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Green Tea Inhibits Human Inducible Nitric-Oxide Synthase Expression by Down-Regulating Signal Transducer and Activator of Transcription-1α Activation

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ABSTRACT

Green tea has been reported to show anti-inflammatory properties because of its inhibitory effects on the expression of several pro-inflammatory genes. Because the inducible nitric-oxide synthase (iNOS) plays an important role in chronic inflammatory diseases, we have focused our attention on the regulation of iNOS expression by green tea in two different human epithelial cell lines, alveolar A549/8 and colon DLD-1 cells. With the use of electrophoretic mobility shift assays, we found a green tea-mediated down-regulation of the DNA binding activity of the transcription factor signal transducer and activator of transcription-1α (STAT-1α), but not of nuclear factor-κB. This down-regulation of the STAT-1α DNA binding was shown to result from reduced tyrosine phosphorylation of the STAT-1α protein and not from antioxidative effects of the green tea extract. Green tea extract inhibited human iNOS expression in a concentration-dependent manner, quantified in terms of iNOS mRNA, iNOS protein, and nitric oxide production in both cell lines. This inhibitory effect of green tea resulted from transcriptional inhibition as shown in reporter gene experiments. These data suggest that green tea extracts may be promising at least as an auxiliary anti-inflammatory principle in chronic inflammatory diseases.

The inducible nitric-oxide synthase (iNOS) is normally absent from resting cells (Kleinert et al., 2000). After activation of cells by different inducers (cytokines, bacterial lipopolysaccharides), iNOS is expressed and active for hours to days as a "high-output" enzyme (MacMicking et al., 1997). The high amounts of NO produced by iNOS may be beneficial for their microbicidal, antiviral, antiparasitic, and antitumoral action (MacMicking et al., 1997; Bogdan, 2001). However, an aberrant iNOS induction is likely to have detrimental consequences and seems to be involved in the pathophysiology of several human diseases (Kröncke et al., 1998) and cancers (Jaiswal et al., 2001). The iNOS enzyme is expressed in various organs, including the lungs and intestine, where an overproduction of NO contributes significantly to vascular failure and end-organ damage during endotoxemia and to diseases such as asthma, short- and long-term lung disease, and septic shock (Barnes and Liew, 1995; Kröncke et al., 1998; Kleinert et al., 2000; Bogdan, 2001). iNOS-derived NO also plays a critical role in the pathogenesis of inflammatory bowel disease along with other pro-inflammatory mediators (Cavicchi and Whittle, 1999). Enhanced iNOS expression is also observed in patients with celiac disease (van Straaten et al., 1999), ulcerative colitis, and Crohn's disease (Guslandi, 1998). Therefore, pharmacological suppression of iNOS-dependent NO production may be helpful in the treatment of these diseases.

The regulation of iNOS expression is cell- and species-specific, and a variety of signal transduction pathways are involved (Kleinert et al., 2000). Regulation of the transcription of the iNOS gene is believed to be the most important control mechanism for iNOS expression. iNOS induction in human cells seems to be partially dependent on activation of the NF-κB signal pathway (Kleinert et al., 1998). In addition

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ABBREVIATIONS: iNOS, inducible nitric-oxide synthase; JAK, Janus kinase; STAT, signal transducer and activator of transcription; kb, kilobase(s); NF-κB, nuclear factor κB; GAS, γ-activated site; tyrphostin AG490, *N*-benzyl-3,4-dihydroxy-benzylidenecyanoacetamide; ECGC, epigallocatechin-3-gallate; LPS, lipopolysaccharide; IFN, interferon; IL, interleukin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NAC, *N*-acetyl-cysteine; ASC, ascorbate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; CM, cytokine mixture; eNOS, endothelial nitric-oxide synthase; GX, green tea extract; TNF, tumor necrosis factor.

to NF-κB activation, the Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway has been shown to be essential for human and murine iNOS expression (Gao et al., 1997; Kleinert et al., 1998; Ganster et al., 2001; Menegazzi et al., 2001; Yao et al., 2003). Transient transfection experiments with human A549/8 or DLD-1 cells have demonstrated that maximal induction of the human iNOS promoter depends on transcription factor binding sites upstream of position −5 kb (de Vera et al., 1996; Ganster et al., 2001; and our unpublished results). The published sequence of the human iNOS promoter exhibits homologies to numerous transcription factor binding sites (GenBank accession no. AC005697). Studies using the human iNOS promoter showed important regulation of the iNOS promoter activity by the transcription factors NF-κB, activator protein 1, and STAT-1α (de Vera et al., 1996; Kleinert et al., 1998; Kleinert et al., 2000; Ganster et al., 2001). Ganster et al. (2001) described the dependence of human iNOS promoter activity on a composite GAS/NF-κB element located at position −5.8 kb in the human iNOS promoter (Ganster et al., 2001). In human A549/8 or DLD-1 cells, the cytokine-induced activity of a stably transfected 16-kb human iNOS promoter fragment was inhibited by tyrphostin AG490, a specific inhibitor of the JAK2- tyrosine kinase (Kleinert et al., 1999), or by fungal metabolites blocking the JAK/STAT pathway (Yao et al., 2003). Therefore, the IFN-γ–JAK–STAT pathway seems to be a good target for the inhibition of human iNOS promoter activation and hence iNOS expression in pathophysiologic situations.

Experimental and epidemiologic studies have demonstrated that green tea can inhibit tumor formation and tumor growth (Fujiki et al., 2002; Lin, 2002). Green tea has an inhibitory effect toward inflammatory diseases such as collagen-induced arthritis (Haqqi et al., 1999), inflammatory bowel disease (Varilek et al., 2001), and the carrageenan-induced paw edema in mice (Das et al., 2002). Only recently have some hints on the possible molecular mechanism of the antitumor or anti-inflammatory effect of green tea emerged. Epigallocatechin gallate (EGCG), the main polyphenol present in green tea (and theaflavin-3,3′-digallate, a polyphenol isolated from black tea) have been shown to inhibit LPS/cytokine-induced iNOS expression in human chondrocytes (Singh et al., 2002) and murine macrophages (Lin and Lin, 1997) by blocking LPS/cytokine-induced NF-κB activation. In addition, ECGC was recently reported to be a specific and potent inhibitor of IFN-γ–elicited STAT-1α activation in a number of human carcinoma cell lines (Menegazzi et al., 2001).

In the current study, we analyzed the effect of green tea extract on the cytokine-induced activation of NF-κB and STAT-1α and on the expression of iNOS in two human epithelial carcinoma cell lines, A549/8 and DLD-1. Our data show that green tea extract exerts an efficient inhibitory action on STAT-1α activation (but not NF-κB activation) and on iNOS expression. This leads to a drastic reduction in NO production in these cells.

Materials and Methods

Reagents

Trypsin, glutamine, and pyruvate solutions, agarose, tRNA, *N*-acetyl-cysteine (NAC), ascorbate (ASC), and bovine serum albumin were purchased from Sigma (Deisenhofen, Germany). Isotopes were obtained from ICN Biomedicals (Eschwege, Germany). T3 and T7 RNA polymerase, RNase A, RNase T1, the cell proliferation reagent WST-1, and DNase I were obtained from Roche Diagnostics (Mannheim, Germany). Human interferon-γ (IFN-γ), interleukin (IL) 1β and tumor necrosis factor-α were from Strathmann, Hannover, Germany. Fetal calf serum (FCS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from PAN-Systems, Nürnberg, Germany. The Dual-Luciferase Reporter Assay System and Passive Lysis buffer were purchased from Promega, Heidelberg, Germany. Restriction enzymes, Klenow DNA polymerase, dNTPs, NTPs and the PhosphoPlus® STAT-1α (Tyr701) Antibody Kit were from New England Biolabs, Frankfurt a. M., Germany. The anti-STAT3 (C20) was purchased from Santa Cruz Biotechnology, Heidelberg, Germany. The polyclonal anti-iNOS and anti-actin antibodies were obtained from Transduction Laboratories, Lexington, KY. The green tea extract was obtained from Indena, Milan, Italy.

