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Caffeic Acid Phenethyl Ester Induces Leukocyte Apoptosis, Modulates Nuclear Factor-Kappa B and Suppresses Acute Inflammation

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Key Words
NF-ÎB · p65 · Apoptosis · Acute inflammation · Caffeic acid phenethyl ester

Abstract
Nuclear factor kappa-B (NF-ÎB) is a heterodimeric transcription factor with a pivotal role in orchestrating immune and inflammatory processes. Inflammatory cytokines and prostanoids activate NF-ÎB, which, in turn, stimulates expression of cytokines, proteases, adhesion molecules and other inflammatory mediators. Caffeic acid phenethyl ester (CAPE) is a compound that modulates nuclear binding of the NF-ÎB p65 subunit (RelA). To determine whether CAPE decreases the viability of cells participating in host defense, we first tested its in vitro effect on a glucocorticoid-sensitive and -resistant cell line of lymphoid origin. CAPE induced apoptotic cell death in a dose-dependent fashion and to a similar extent in both cell lines. Furthermore, a low concentration of CAPE decreased the LD_{50} of dexamethasone by 3- to 5-fold. Since therapeutic induction of apoptosis of activated inflammatory cells holds the attraction of destroying effector cells safely without secondary tissue damage, we examined the effects of CAPE in a rat model of carrageenin-induced subcutaneous inflammation. Local administration of CAPE resulted in increased leukocyte apoptosis and marked reduction in exudate leukocyte, neutrophil and monocyte concentrations at the inflammatory site. CAPE decreased expression of cytosolic IÎB· and increased nuclear translocation of p65. These findings may suggest that novel anti-inflammatory therapies can be based upon activation of NF-ÎB-mediated transcription of genes curbing the inflammatory response and that CAPE or its analogs hold therapeutic promise.

Introduction

Nuclear factor kappa-B (NF-ÎB) was first identified in 1986 as a constitutively active transcription factor binding the kappa light chain immunoglobulin enhancer in B cells; soon thereafter, it was found to be ubiquitously present and to participate in a major fashion in immune and inflammatory processes [1, 2]. NF-ÎB is not a single protein but a collection of dimers composed of members of the Rel family of DNA-binding proteins (transcription factors) that contain five distinct members: c-Rel, RelB, p50, p52, and p65 (RelA). These proteins form a combinatorially diverse array of hetero- and homodimers that regulate the expression of a large number of genes. The most common inducible NF-ÎB form is a p50:p65 heterodimer which is held in the cytoplasm in an inactive state, bound to ÎB inhibitor proteins (IÎBs). Upon activation of the cell by various stimuli, specific kinases (IKK-1, IKK-2) phosphorylate IÎBs in a site-specific manner [3],

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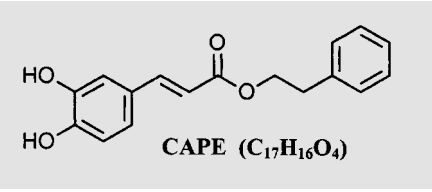

Fig. 1. Molecular structure of CAPE.

causing detachment from NF-ÎB, ubiquitination and proteasome-mediated degradation. The liberated NF-ÎB dimers translocate to the nucleus where they bind DNA at consensus decameric sequences, the ÎB sites, in the regulatory regions of target genes. The biological functions of NF-ÎB are diverse and include embryonal development, response to stress, and inflammation. NF-ÎB activation occurs in response to various nonimmune and immune stimuli, and is rapid and highly flexible. This activation has a dual function in the regulation of various immune and inflammatory processes: not only does it lead to coordinated expression of genes whose products promote inflammatory responses [4–6], but it also induces apoptosis of activated immune cells [3, 7, 8], thereby limiting the extent and duration of inflammation.

