seem to affect NF-κB activation in these H–RS cells. These findings suggest that EC is inhibiting NF-κB by a mechanism different from its antioxidant properties

in both H–RS cell lines (Figure 4B). We further studied the possible mechanism of EC action, characterizing p65 phosphorylation in nuclear fractions. The phosphorylation of p65 can occur at the cytoplasm or at the nuclei, is mediated by different cellular kinases and increases the transactivating activity of p65 [56]. We did not observe changes in p65 phosphorylation in cells incubated with EC nor in cells incubated with LA, compared to untreated cells (Figure 4C). These results indicate that, in L-428 and KM-H2 cells, EC-mediated inhibition of NF-κB does not occur through its antioxidant activity or by affecting the activation of p65 through phosphorylation. The content of p65 and p50 in nuclear fractions was similar in L-428 and KM-H2 cells incubated in the absence or the presence of EC or LA (Figure 4C). This finding further supports our previous finding that EC inhibition of NF-κB binding to DNA could be secondary to a direct interaction of EC at regions of the NF-κB proteins involved in the binding to DNA [32].

EC sensitizes SN-50-induced decrease in cell viability in L-428 and KM-H2 cells

Flavonoids from different families have been described to have the capacity to inhibit cell proliferation and induce apoptosis in different cancers [24–27]. Flavonoids could also sensitize cancer cells to traditional anticancer therapies [57].

We next evaluated if EC could affect H–RS cell viability and sensitize H–RS cells to SN-50-induced decrease in cell viability. Cells were incubated for 24–48 h under the different treatments (EC, SN-50 and/or LA). Treatment with the antioxidant LA did not affect cell viability (evaluated by measuring the ATP cell content) (Figure 5). After 24 h of incubation, EC caused a modest but significant decrease (14 and 10% in L-428 and KM-H2 cells, respectively) in cell viability (Figure 5). Since flavanols have been shown to generate hydrogen peroxide in cell culture media [58], an additional group was treated with EC and catalase (100 U/ml). Catalase did not affect EC-induced decrease in cell viability indicating that this effect was not secondary to the production of H_2O_2 in the culture media. SN-50 significantly decreased cell viability in both cell lines (28 and 26% in L-428 and KM-H2 cells, respectively). The combined treatment of cells with EC and SN-50 caused an additive effect on the decrease in cell viability (37 and 35% in L-428 and KM-H2 cells, respectively). After 48 h of incubation, the effects of EC, SN-50 and LA were similar to those observed at 24 h of treatment (data not shown). Similarly, the combined effects of EC and SN-50 on NF-κB–DNA binding activity, NF-κB-dependent gene expression and cell viability were additive. These results suggest that, in the current experimental conditions, the decreased cell viability in H–RS cells is mostly associated with the down regulation of NF-κB.

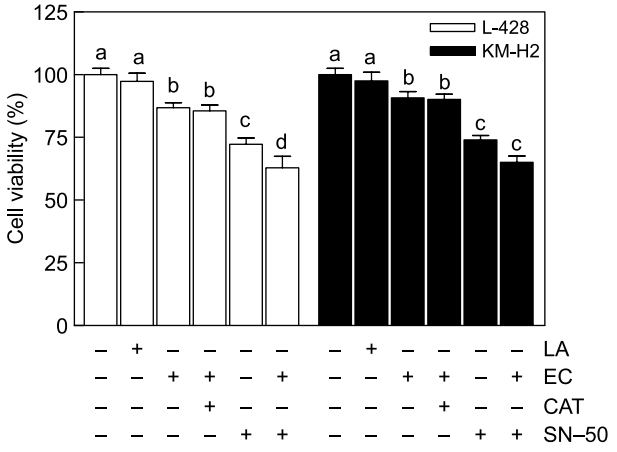

Figure 5. Effect of EC and SN-50 on cell viability. Cell viability was measured in L-428 and KM-H2 cells after 24 h of incubation without or with 25 μM EC, 25 μM EC and 100 U/ml catalase (CAT) (EC + CAT), 20 μM SN-50 (SN-50), 25 μM EC and 20 μM SN-50 (EC + SN-50) or 0.5 mM LA. Cell viability was measured as described under Materials and Methods. After quantitation, results are shown as means ± SEM of three independent experiments. a is significantly different from b, c and d; b is significantly different from c and d; c is significantly different from d. ($p < 0.05$, one way ANOVA test).

Summary

The findings that in L-428 and KM-H2 cells EC: (i) does not inhibit NF-κB through its antioxidant activity; (ii) does not affect NF-κB activation at the level of p65 phosphorylation; (iii) does not affect NF-κB translocation; and (iii) at the nucleus, inhibits NF-κB–DNA binding not affecting p65 and p50 nuclear content; further support our previously proposed mechanism of a direct interaction of EC with NF-κB proteins and subsequent inhibition of NF-κB binding to DNA [32].