Cell Culture, Cytokine Treatment, RNA Isolation and Nitrite Measurement

The human alveolar epithelial A549/8 cells, human colon carcinoma DLD-1 cells and human ECV304 cells were grown in DMEM with 5 to 10% fetal bovine serum, 2 mM L-glutamine, penicillin and streptomycin. For RNA isolation, they were plated onto 10 cm-diameter (58 cm^2/well) dishes, whereas those experiments involving luciferase activity determinations or NO production studies were performed with cells plated onto 6-well plates (9.6 cm^2/well) or 24-well plates (1.75 cm^2/well). Eighteen hours beforecytokine induction, cells were washed with PBS solution and incubated with DMEM containing 2 mM L-glutamine in the absence of serum and phenol red. After this preincubation time cells were pretreated with different concentrations of green tea extract for 1 h. A549/8 and DLD-1 cells were induced with a cytokine mixture (CM) composed of IFN-γ (100 U/ml), IL-1β (50 U/ml) and tumor necrosis factor-α (10 ng/ml) for the time periods indicated in the figure legends. Then the supernatant of the cells (300 μl) was used to measure NO_2^- by the Griess reaction, and cells were processed for RNA isolation by guanidinium thiocyanate/phenol/chloroform extraction as described previously (Chomczynski and Sacchi, 1987; Kleinert et al., 1998).

RNase Protection Analysis

For the generation of radiolabeled human iNOS-, human β-actin, and human GAPDH-antisense probes for RNase protection assays, 0.5 μg of the linearized plasmids pCR_iNOS_human (Kleinert et al., 1996), pCR_β-actin_human (Kleinert et al., 1996), or pXcm_GAPDH-_human (Witteck et al., 2003) were in vitro-transcribed using T3 or T7 RNA polymerase and [α-^{32}P]UTP. To quantify human iNOS mRNA levels, RNase protection experiments were performed as described previously (Kleinert et al., 1998). In all experiments, GAPDH or β-actin mRNA expression was determined for normalization purposes. Densitometric analyses were performed using a Molecular Imager FX Pro (Bio-Rad, Munich, Germany). The protected fragments of human iNOS, β-actin, and human GAPDH-mRNA were 386 nt or 195 nt, 108 nt, and 105 nt, respectively.

Analysis of the Human iNOS Promoter Activity and Human eNOS Promoter Activity

To investigate the effect of green tea extract or antioxidants, such as NAC or ASC, on cytokine-induced iNOS promoter activity or GAS-dependent promoter activity, pools of stably transfected A549/8 or DLD-1 cells [containing a 16-kb fragment of the human iNOS promoter cloned in front of a luciferase reporter gene or pGAS-Luc (Yao et al., 2003)] were incubated for 18 h with DMEM without FCS and without phenol red. Before cytokine induction, the cells were pretreated with green tea extract, NAC, or ASC in the concentrations indicated. After CM incubation for 4.5 h in the presence or absence of

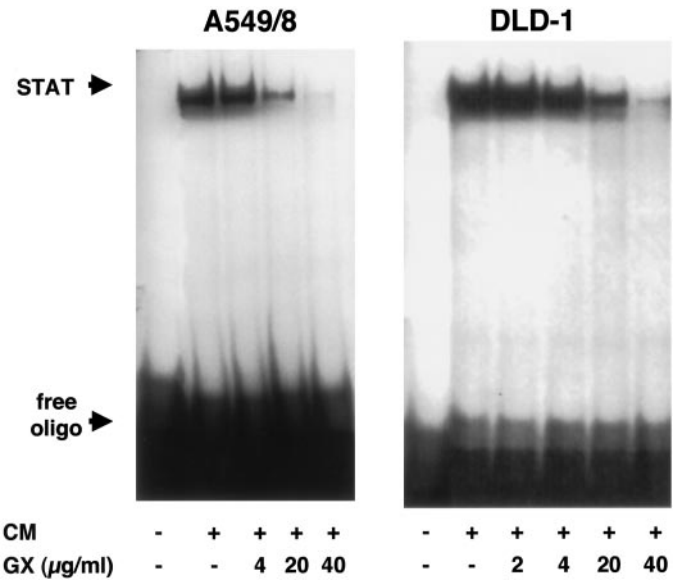

Fig. 1. Effect on green tea extracts on cytokine-induced STAT-1α DNA binding activity n human A549/8 and DLD-1 cells. Gel shift experiments using a radiolabeled oligonucleotide containing a consensus STAT-1α binding site and nuclear extracts from untreated A549/8 and DLD-1 cells, or A549/8 and DLD-1 cells stimulated with a CM, consisting of 100 U/ml IFN-γ, 50 U/ml IL-1β and 10 ng/ml TNF-α, in the presence or absence of GX (2–40 μg/ml). The position of the DNA protein complex (STAT) and the free oligonucleotide is indicated. The gel is representative of four gels showing similar results.

green tea extract, NAC or ASC cells were lysed in 1× passive lysis buffer.

To investigate the effect of green tea extract on the constitutive human eNOS promoter activity, pools of stably transfected ECV 304 cells [containing a 3.5-kb fragment of the human eNOS promoter cloned in front of a luciferase reporter gene; (Yao et al., 2003)] were incubated for 24 h with DMEM without FCS and without phenol red. Then the cells were incubated with or without different concentrations of green tea extract for 4 to 5 h, before being lysed in 1× passive lysis buffer.

Firefly luciferase activity was determined using the dual-luciferase assay kit. Protein concentrations of the extracts were determined by Bradford reagent using bovine serum albumin as standard. Protein content of the extracts was used for normalization of the luciferase activity.

Western Blot Experiments

Detection of STAT-1α Phosphorylation. A549/8 and DLD-1 cells incubated with or without CM in the presence or absence of green tea extract for 0.5 h were lysed on ice with 20 mM HEPES, pH 7.4, 420 mM NaCl, 1% Nonidet P40, 1 mM EGTA, and 1 mM EDTA for 15 min. After centrifugation for 15 min at 12,000 rpm, proteins (50 μg/lane) were fractionated by SDS-polyacrylamide gel electrophoresis in a 7.5% gel, electroblotted onto polyvinylidene difluoride membrane (Millipore S.p.A., Rome, Italy), and reacted with anti-STAT-1α phosphotyrosine701 diluted 1:700 (New England Biolabs, Hetchin, England) and after stripping with an anti-STAT-1α or an anti-actin antibody diluted 1:1000 according to standard procedures. Immune complexes were detected by using anti-rabbit horseradish peroxidase-conjugated immunoglobulin for detection of the primary antibody. The immunoreactive proteins on the blot were detected by the enhanced chemiluminescence detection system (ECL; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Detection of iNOS Protein. DLD-1 cells incubated with or without CM in the presence or absence of green tea extract for 24 h were lysed on ice with radioimmunoprecipitation assay buffer (20 mM HEPES, pH 7.4, 420 mM NaCl, 1% Nonidet P40, 1 mM EGTA, and 1 mM EDTA) for 20 min. After centrifugation for 20 min at 12,000 rpm, proteins (50 μg/lane) were fractionated by SDS-polyacrylamide gel electrophoresis in a 7.5% gel, electroblotted onto polyvinylidene difluoride membrane (Millipore S.p.A.), and reacted with a polyclonal anti-iNOS antibody (BD Biosciences Transduction Laborato-