Neutrophils play a pivotal role in inflammation. Trapped at the inflammatory site, these cells eventually undergo apoptosis [9], during which a series of sequential changes occur: chromatin fragmentation, nuclear condensation, cytoplasmic shrinkage and alteration of plasma membrane. Our understanding of programmed cell death in health and disease is far from complete, and the challenge of converting that understanding into new therapeutic modalities has only begun to be approached. Although the role of NF-ÎB in the induction of apoptosis during inflammation is complex, it is certain that both B- and T-cell immune responses require NF-ÎB for their activation. Modulation of NF-ÎB activity in either direction, either inhibition or stimulation, may result in impaired cellular functions and apoptotic cell death [10–12]. In light of the apparent role of p65 in the onset and propagation of acute inflammation and of previous work showing a requirement for p65 in the initiation of immune responses through activation of antigen-presenting cells [13], we investigated whether administration of caffeic acid phenethyl ester (CAPE), a hydroxylated flavonoid relative (fig. 1), could lead to attenuation of the inflammatory response. This compound was extensively evaluated previously and was found to decrease IÎB resynthesis and to modulate nuclear p65 translocation; its effects on NF-ÎB activation are specific and not seen with other essential transcription factors such as AP-1, TFIID or Oct-1 [14]. In this study, we report on the effects of CAPE on cell survival and induction of apoptosis in cell lines of lymphoid origin in vitro, as well as its ability to suppress the acute inflammatory response in vivo.

Materials and Methods

Reagents and Animals. CAPE was synthesized as previously described [15]. RPMI-1640, phosphate buffered saline (PBS) and fetal bovine serum were obtained from Life Technologies, Gaithersburg, Md., USA. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay and diaminobenzidine were obtained from Sigma, St. Louis, Mo., USA. The in situ Cell Death Detection Kit was supplied by Boehringer-Mannheim, Indianapolis, Ind., USA. Proteinase K was obtained from Gibco BRL, Gaithersburg, Md., USA. Mararaglas 55 was from Ladd Industries, Burlington, Vt., USA. Polyclonal anti-IÎB· and anti-p65 antibodies were supplied by Oncogene Research, Cambridge, Mass., USA. Adult (130–150 g) male jugular vein-cannulated Sprague-Dawley rats were obtained from Taconic Farms, Germantown, N.Y., USA. The study was approved by the NICHD Animal Care and Use Committee. Animals were treated in accordance with guidelines contained in 'The care and use of laboratory animals, NIH, Washington, D.C., 1989'.

In vitro Studies. To evaluate the effects of CAPE on the viability of cell lines of lymphoid origin, 4×10^6 cells of the glucocorticoid sensitive C7-14 and -resistant CEM lymphoblastic leukemia cell lines were plated in 12-well plates with 4 ml of RPMI-1640 containing 10% FBS. Resistance in the CEM cell line is due to insufficient expression of the glucocorticoid receptor; signal transduction pathways distal from the glucocorticoid receptor are intact [16]. Cells were exposed to CAPE for 48 h at concentrations of 1, 2.5, 5, 20 and 50 ì*M*. For this, CAPE was dissolved in 70% ethyl alcohol and 10–20 ìl of stock solution were added to 2 ml medium. To evaluate whether CAPE potentiates the effect of dexamethasone on cell survival, cells were treated with increasing concentrations of dexamethasone (0.01, 0.1, 0.5 and 1 ì*M*) in the presence of 2.5 ì*M* of CAPE or vehicle. Dexamethasone stock solutions were prepared in 10% ethyl alcohol, and similarly to CAPE, the volume of the dexamethasone stock solution added to 2 ml of culture medium was 10–20 ìl. Cell survival was assessed at 48 h. In both experiments, quantification of cell death was performed by the MTT assay [17] in quadruplicate within each experiment. Cells were washed two times in PBS and incubated for 4 h at 37 °C with 1 mg/ml MTT. Formazan crystals were dissolved in a mixture of isopropanol and 1 *N* HCl. Absorbance (A) was measured at 570 nm, with 630 nm as reference wavelength and cell death was estimated as a proportion $[(A_{placebo} - A_{CAPE})/A_{placebo}]$. Apoptosis was detected on cytospin preparations using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), as previously described [18]. Air-dried cytospins were labeled according to the manufacturer's instructions and were viewed with a fluorescence microscope equipped with a FITC narrow-band filter. Electron microscopy was performed after cell pellets were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) for 24 h, postfixed in OsO_4 and embedded in Mararaglas 55. Sections were stained with uranyl acetate and lead citrate and examined in a Philips CM10 electron microscope.