Taking into consideration the high concentrations of EC necessary to inhibit NF-κB in H–RS cells and the modest effects on cell viability, even in combination with the NF-κB inhibitor SN-50, indicates that EC can not be considered as a possible therapeutic approach in the treatment of Hodgkin's lymphoma. However, the capacity of EC to inhibit NF-κB could be relevant in other pathological conditions (i.e. inflammatory processes).

It is very important to point out that even when the combined treatment with EC and SN-50 led to an almost complete inhibition of NF-κB–DNA binding activity, this translated in a partial effect on cell viability. Thus, the inhibition of additional signaling pathways should be also considered when designing combined therapeutic approaches for Hodgkin's lymphoma.

Acknowledgements

This work was supported by grants from the University of California Cancer Research Coordination Committee (CRCC), and the University of California, Davis, USA.

References

[1] Karin M, Cao Y, Greten FR, Li ZW. NF-kappaB in cancer: From innocent bystander to major culprit. Nat Rev Cancer 2002;2:301–310.

[2] Rayet B, Gelinas C. Aberrant *rel/nfkb* genes and activity in human cancer. Oncogene 1999;18:6938–6947.

[3] Cahir McFarland ED, Izumi KM, Mosialos G. Epstein-barr virus transformation: Involvement of latent membrane protein 1-mediated activation of NF-kappaB. Oncogene 1999; 18: 6959–6964.

[4] Baeuerle PA, Baltimore D. I kappa B: A specific inhibitor of the NF-kappa B transcription factor. Science 1988;242:540–546.

[5] Delhase M, Hayakawa M, Chen Y, Karin M. Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation. Science 1999; 284: 309–313.

[6] Karin M, How M. NF-kappaB is activated: The role of the IkappaB kinase (IKK) complex. Oncogene 1999; 18: 6867–6874.

[7] Aggarwal BB. Nuclear factor-kappaB: The enemy within. Cancer Cell 2004;6:203–208.

[8] Karin M, Greten FR. NF-kappaB: Linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 2005;5:749–759.

[9] Kucharczak J, Simmons MJ, Fan Y, Gelinas C. To be, or not to be: NF-kappaB is the answer—role of Rel/NF-kappaB in the regulation of apoptosis. Oncogene 2003;22:8961–8982.

[10] Lin A, Karin M. NF-kappaB in cancer: A marked target. Semin Cancer Biol 2003;13:107–114.

[11] Pahl HL. Activators and target genes of Rel/NF-κB transcription factors. Oncogene 1999;18:6853–6866.

[12] Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, Ben-Neriah Y. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. Nature 2004;431:461–466.

[13] Bargou RC, Emmerich F, Krappmann D, Bommert K, Mapara MY, Arnold W, Royer HD, Grinstein E, Greiner A, Scheidereit C, Dorken B. Constitutive nuclear factor-kappaB–RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. J Clin Invest 1997; 100: 2961–2969.

[14] Horie R, Watanabe T. The biological basis of Hodgkin's lymphoma. Drug News Perspect 2003;16:649–656.

[15] Krappmann D, Emmerich F, Kordes U, Scharschmidt E, Dorken B, Scheidereit C. Molecular mechanisms of constitutive NF-kappaB/Rel activation in Hodgkin/Reed-Sternberg cells. Oncogene 1999;18:943–953.

[16] Emmerich F, Meiser M, Hummel M, Demel G, Foss HD, Jundt F, Mathas S, Krappmann D, Scheidereit C, Stein H, Dorken B. Overexpression of I kappa B alpha without inhibition of NF-kappaB activity and mutations in the *IkappaB alpha* gene in Reed-Sternberg cells. Blood 1999; 94: 3129–3134.

[17] Cabannes E, Khan G, Aillet F, Jarrett RF, Hay RT. Mutations in the *IkBa* gene in Hodgkin's disease suggest a tumour suppressor role for IkappaBalpha. Oncogene 1999; 18: 3063–3070.

[18] Jungnickel B, Staratschek-Jox A, Brauninger A, Spieker T, Wolf J, Diehl V, Hansmann ML, Rajewsky K, Kuppers R. Clonal deleterious mutations in the *IkappaBalpha* gene in the malignant cells in Hodgkin's lymphoma. J Exp Med 2000; 191:395–402.

[19] Emmerich F, Theurich S, Hummel M, Haeffker A, Vry MS, Dohner K, Bommert K, Stein H, Dorken B. Inactivating IkappaBepsilon mutations in Hodgkin/Reed-Sternberg cells. J Pathol 2003;201:413–420.