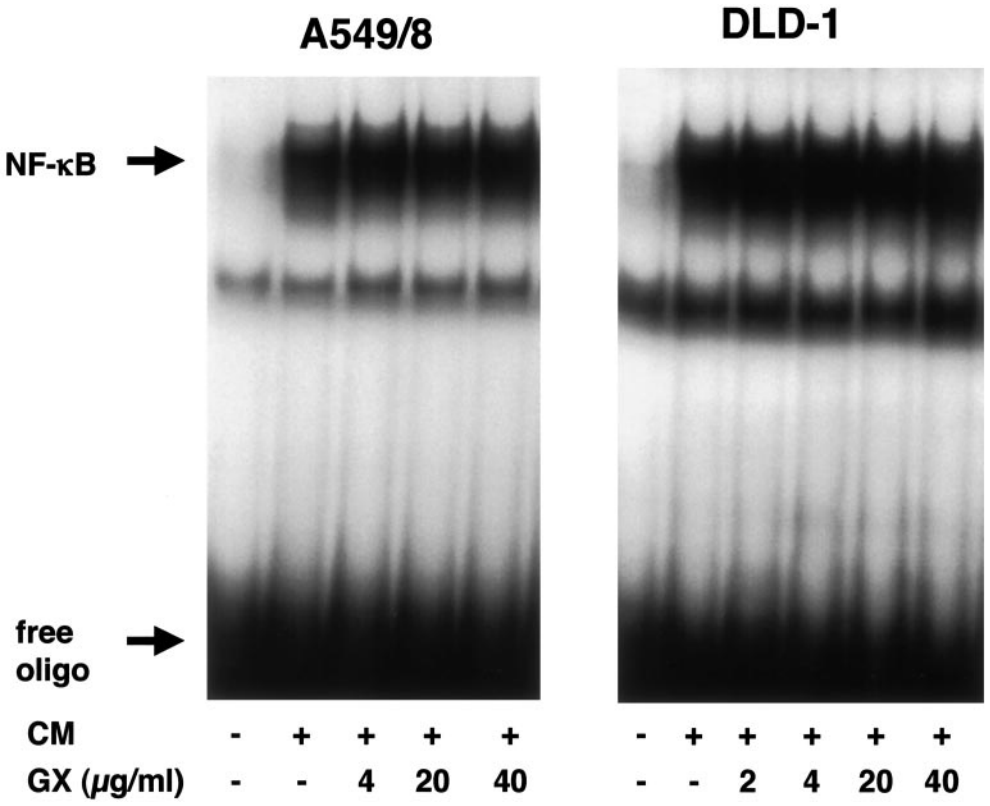

Fig. 2. Effect on green tea extracts on cytokine-induced NF-κB DNA binding activity in human A549/8 and DLD-1 cells. Gel shift experiments using a radiolabeled oligonucleotide containing a consensus NF-κB binding site and nuclear extracts from untreated A549/8 and DLD-1 cells or A549/8 and DLD-1 cells stimulated with a CM in the presence or absence of GX (2–40 μg/ml). The position of the DNA protein complex (NF-κB) and the free oligonucleotide is indicated. The gel is representative of three gels showing similar results.

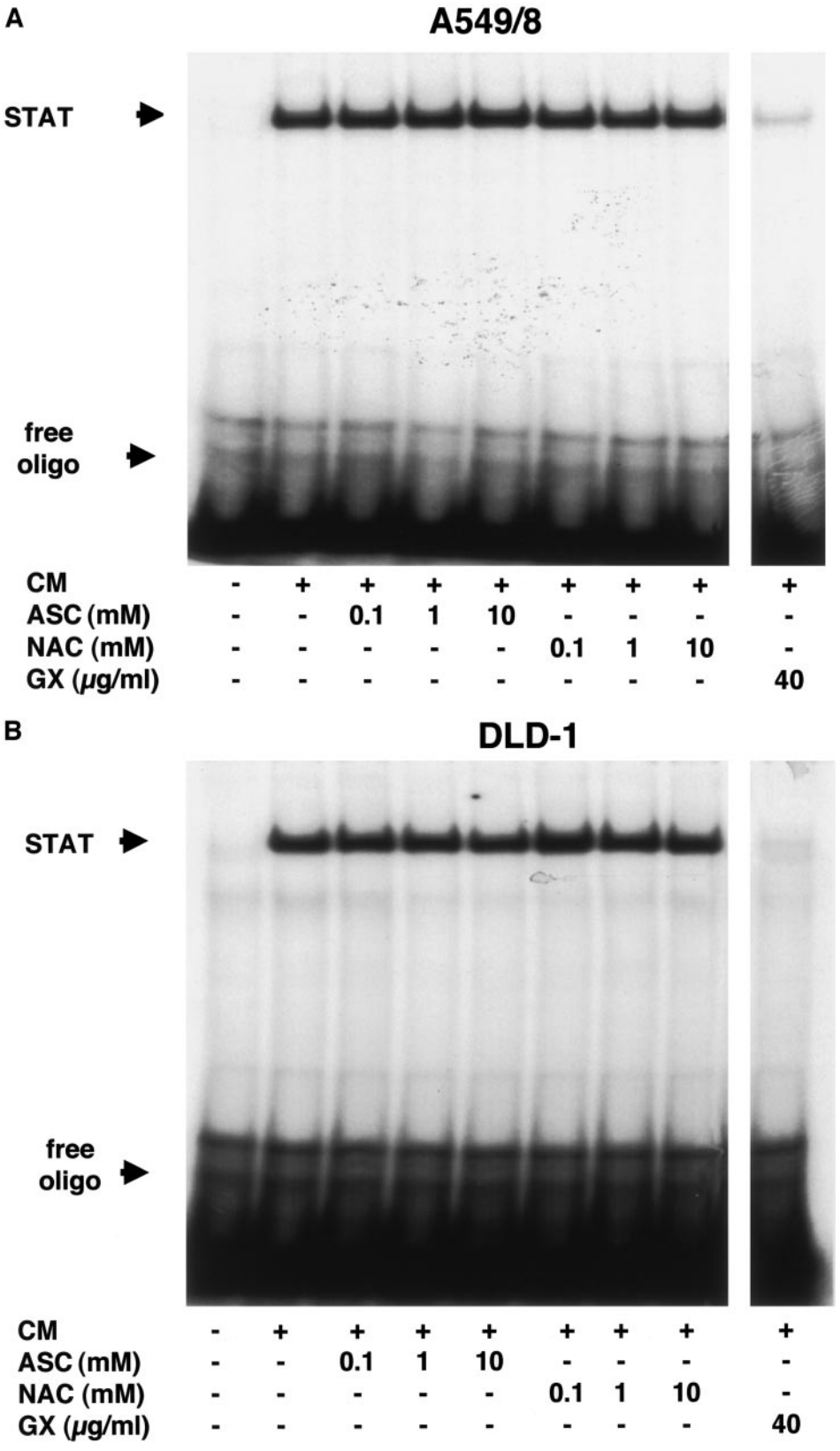

Fig. 3. Effect of antioxidants on cytokine-induced STAT-1α DNA binding activity in human A549/8 and DLD-1 cells. Gel shift experiments using a radiolabeled oligonucleotide containing a consensus STAT-1α binding site and nuclear extracts from untreated A549/8 (A) and DLD-1 cells (B), or A549/8 and DLD-1 cells stimulated with a CM in the presence or absence of ASC (0.1–10 mM), NAC (0.1–10 mM), or GX (40 μg/ml). The position of the DNA protein complex (STAT) and the free oligonucleotide is indicated. Each gel is representative of three gels showing similar results.

ries, Lexington, KY) diluted 1:200 and after stripping with anti-STAT-1α or anti-STAT3 antibody diluted 1:1000 according to standard procedures. Immune complexes were detected by using anti-rabbit horseradish peroxidase-conjugated immunoglobulin for detection of the primary antibody. The immunoreactive proteins on the blot were detected by ECL detection system (Amersham Biosciences).