Table 1. The effect of dexamethasone and CAPE on cellular viability in a glucocorticoid-sensitive (C7-14) and -resistant (CEM) lymphoblastic leukemia cell line

Dexamethasone concentration (ÌM)	CAPE concentration (ÌM)	Mean survival (± SEM), %	
		C7-14	CEM
–	0	100 ± 3.1	100 ± 0.5
–	1	65 ± 0.4	64 ± 0.5
–	2.5	49 ± 0.3	47 ± 1.1
–	5	30 ± 0.3*	21 ± 0.7
–	20	21 ± 0.5*	12 ± 0.6
–	50	8 ± 0.2*	4 ± 0.3
0	–	100 ± 1.2	100 ± 0.8
0.01	–	78 ± 1.1*	85 ± 0.9
0.1	–	25 ± 0.7*	59 ± 0.3
0.5	–	14 ± 0.2*	49 ± 0.5
1	–	16 ± 0.3*	41 ± 0.9
0	2.5	100 ± 0.1	100 ± 1.9
0.01	2.5	45 ± 0.4*	91 ± 1.5
0.1	2.5	3 ± 0.5*	52 ± 1
0.5	2.5	3 ± 0.3*	39 ± 0.9
1	2.5	3 ± 0.5*	39 ± 2.6

* $p < 0.05$ C7-14 vs. CEM.

Animal Studies. Rats were housed individually under controlled illumination (14-hour light, 10-hour dark cycle), with food and water available ad libitum. Subcutaneous inflammation was induced by carrageenin, as previously described [19]. Briefly, 4 ml of a 2% carrageenin suspension were administered into an air pouch generated 24 h previously by injection of 10 ml of air in the subcutaneous space of the interscapular area. The intravenous CAPE-treated group (6 rats) received an identical dose of CAPE administered through the jugular vein. For the intravenous treatment, a 10 mg/ml CAPE suspension was prepared in 50% ethyl alcohol and ~ 0.8 ml of this stock were injected in order to achieve a total dose of 40 mg/kg. In the locally treated group (6 rats), CAPE was administered at the dose of 40 mg/kg at the inflammatory site (suspended in carrageenin). Both the local-CAPE-treated and the control group (6 rats) received 0.8 ml vehicle intravenously. The inflammatory exudate was collected 16 h after induction of inflammation. Exudate volume, leukocyte and differential counts were performed by a commercial laboratory (AniLytics, Gaithersburg, Md., USA). Leukocyte cytoplasmic and nuclear fractions were prepared immediately after the animals were sacrificed. Detection of apoptosis in inflammatory cells was performed using TUNEL. Photomicrographs of randomly selected fields were taken at high resolution and apoptosis was evaluated by scoring 400 cells derived from the local CAPE-treated and the untreated groups of rats. Apoptosis was expressed as a ratio of positive nuclei per total number of leukocyte nuclei.

NF-ÎB p65 Subunit and IÎB· Western Blots. 3×10^6 cells were washed with ice-cold PBS and resuspended in 5 volumes of hypotonic cell lysis buffer A (10 m*M* KCl, 1.5 m*M* $MgCl_2$, 4 m*M* ß-mercaptoethanol, 0.5 m*M* phenylmethylsulfonyl fluoride, 10 Ìg/ml leupeptin, 10 m*M* Hepes, pH 7.5). The cells were allowed to swell on ice for 10 min followed by homogenization with a Teflon pestle (1,500 rpm). Nuclei were collected by centrifugation at 1,000 *g* for 10 min at 4°C. The supernatant cytosolic fraction was collected and immediately frozen at –70°C. After the isolated nuclei were resuspended in 2.5 volumes of buffer B (0.2 m*M* EDTA, 4 m*M* ß-mercaptoethanol, 0.5 m*M* phenylmethylsulfonyl fluoride, 10 Ìg/ml leupeptin, 20% glycerol, 20 m*M* Hepes, pH 7.5), ammonium sulfate was added to yield a final concentration of 0.3 *M*. The suspension was incubated at 4°C for 30 min, followed by centrifugation at 100,000 *g* for 60 min. The nuclear protein fraction was recovered prior to swelling of the pellet. Detection of IÎB· in the cytosolic fraction and of p65 in the nuclear protein fraction was performed by immunoblotting using polyclonal antibodies [17]. Intensity of specific IÎB· and p65 bands was assessed by visual inspection.

Data Analyses. Data are shown as the means ± SEM. Statistical comparisons between groups were made with the analysis of variance test (ANOVA) and the $^{-2}$ test. p values of 0.05 or less were considered significant.