[20] Martin-Subero JI, Gesk S, Harder L, Sonoki T, Tucker PW, Schlegelberger B, Grote W, Novo FJ, Calasanz MJ, Hansmann ML, Dyer MJ, Siebert R. Recurrent involvement of the REL and BCL11A loci in classical Hodgkin lymphoma. Blood 2002;99:1474–1477.

[21] Horie R, Watanabe T, Morishita Y, Ito K, Ishida T, Kanegae Y, Saito I, Higashihara M, Mori S, Kadin ME, Watanabe T. Ligand-independent signaling by overexpressed CD30 drives NF-kappaB activation in Hodgkin–Reed-Sternberg cells. Oncogene 2002;21:2493–2503.

[22] Hammarskjold ML, Simurda MC. Epstein-Barr virus latent membrane protein transactivates the human immunodeficiency virus type 1 long terminal repeat through induction of NF-kappa B activity. J Virol 1992;66:6496–6501.

[23] Graf BA, Milbury PE, Blumberg JB. Flavonols, flavones, flavanones and human health: epidemiological evidence. J Med Food 2005;8:281–290.

[24] Hou Z, Sang S, You H, Lee MJ, Hong J, Chin KV, Yang CS. Mechanism of action of (−)-epigallocatechin-3-gallate: Auto-oxidation-dependent inactivation of epidermal growth factor receptor and direct effects on growth inhibition in human esophageal cancer KYSE 150 cells. Cancer Res 2005; 65: 8049–8056.

[25] Leone M, Zhai D, Sareth S, Kitada S, Reed JC, Pellecchia M. Cancer prevention by tea polyphenols is linked to their direct inhibition of antiapoptotic Bcl-2-family proteins. Cancer Res 2003;63:8118–8121.

[26] Vayalil PK, Mittal A, Katiyar SK. Proanthocyanidins from grape seeds inhibit expression of matrix metalloproteinases in human prostate carcinoma cells, which is associated with the inhibition of activation of MAPK and NFkappaB. Carcinogenesis 2004;25:987–995.

[27] Zhao J, Wang J, Chen Y, Agarwal R. Anti-tumor-promoting activity of a polyphenolic fraction isolated from grape seeds in the mouse skin two-stage initiation–promotion protocol and identification of procyanidin B5-3′-gallate as the most effective antioxidant constituent. Carcinogenesis 1999;20:1737–1745.

[28] Park YC, Rimbach G, Saliou G, Valacchi G, Packer L. Activity of monomeric, dimeric and trimeric flavonoids on NO production, TNF-alpha secretion, and NF-kappaB-dependent gene expression in RAW 264.7 macrophages. FEBS Lett 2000; 465:93–97.

[29] Saliou C, Valacchi G, Rimbach G. Assessing bioflavonoids as regulators of NF-kappaB activity and inflammatory gene expression in mammalian cells. Meth Enzymol 2001; 335: 380–387.

[30] Surh YJ, Kundu JK, Na HK, Lee JS. Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals. J Nutr 2005;135:2993S–3001S.

[31] Yang F, de Villiers WJ, McClain CJ, Varilek GW. Green tea polyphenols block endotoxin-induced tumor necrosis factor-production and lethality in a murine model. J Nutr 1998; 128: 2334–2340.

[32] Mackenzie GG, Carrasquedo F, Delfino JM, Keen CL, Fraga CG, Oteiza PI. Epicatechin, catechin and dimeric procyanidins inhibit PMA-induced NF-kappaB activation at multiple steps in Jurkat T cells. Faseb J 2004;18:167–169.

[33] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248–254.

[34] Nomura M, Ma W, Chen N, Bode AM, Dong Z. Inhibition of 12-*O*-tetradecanoylphorbol-13-acetate-induced NF-kappaB activation by tea polyphenols, (−)-epigallocatechin gallate and theaflavins. Carcinogenesis 2000;21:1885–1890.

[35] Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, Sies H, Kwik-Uribe C, Schmitz HH, Kelm M. (−)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. Proc Natl Acad Sci U S A 2006;103:1024–1029.

[36] Tsang C, Auger C, Mullen W, Bornet A, Rouanet JM, Crozier A, Teissedre PL. The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. Br J Nutr 2005;94:170–181.

[37] Natsume M, Osakabe N, Yasuda A, Baba S, Tokunaga T, Kondo K, Osawa T, Terao J. *In vitro* antioxidative activity of (−)-epicatechin glucuronide metabolites present in human and rat plasma. Free Radic Res 2004;38:1341–1348.