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared from cells treated with or without cytokines in the presence or absence of green tea extract, NAC, or ASC according to Osborne et al. (1997) The protease inhibitors leupeptin (10 μg/ml), antipain (5 μg/ml), and pepstatin (5 μg/ml) and phenylmethylsulfonyl fluoride (1 mM) were present. Protein concentration in the nuclear extracts was determined by using the method of Bradford (1976). Nuclear extract (10 μg) were incubated at room temperature for 20 min with a ^{32}P-labeled double-stranded oligonucleotide containing the STAT-1α binding site (sis-inducible factor-binding recognition element, SIE/m67) from the c-fos promoter (5′-gtcgaCATTTCCCGTAAATCg-3′; lower case letters represent additional sequences not present in these promoters) (Wagner et al., 1990), or the NF-κB binding sequence from the IL-6 promoter (5′-gatcCAGAGGGGACTTTCCGAGt-3′) (Promega, Milan, Italy), in a 15-μl reaction mixture containing 20 mM HEPES, pH 7.9, 50 mM KCl, 10% glycerol, 0.5 mM dithiothreitol, 0.1 mM EDTA, 2 μg of poly(dI-dC), and 1 μg of salmon sperm DNA. Products were fractioned on a nondenaturing 5% polyacrylamide gel in Tris-borate/EDTA buffer (1.08% Tris, pH 8.3, 0.55% boric acid, and 20 mM EDTA).

Determination of Cellular Viability

The effect of green tea extract on cell viability was measured by a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases (cell proliferation reagent WST-1; Roche).

Cells were seeded at the concentration of 10^3 cells per well in flat-bottomed, 96-well plates in 0.5 ml of medium. Cells were preincubated in medium containing 2% FCS for 18 h. Then cells were treated with or without a CM in the presence or absence of green tea extract. After 24 h of incubation, 10 μl of the WST-1 solution was added, and the cells were incubated for additional 4 h. Afterward, the optical density at 450 nm was determined.

Calculations

All data are presented as mean ± S.E.M. Differences between means were tested for statistical significance using factorial analysis of variance followed by Fisher's protected least significant difference test as the post hoc test (StatView software; SAS Institute, Cary, NC). Concentrations of compounds producing half-maximal inhibition were determined using Prism 3 software (GraphPad Software Inc., San Diego, CA).

Results

Green Tea Extract Inhibited STAT-1α DNA Binding, but Did Not Affect NF-κB DNA Binding. To study the effect of green tea extract on the DNA binding activity of inflammatory transcription factors in A549/8 and DLD-1 cells, we performed electrophoretic mobility shift assays for STAT-1α and NF-κB, transcription factors known to play a critical role in human iNOS expression. As shown in Figs. 1 and 2, in both cell lines, cytokine treatment rapidly induced both STAT-1α and NF-κB DNA binding activity. However, only STAT-1α DNA binding was reduced in a concentration-dependent manner by coincubation of cytokine-treated cells with green tea extract (Fig. 1); activation of NF-κB DNA binding remained unchanged (Fig. 2).

Incubation of A549/8 or DLD-1 Cells with the Potent Antioxidants NAC and ASC Did Not Change Cytokine-Induced STAT-1α DNA Binding. Because green tea extract is known to contain different compounds with antioxidative properties, we analyzed the effect of the potent antioxidative compounds NAC (0.01–10 mM) and ASC (0.01–10 mM) on cytokine-induced activation of STAT-1α DNA binding activity. As shown in Fig. 3, neither NAC nor ASC incubation changed the STAT-1α DNA binding activity induced by cytokine treatment in A549/8 (Fig. 3A) or DLD-1 cells (Fig. 3B). NAC and ASC also did not modify cytokine-induced activity of an INF-γ–dependent promoter containing five γ-activated sites (GAS, binding site of STAT-1α) stably

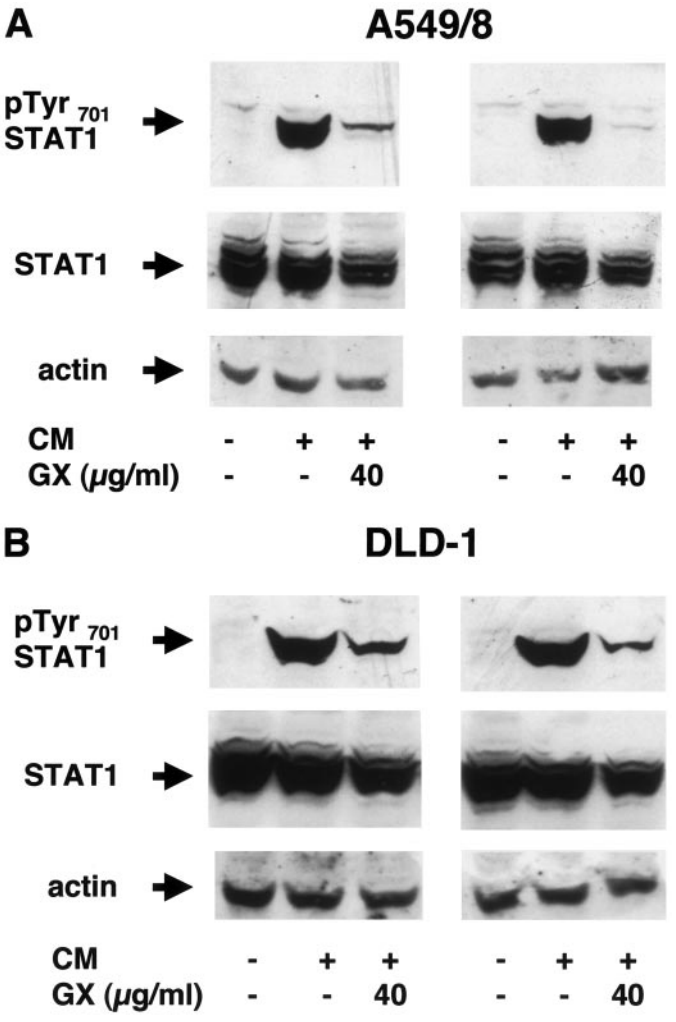

Fig. 4. Effect of green tea extract on cytokine-induced activation of STAT-1α in human A549/8 and DLD-1 cells. A, A549/8 cells were preincubated for 18 h with DMEM (without FCS and phenol red). Then, GX (40 μg/ml) was added for 1 h. Subsequently, the cells were incubated for an additional 30 min with or without a CM in the presence or absence of green tea extract. Nuclear extracts were prepared and 50 μg of nuclear proteins were analyzed for STAT-1α tyrosine-(Tyr701) phosphorylation by Western blotting using the PhosphoPlus STAT-1α (Tyr701) antibody kit as described under *Materials and Methods*. The blots shown are representative for four experiments showing the same results. Top, a Western blot using a STAT-1α-tyrosine-(Tyr701)-phosphate-specific antibody. Middle, the same blot (after stripping) using a STAT-1α antibody, which detects phosphorylated and nonphosphorylated STAT-1α. Bottom, the same blot (after stripping) using an actin antibody. B, experiments similar to those in A. Results are generated with nuclear extracts obtained from DLD-1 cells.

transfected in A549/8 cells or human iNOS promoter activity (data not shown).