Results

In vitro Studies of Cell Survival

We evaluated the effect of CAPE on cellular viability in a glucocorticoid-sensitive (C7-14) and -resistant (CEM) lymphoblastic leukemia cell line at 48 h. Cell survival in the presence of CAPE, dexamethasone and dexamethasone plus CAPE is shown in table 1 and in figure 2a. Exposure to CAPE in a low concentration (2.5 Ì*M*) for 48 h decreased the LD_{50} of dexamethasone from 4×10^{-8} *M* to 0.8×10^{-8} *M* in the C7-14 cell line and from 40×10^{-8} to 15×10^{-8} *M* in the CEM cell line (fig. 2b). Cell death induced by CAPE was shown to be apoptotic by in situ labeling of DNA strand breaks (fig. 2c). Morphologic analysis revealed that CAPE induced features consistent with apoptosis, including cellular disruption and nuclear chromatin condensation and fragmentation, not seen in untreated cells (fig. 2d).

In vivo Studies

We evaluated the effect of CAPE on carrageenin-induced acute inflammation (table 2). Mean exudate volumes were similar in the local and intravenous-CAPE-treated groups and the controls (fig. 3a). Exudate leukocyte, neutrophil and monocyte concentrations were less in the local-CAPE-treated, but not the intravenous-CAPE-treated group compared with the controls. Exudate lymphocyte and eosinophil concentrations were not different among the three groups. Apoptosis was detected in 18% of the inflammatory exudate leukocytes in the local CAPE-treated group versus 5% of leukocytes in the untreated group (fig. 3c, $p < 0.001$). Local CAPE treatment resulted