[38] Williamson G, Barron D, Shimoi K, Terao J. *In vitro* biological properties of flavonoid conjugates found *in vivo*. Free Radic Res 2005;39:457–469.

[39] Hinz M, Lemke P, Anagnostopoulos I, Hacker C, Krappmann D, Mathas S, Dorken B, Zenke M, Stein H, Scheidereit C. Nuclear factor kappaB-dependent gene expression profiling of Hodgkin's disease tumor cells, pathogenetic significance, and link to constitutive signal transducer and activator of transcription 5a activity. J Exp Med 2002;196:605–617.

[40] Hinz M, Loser P, Mathas S, Krappmann D, Dorken B, Scheidereit C. Constitutive NF-kappaB maintains high expression of a characteristic gene network, including CD40, CD86, and a set of antiapoptotic genes in Hodgkin/Reed-Sternberg cells. Blood 2001;97:2798–2807.

[41] Lin YZ, Yao SY, Veach RA, Torgerson TR, Hawiger J. Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. J Biol Chem 1995;270:14255–14258.

[42] Song A, Nikolcheva T, Krensky AM. Transcriptional regulation of RANTES expression in T lymphocytes. Immunol Rev 2000;177:236–245.

[43] Falvo JV, Uglialoro AM, Brinkman BM, Merika M, Parekh BS, Tsai EY, King HC, Morielli AD, Peralta EG, Maniatis T, Thanos D, Goldfeld AE. Stimulus-specific assembly of enhancer complexes on the tumor necrosis factor alpha gene promoter. Mol Cell Biol 2000;20:2239–2247.

[44] Bowie A, O'Neill LA. Oxidative stress and nuclear factor-kappaB activation: A reassessment of the evidence in the light of recent discoveries. Biochem Pharmacol 2000;59:13–23.

[45] Ginn-Pease ME, Whisler RL. Redox signals and NF-kappaB activation in T cells. Free Radic Biol Med 1998;25:346–361.

[46] Li N, Karin M. Is NF-kB the sensor of oxidative stress? FASEB J. 1999;13:1137–1143.

[47] Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. Faseb J 1996;10:709–720.

[48] Moran LK, Gutteridge JM, Quinlan GJ. Thiols in cellular redox signalling and control. Curr Med Chem 2001; 8: 763–772.

[49] Arrigo AP. Gene expression and the thiol redox state. Free Radic Biol Med 1999;27:936–944.

[50] Singh R, Ahmed S, Islam N, Goldberg VM, Haqqi TM. Epigallocatechin-3-gallate inhibits interleukin-1beta-induced expression of nitric oxide synthase and production of nitric oxide in human chondrocytes: Suppression of nuclear factor kappaB activation by degradation of the inhibitor of nuclear factor kappaB. Arthritis Rheum 2002;46:2079–2086.

[51] Sen P, Chakraborty PK, Raha S. Tea polyphenol epigallocatechin 3-gallate impedes the anti-apoptotic effects of low-grade repetitive stress through inhibition of Akt and NFkappaB survival pathways. FEBS Lett 2006;580:278–284.

[52] Lotito SB, Actis-Goretta L, Renart ML, Caligiuri M, Rein D, Schmitz HH, Steinberg FM, Keen CL, Fraga CG. Influence of oligomer chain length on the antioxidant activity of procyanidins. Biochem Biophys Res Commun 2000; 276: 945–951.

[53] Han D, Handelman G, Marcocci L, Sen CK, Roy S, Kobuchi H, Tritschler HJ, Flohe L, Packer L. Lipoic acid increases de novo synthesis of cellular glutathione by improving cystine utilization. Biofactors 1997;6:321–338.

[54] Packer L. alpha-Lipoic acid: A metabolic antioxidant which regulates NF-kappaB signal transduction and protects against oxidative injury. Drug Metab Rev 1998;30:245–275.

[55] Roy S, Packer L. Redox regulation of cell functions by alpha-lipoate: Biochemical and molecular aspects. Biofactors 1998;7:263–267.

[56] Viatour P, Merville MP, Bours V, Chariot A. Phosphorylation of NF-kappaB and IkappaB proteins: Implications in cancer and inflammation. Trends Biochem Sci 2005;30:43–52.

[57] Garg AK, Buchholz TA, Aggarwal BB. Chemosensitization and radiosensitization of tumors by plant polyphenols. Antioxid Redox Signal 2005;7:1630–1647.

[58] Long LH, Clement MV, Halliwell B. Artifacts in cell culture: Rapid generation of hydrogen peroxide on addition of (−)-epigallocatechin, (−)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. Biochem Biophys Res Commun 2000;273:50–53.