Green Tea Extract Inhibited the Activation of STAT-1α in A549/8 Cells and DLD-1 Cells. To further confirm the mechanism of action of the green tea extract on DNA binding activity in DLD-1 and A548/9 cells, we studied STAT-1α activation by immunoblot analyses using an antibody that specifically detects the tyrosine (Tyr701)-phosphorylated form of STAT-1α (Darnell et al., 1994; Ihle et al., 1994; Ihle, 1995). This phosphorylation is critical for the activation of the STAT-1α protein and enables it to dimerize, to migrate into the nucleus, and to bind to specific STAT-1α binding sites on the DNA (the GAS elements). Incubation of A549/8 and DLD-1 cells for 30 min with a CM consisting of 100 U/ml interferon-γ, 50 U/ml IL-1β, and 10 ng/ml tumor necrosis factor-α, resulted in a marked enhancement of STAT-1α tyrosine phosphorylation (Fig. 4). Coincubation of CM-treated A549/8 and DLD-1 cells with green tea extract (40 μg/ml) markedly inhibits the Tyr701 phosphorylation, in line with the inhibition of DNA binding activity seen upon treatment with green tea (Fig. 1).

Green Tea Extract Inhibited Human iNOS mRNA- and Protein Expression and Nitrite Production in A549/8 and DLD-1 Cells. As shown in Fig. 5, green tea extract (GX) inhibited CM-elicited iNOS mRNA expression in A549/8 and DLD-1 cells in a concentration-dependent manner, as measured by RNase protection assay. In addition, the cytokine-induced expression of iNOS protein was inhibited

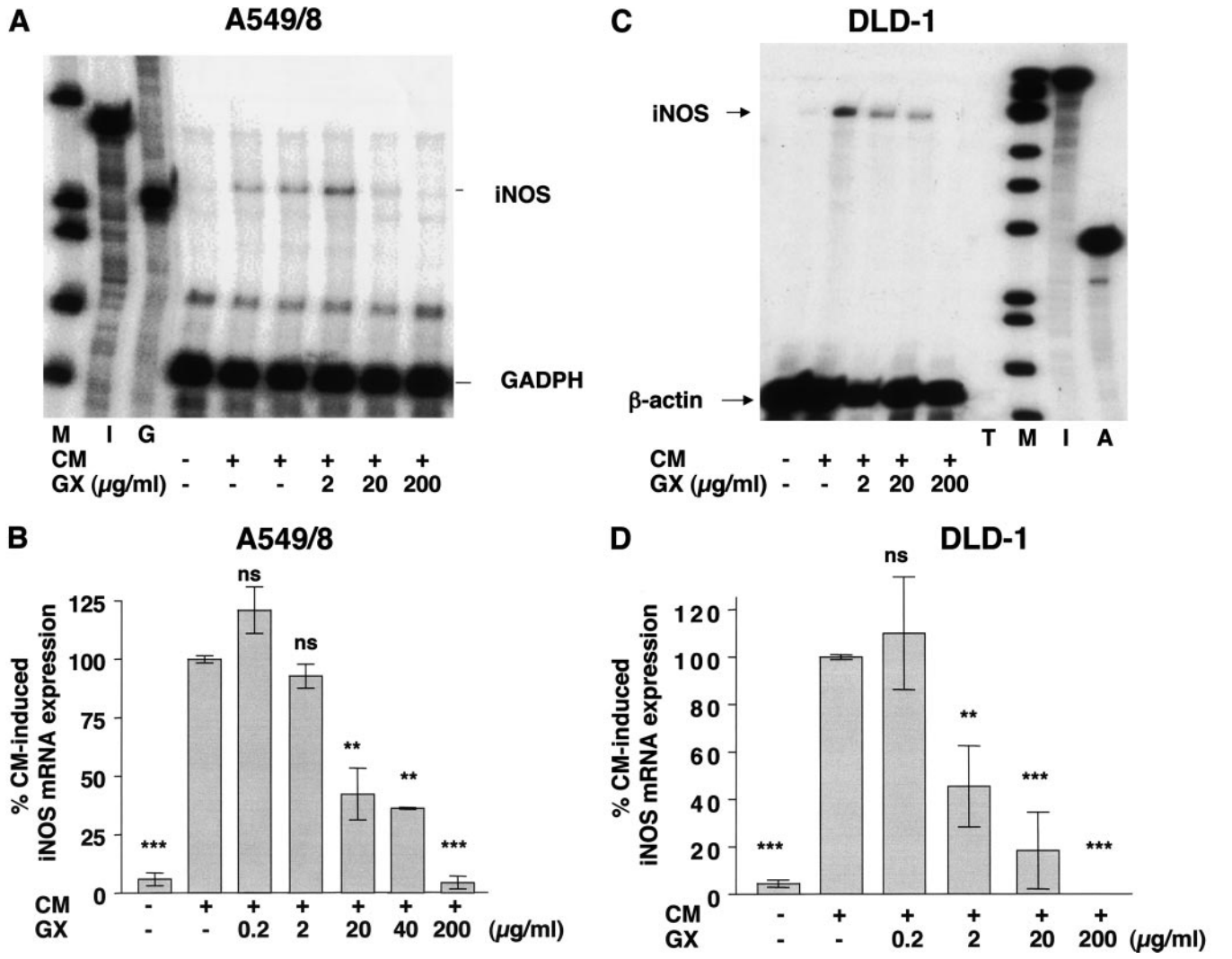

Fig. 5. Effect of green tea extract on cytokine-induced human iNOS mRNA expression in epithelial A549/8 and DLD-1 cells A, representative RNase protection assay performed with total RNA from human A549/8 cells incubated for 8 h in serum-free medium alone or in serum-free medium containing a CM in the presence or absence of GX (2–200 μg/ml). Cells were preincubated for 18 h in serum-free medium and for an additional 1 h with the different concentrations of GX. Experiments were performed using antisense RNA probes for human iNOS and GAPDH, which was used for normalization. The positions of the protected iNOS- and GAPDH fragments are indicated. M, molecular weight standard; ΦX174-restricted with HinFI; I, iNOS antisense probe; G, GAPDH antisense probe. B, densitometric analyses of six different gels. Columns and symbols (means ± S.E.M.) represent relative iNOS mRNA levels at the different concentrations of green tea extract (***, $p < 0.001$; ns, not significant versus CM). Half-maximal inhibition was achieved with 15.1 μg/ml. C, representative RNase protection assay performed with total RNA from human DLD-1 cells treated as described above for A549/8 cells. Experiments were performed using antisense RNA probes for human iNOS and β-actin, which was used for normalization. The positions of the protected iNOS- and β-actin fragments are indicated. T, tRNA control; M, molecular weight standard, ΦX174-restricted with HinFI; I, iNOS antisense probe; A, β-actin antisense probe. D, data obtained using RNA isolated from human DLD-1 cells. Columns and symbols (means ± S.E.M., $n = 5$) represent relative iNOS mRNA levels at the different concentrations of green tea extract (***, $p < 0.001$; ns, not significant versus CM). Half-maximal inhibition was achieved with 2.2 μg/ml.

by green tea extract (Fig. 6). Accordingly, inhibition of cytokine-induced NO production was also observed (Fig. 7) by measuring nitrite in cell supernatants using the Griess assay.