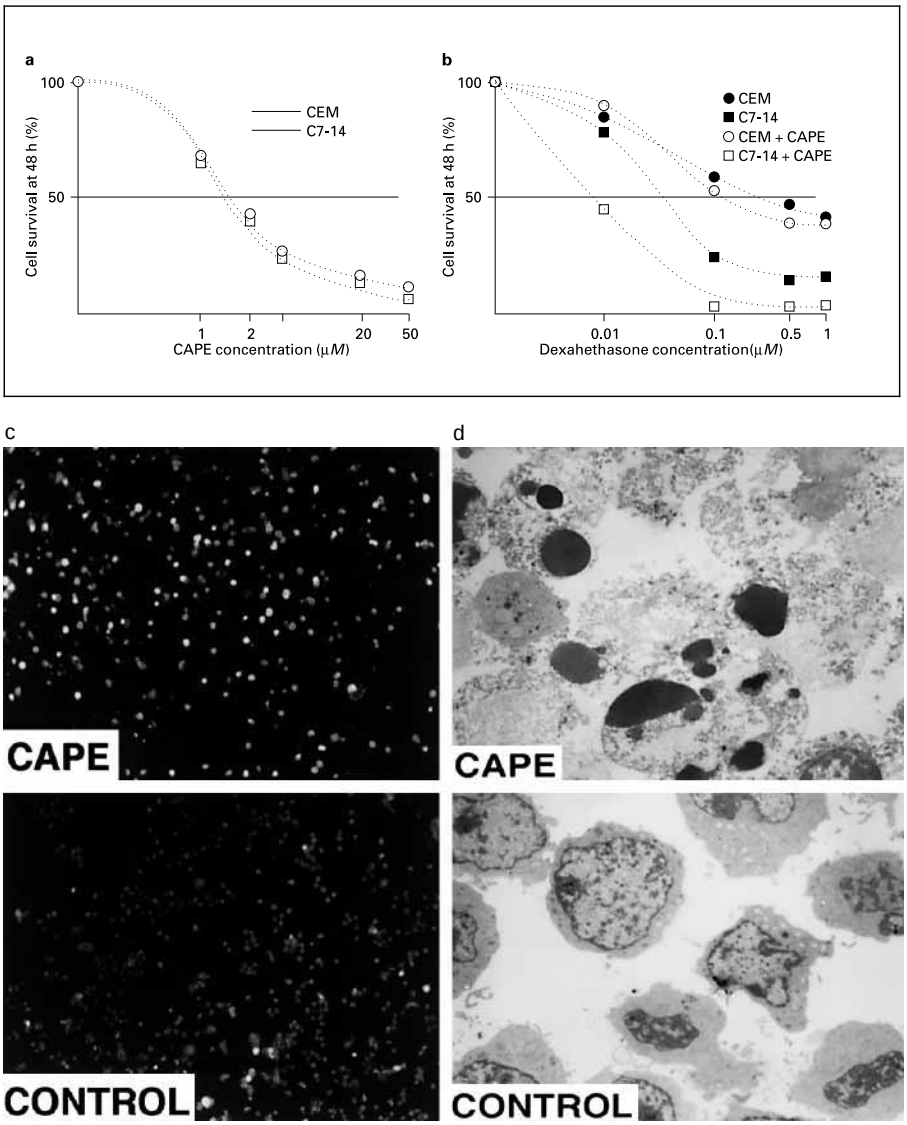

Fig. 2. a CAPE induced cell death in a dose-dependent fashion in a glucocorticoid-sensitive (C7-14) and -resistant (CEM) lymphoblastic leukemia cell line. Cell survival at 48 h in the presence of 0, 1, 2.5, 5, 20 and 50 Ì*M* of CAPE. Error bars too small to be seen. b CAPE had an additive effect on dexamethasone-induced cell death. c CAPE-induced cell death was shown to be apoptotic by in situ labeling of DNA strand breaks. Apoptosis in the presence of 20 Ì*M* CAPE or vehicle in the CEM cell line. d CAPE induced morphologic features consistent with apoptosis. Electron-microscopic studies demonstrated irregular nuclear envelope, chromatin condensation and fragmentation, and the presence of autophagic vesicles in the CAPE treated cells, not present in the vehicle treated cells (× 4,000).

in an increase in nuclear p65 expression, while cytoplasmic IÎB· expression was decreased compared to the placebo treated animals (fig. 3d). Intravenous CAPE treatment did not result in significant changes with regard to nuclear p65 and cytoplasmic IÎB· levels in the leukocytes derived from the inflammatory exudate.

Discussion

Nuclear translocation of NF-ÎB dimers and subsequent transcriptional events underlie the coordinated expression of cytokines (TNF-·, IL-1ß, IL-6 and GM-CSF), enzymes (e.g. nitric oxide synthase and cyclooxygenase-2) and adhesion molecules (e.g. intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and E-selectin) [4, 20–22] that initiate and propagate the inflammatory response. The basis for the NF-ÎB-mediated modulation of transcriptional events is that genes encoding these cytokines feature ÎB or ÎB-like binding motifs in their 5′ regulatory region. Binding of NF-ÎB dimers to these motifs either suffices to confer transcriptional inducibility or is required for full promoter activation. Stimuli known to be potent activators of NF-ÎB induce expression of transcripts encoding these inflammatory mediator molecules. Targeted disruption in mice of genes encoding different