Green Tea Extract Inhibited Human iNOS Promoter Activity in Stably Transfected A549/8 and DLD-1 Cells. To analyze the effect of green tea extract on human iNOS promoter activity, we incubated A549/8 and DLD-1 cells stably transfected with pNOSII(16)Luc (de Vera et al., 1996; Hausding et al., 2000), with different concentrations of green tea extract. The plasmid pNOSII(16)Luc contains a 16-kb fragment of the human iNOS promoter, cloned in front of a luciferase reporter gene. In line with previous reports (Hausding et al., 2000; Witteck et al., 2003; Yao et al., 2003), CM incubation of these pools of stably transfected A549/8 and DLD-1 cells (Fig. 8) resulted in a 5-fold enhancement of luciferase expression. Green tea extract concentration-dependently inhibited the CM-induced iNOS promoter activity in these cells (Fig. 8).

Green Tea Extract Has No Effect on the Viability of Human A549/8 and DLD-1 Cells. To determine whether the inhibition of iNOS expression was a result of a cytotoxic action of the green tea extract, the effect of green tea extract on the cellular viability of A549/8 and DLD-1 cells was analyzed using the WST-1 cell viability assay (Roche). Incubation of A549/8 and DLD-1 cells with different concentrations of green tea extract resulted in no reduction of cell viability (see Fig. 9).

Green Tea Extract Had Only a Modest Effect on the Activity of the Human 3.5-kb eNOS Promoter. To determine whether the inhibition of iNOS expression was a result of the inhibition of general transcription factors, the effect of green tea extract on a constitutively active promoter was analyzed. For this purpose ECV-pNOS III-Hu-3500-Luc-neo cells (Yao et al., 2003) were used. These cells derived from human ECV304 cells, which had been transfected with the plasmid pNOS III-Hu-3500-Luc-neo, containing a 3.5-kb fragment of the human eNOS promoter, cloned in front of a luciferase reporter gene. This promoter displayed a significant constitutive activity in the stably transfected cells (see Fig. 10). Incubation of ECV-pNOS III-Hu-3500-Luc-neo cells

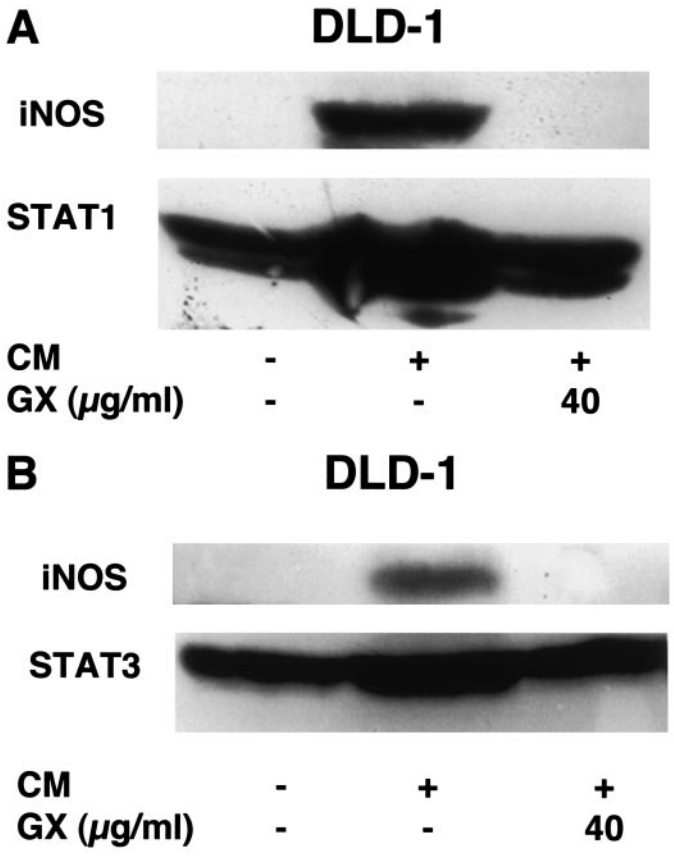

Fig. 6. Effect of green tea extract cytokine-induced iNOS protein expression in human DLD-1 cells. DLD-1 cells were preincubated for 18 h with DMEM (without FCS and phenol red). Then cells were incubated for 24 h with or without a CM in the presence or absence of GX (40 μg/ml). Total cell extracts (80 μg of total proteins) were analyzed for iNOS- and (for normalization) for STAT-1α and STAT3 protein expression by Western blotting using a polyclonal anti-iNOS antibody, a monoclonal STAT-1α antibody, and a polyclonal STAT3 antibody, respectively, as described under *Materials and Methods*. The blot shown is representative for three experiments showing the same results.

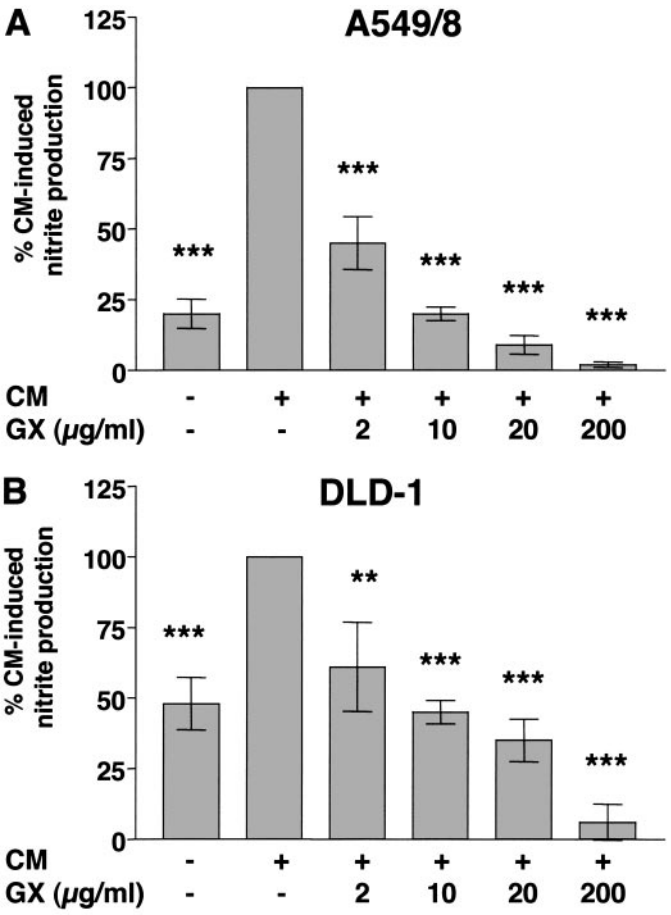

Fig. 7. Effect of green tea extract on cytokine-induced nitrite production in human epithelial A549/8 and DLD-1cells. A, statistical analysis of 9 different Griess assays performed with supernatants from untreated A549 cells or cells stimulated for 24 h with a CM in the presence or absence of GX (2–200 μg/ml). Cells were preincubated for 18 h in serum-free medium and additionally for 1 h with or without GX. Columns and symbols (means ± S.E.M.) represent the relative nitrite levels determined under the conditions indicated (100% corresponds to 400 pmol/ml/24 h; ***, $p < 0.001$ versus CM). Half-maximal inhibition was achieved with 1.5 μg/ml. B, data generated with DLD-1 cells. Columns and symbols (means ± S.E.M.) represent the relative nitrite levels determined under the conditions indicated (100% corresponds to 400 pmol/ml/24 h; **, $p < 0.01$; ***, $p < 0.001$ versus CM). Half-maximal inhibition was achieved with 4.7 μg/ml.

with the highest concentrations of green tea extract resulted in only a moderate reduction in eNOS promoter activity at high concentrations of green tea extract (control cells, 100%; green tea extract, 200 μg/ml, 58.3 $\pm$ 6.5%, n = 12).