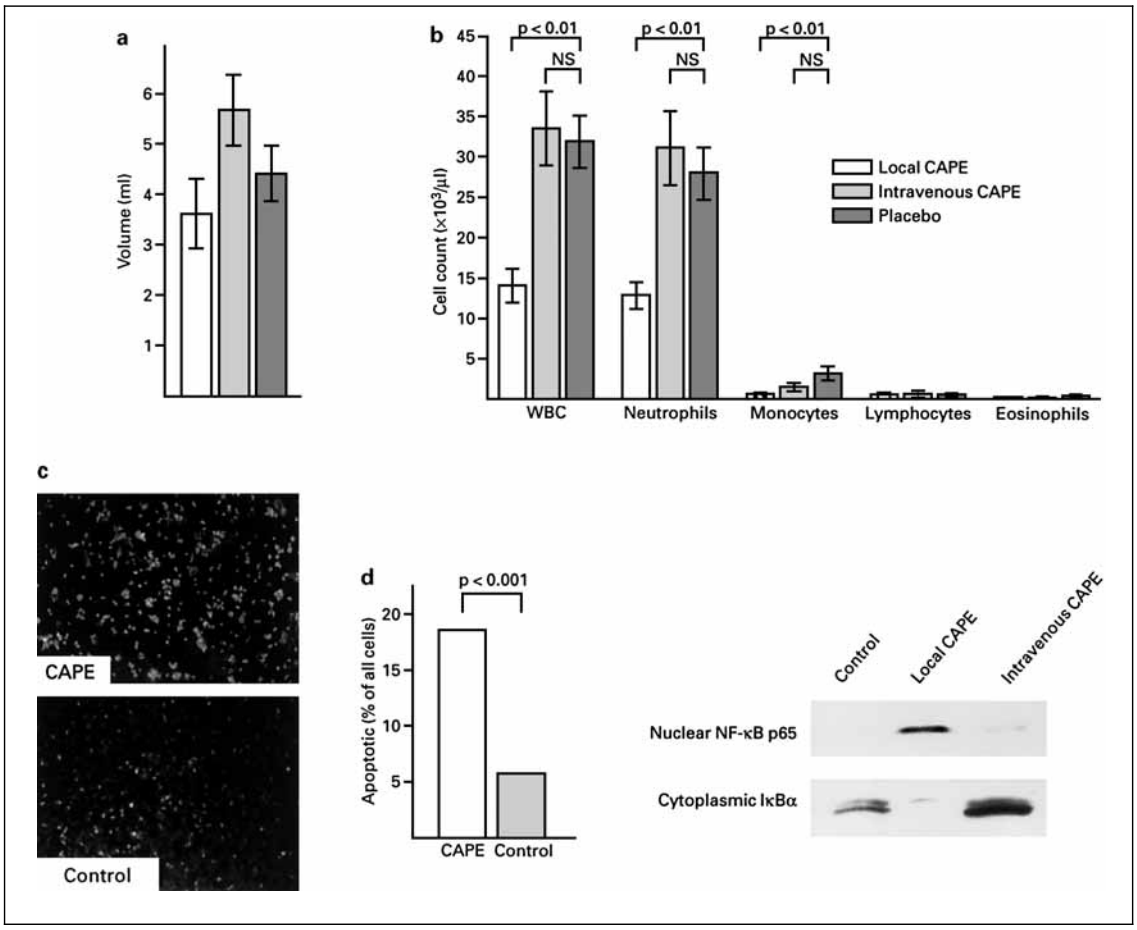

Fig. 3. a CAPE treatment produced no change in inflammatory exudate volume between local CAPE-treated (white bars), intravenous-treated (gray bars) or placebo-treated (dark bars) rats (n = 6). b CAPE treatment led to significant decreases in exudate leukocyte, neutrophil and monocyte concentrations, while lymphocyte and eosinophil concentrations were unchanged. c CAPE induced apoptotic cell death in vivo. Apoptosis was detected in 18% of the inflammatory exudate leukocytes in the local-CAPE-treated group versus 5% of leukocytes in the untreated group. d CAPE treatment resulted in a marked increase in leukocyte nuclear NF-ÎB p65 protein expression, while cytoplasmic IÎB· expression was decreased compared to leukocytes derived from placebo-treated animals.

subunits of the NF-ÎB/Rel protein family (p105, RelB, c-Rel and p65) result in impaired cellular functions, failure of differentiation of immune cells and lymph nodes, immune defects and embryonal apoptosis [10, 11, 23, 24]. In various animal models, inhibition of inflammation can be achieved by prevention of the degradation of IÎBs by specific inhibition of ubiquitin ligase [25], stable gene transfer of IÎB· cDNA [26] or local administration of antisense oligonucleotides to the p65 subunit of NF-ÎB [27].

We found that CAPE elicited apoptosis of immune cells in a dose-dependent manner in vitro. This effect was similar in glucocorticoid-sensitive and -resistant cell lines,

Table 2. The effect of intravenously and locally administered CAPE on exudate volume and cellularity

	Control (no CAPE)	Intravenous CAPE	Local CAPE
Exudate volume, ml	4.5 ± 0.5	5.8 ± 0.7	3.7 ± 0.7
White blood cells, × 10³/Ìl	31.9 ± 3.2	33.5 ± 4.7	13.9 ± 2*
Neutrophils, × 10³/Ìl	28 ± 2.9	31.2 ± 4.5	12.7 ± 1.9*
Monocytes, × 10³/Ìl	3 ± 0.9	1.5 ± 0.3	0.6 ± 0.1*
Lymphocytes, × 10³/Ìl	0.4 ± 0.1	0.5 ± 0.3	0.4 ± 0.2
Eosinophils, × 10³/Ìl	0.5 ± 0.2	0.2 ± 0.1	0.2 ± 0.1

* $p < 0.05$ control vs. local CAPE group.

suggesting that impairment of the glucocorticoid signaling pathway does not confer protection from NF-ÎB-mediated apoptotic cell death. Administration of glucocorticoids produced an additive, but not synergistic effect, which implies that CAPE has exerted its effect independently of the glucocorticoid receptor. There may be clinical implications of this finding in situations associated with glucocorticoid resistance (such as in septic shock or during treatment for certain hematologic malignancies). In certain leukemias, for example, therapeutic response to glucocorticoids predictably declines with time which prevents these malignant cells from undergoing apoptosis [28]. In this situation, induction of apoptosis through the NF-ÎB-mediated signal transduction pathway may impair cellular proliferation.