Discussion

An aberrant expression of iNOS leading to inappropriate NO production has been implicated in human autoimmune and pro-inflammatory diseases, such as rheumatoid arthritis, asthma, short-term and chronic lung diseases, neurodegenerative diseases (such as Alzheimer's and Parkinson's diseases), as well as inflammatory bowel diseases, tumor development, and septic shock (Kröncke et al., 1998). One possible way to inhibit excessive NO production by iNOS is specific enzyme inhibition. However, the use of inhibitors of the iNOS enzyme activity is reported to provoke secondary undesired effects (Husain, 2002; Kang et al., 2002). An alternative approach to block iNOS-dependent NO production is the suppression of iNOS induction (Yao et al., 2003). In contrast to eNOS and neuronal nitric-oxide synthase, which are constitutively expressed, expression of iNOS has to be induced (Kleinert et al., 2000). The transcription factors involved in the expression of the three nitric-oxide synthase isoforms differ markedly (Kleinert et al., 2000). Therefore, it seems likely that specific inhibitors of iNOS expression can be generated. In recent years, several authors described the dependence of human iNOS induction on the IFN-γ–JAK–STAT pathway (Kleinert et al., 1998, 2000; Dell'Albani et al., 2001; Ganster et al., 2001; Ohmori and Hamilton, 2001). Therefore, the IFN-γ–JAK–STAT pathway seems to be a reasonable target for the development of inhibitors of iNOS expression.

EGCG, the main component of green tea, has been shown to inhibit the activation of the STAT-1α transcription factor

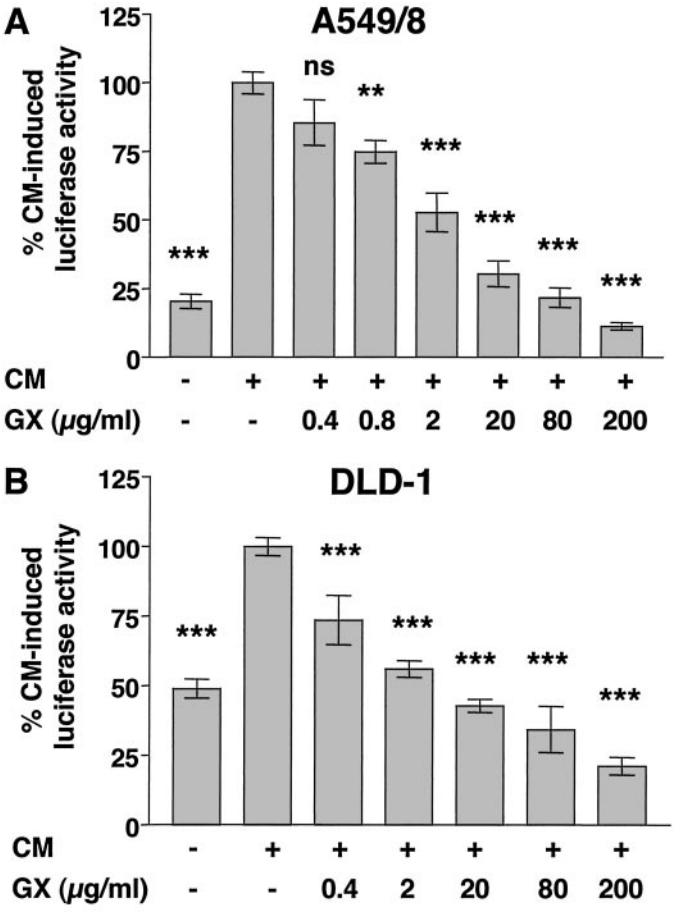

Fig. 8. Effect of green tea extract cytokine-induced human iNOS promoter activity in stably transfected A549/8 and DLD-1 cells A549/8 and DLD-1 cells, stably transfected with pNOSII(16)Luc (de Vera et al., 1996), a construct containing a 16-kb fragment of the human iNOS promoter in front of a luciferase reporter gene, were preincubated in serum-free medium for 18 h and then with GX (0.4–200 μg/ml) for an additional 1 h. Then, cells were incubated with a cytokine mixture (CM) for another 4 h in the presence or absence of the green tea extract. Luciferase activity and protein content in cell extracts were determined. A, columns and symbols (means $\pm$ S.E.M.; n = 18) represent the relative luciferase activities (percentage of the CM effect) in extracts of stably transfected A549/8 cells, incubated with GX (***, $p < 0.001$; **, $p < 0.01$; ns, not significant versus CM). Half-maximal inhibition was achieved with 1.5 μg/ml. B, columns and symbols (means $\pm$ S.E.M.; n = 16) represent the relative luciferase activities (percentage of the CM effect) in extracts of stably transfected DLD-1 cells, incubated with GX (***, $p <$ 0.001; ns, not significant versus CM). Half-maximal inhibition was achieved with 1.3 μg/ml.

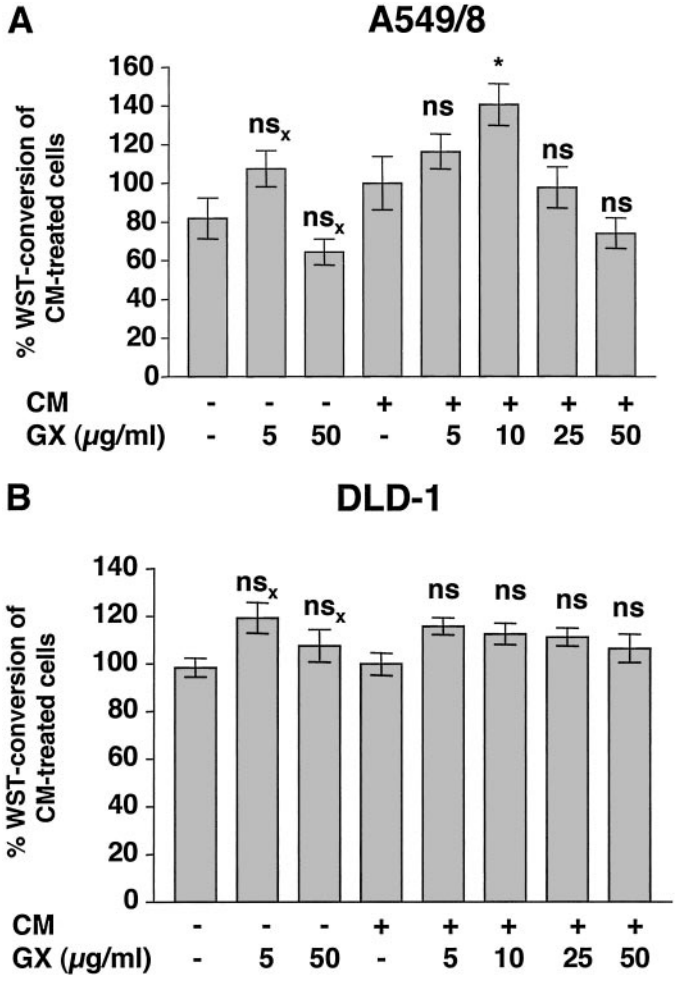

Fig. 9. Effect of green tea extract on the cell viability of A549/8 and DLD-1 cells. A549/8 and DLD-1 cells were preincubated in medium containing 2% FCS for 18 h and then with or without a CM in the presence or absence of GX (5–50 μg/ml) for an additional 24 h. Then, 10 μl of the WST-1 solution (Roche) was added and the optical density at 450 nm was analyzed after an additional 4 h. Columns and symbols (means $\pm$ S.E.M.; n = 24) represent relative optical densities at 450 nm (percentage of CM-treated) in the presence of the different concentrations of the green tea extract (*, $p < 0.05$; ns, not significant versus CM; ns$_x$, not significant versus control).

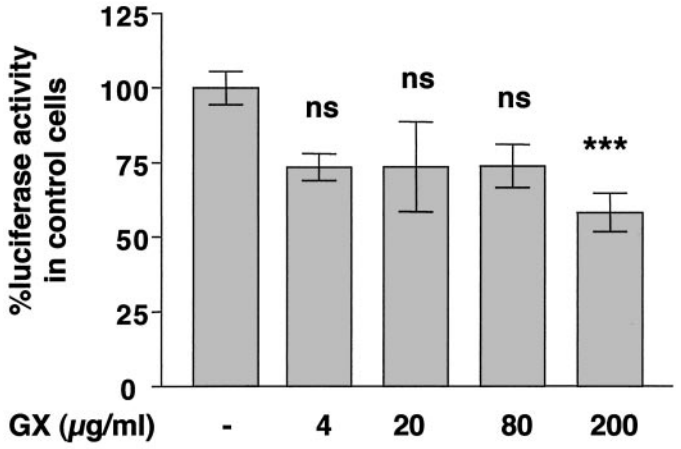

Fig. 10. Effect of green tea extract on constitutive human eNOS promoter activity in stably transfected ECV-pNOS III-Hu-3500-Luc-neo cells ECV-pNOS III-Hu-3500-Luc-neo cells (Yao et al., 2003) stably transfected with pNOS III-Hu-3500-Luc-neo, a construct containing a 3.5-kb human eNOS promoter fragment in front of a luciferase reporter gene were preincubated with serum-free medium for 18 h. Then, cells were incubated with or without GX (4–200 μg/ml) for an additional 6 h in serum-free medium. Cell extracts were prepared and luciferase activity and protein content of the extracts were determined. Columns and symbols (means ± S.E.M.; $n = 12$) represent relative luciferase activities (percentage of control cells) in the presence of the different concentrations of the green tea extract (***, $p < 0.001$; ns, not significant versus control). With 200 μg/ml green tea extract, the human eNOS promoter activity was reduced to 60% of control.

by suppressing Tyr701 phosphorylation of the STAT-1α protein (Menegazzi et al., 2001). Therefore, we focused our attention on green tea extract, a natural product whose main component is EGCG, to identify a new principle to inhibit iNOS expression. Green tea extract is the most used ingredient for all the food and cosmetic green tea-flavored products on the market. The maximum green tea extract concentrations investigated are in the range of the plasma concentrations found in humans drinking 6 to 10 cups of green tea per day (Yang et al., 1997).

As a cellular model, we choose two epithelium-derived human cell lines: DLD-1 colon carcinoma and A548/9 alveolar carcinoma cells. As reported already, there are many diseases in the colon and in the lung that seem to be correlated to an aberrant expression of the iNOS enzyme (Barnes and Liew, 1995; Guslandi, 1998; Kröncke et al., 1998; Cavicchi and Whittle, 1999; van Straaten et al., 1999; Kleinert et al., 2000; Bogdan, 2001).

Data presented in the current work clearly show that, in line with previous reports on EGCG, green tea extract also exerts a concentration-dependent, specific inhibitory action toward IFN-γ–elicited STAT-1α activation in epithelial cell lines (Figs. 1 and 4). Because the potent antioxidative compounds *N*-acetyl-cysteine and ascorbate did not influence cytokine-induced STAT-1α DNA binding, this green tea-mediated inhibition of STAT-1α activity is unlikely to result from a general antioxidative effect of the extract. The mechanism of the green tea extract-mediated inhibition of STAT-1α activation seems to operate mainly via inhibition of cytokine induced STAT-1α tyrosine phosphorylation (see Fig. 4). In addition, however, a green tea extract-related reduction of STAT-1α expression may partly explain the effect. Furthermore, green tea-mediated inhibition of cytokine-induced iNOS promoter activity (Fig. 8) leads to the inhibition of iNOS synthesis (mRNA and protein; Figs. 5 and 6) and NO production (Fig. 7) in these cell lines. In all cases, the concentrations of green tea extract required for half-maximal inhibition displayed no cytotoxicity in A549/8 and DLD-1 cells (Fig. 9) and were below the concentrations showing a significant effect on the activity of the constitutively active eNOS promoter (Fig. 10). Only at very high concentrations of green tea extract was a relevant effect on eNOS promoter activity seen (Fig. 10). These results show that the inhibitory effect of green tea extract on iNOS expression is unlikely to result from inhibition of general transcription or cell viability. Inhibition of STAT-1α activation and thereby iNOS expression by the green tea seems to be a rather specific effect. Inhibition of the INF-γ–JAK–STAT-1α pathway by green tea extract may be an efficient way to reduce massive iNOS-derived NO production. In view of the fact that drugs currently used in the treatment of inflammatory bowel disease, such as steroids, do not substantially reduce intestinal NO synthesis (Leonard et al., 1998), the above effect of green tea may be of therapeutic relevance. Furthermore, in chronic inflammatory lung diseases, especially in neonates, the use of steroids has a number of undesired effects (Shanley et al., 2002). Therefore, in inflammatory diseases, green tea may be a suitable adjuvant to reduce the doses of steroids needed.

In contrast to other reports (Lin and Lin, 1997; Singh et al., 2002), we observed no inhibitory effect of green tea extract on the LPS/cytokine-induced NF-κB DNA binding activity. Therefore, in human epithelial A549/8 or DLD-1 cells, inhibition of NF-κB activity is unlikely to be involved in the inhibition of iNOS expression by green tea extract as described for human chondrocytes or murine macrophages. In addition, as shown in our previous reports, induction of iNOS expression depends only partially on NF-κB activation in A549/8 or DLD-1 cells (Kleinert et al., 1998). Therefore, the effect of green tea extract (or ECGC) on iNOS expression seems to be cell-specific.

In conclusion, green tea extract may be as effective as EGCG in inhibiting iNOS expression by suppressing IFN-γ–elicited STAT-1α activation. As a possible anti-inflammatory drug, green tea extract may be more suitable than EGCG because green tea extract is far more economical than EGCG and, more important, is more stable because of the presence of different antioxidants in the extract.

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