CAPE is a structural relative of the flavonoids. Interest in the compound arose when it was found to display specific cytotoxicity towards tumor cells [29] and to inhibit the HIV-1 integrase enzyme [15]. It has antioxidant, anti-inflammatory and immunomodulatory properties and inhibits cellular proliferation in vitro by altering the intracellular redox state [30]. Local administration of CAPE effectively suppressed acute inflammation in vivo by inducing apoptosis of leukocytes. Programmed cell death of these effector cells of inflammation would prevent release of cytokines and other molecules [4, 21, 22] which would lead to further leukocyte recruitment, thus perpetuating the inflammatory process. Apoptosis also facilitates subsequent elimination of these cells by phagocytosis.

We found that locally administered CAPE simultaneoulsy increased nuclear translocation of p65, while it decreased cytoplasmic IÎB· levels. These phenomena can be explained by increased phosphorylation and subsequent degradation of IÎB·, an event which ordinarily leads to detachment of NF-ÎB, followed by its nuclear translocation. It is also possible that decreased IÎB· resynthesis contributed to this diminution of cytoplasmic IÎB· levels, as was shown previously in vitro [14]. That such a modulation of NF-ÎB-mediated transcriptional events exerts potent negative effects on bone-marrow-derived immune and inflammatory cells is consistent with the finding that NF-ÎB is one of the main regulators of cytokine expression in human neutrophils [13], and monocytes/macrophages [20]. Contrary to our findings in the rats treated with local CAPE, we observed a paradoxical increase in cytoplasmic IÎB· expression in the intravenous-CAPE-treated rats. We think this effect may be attributable to the expected inflammatory stress-induced corticosterone elevation in those animals.

Although nuclear translocation of p65 and upregulation of NF-ÎB-mediated transactivation are associated with induction of apoptosis in some in vitro models, to our knowledge this study represents the first observation that it can also induce apoptosis-mediated anti-inflammatory effects in vivo. The decrease in the inflammatory exudate neutrophil concentration may suggest that modulation of the NF-ÎB-dependent inflammatory process could have an inhibitory effect on cytokine or complement-mediated neutrophil recruitment from the bone marrow. The lack of effect of CAPE on the volume of the inflammatory exudate argues that its anti-inflammatory effects do not result from alterations in vascular permeability (unlike glucocorticoids), but are solely due to decreases in the number and function of effector cells participating in the inflammatory response. In contrast to the potent local effects of CAPE, we observed no significant effects of intravenously administered CAPE on exudate cellularity, and leukocyte nuclear p65 and cytoplasmic IÎB· levels; this may be due to rapid systemic clearance of the compound and/or its impaired penetration to the site of inflammation.

Activation of NF-ÎB has been shown to occur as a response to both acute and chronic inflammatory diseases in humans, such as septic shock, arthritis, systemic lupus erythematosus, gastroenteritis, asthma and adult respiratory distress syndrome [31–33]. Whether this activation is the cause of the underlying inflammatory process or develops as a response to curb these processes is not completely clear, however, our results are more compatible with the former. Although our data as well as those of others [7] show evidence of NF-ÎB activation (i.e. nuclear translocation) results in apoptosis of inflammatory cells, several other in vivo studies associated anti-inflammatory effects with inhibition of NF-ÎB activation. NF-ÎB concentration-, tissue-, and model-dependent differences in NF-ÎB activation may exist and may, in part, be determined by the prevailing cellular redox state [34].

NF-ÎB activation is a critical step in the inflammatory cascade and agents that modulate NF-ÎB activity have potential for in vivo therapeutic interventions. CAPE is a potent inducer of inflammatory cell apoptosis through a glucocorticoid receptor-independent mechanism and its proapoptotic and anti-inflammatory properties may provide a novel approach to the treatment of inflammatory conditions. Analogs of CAPE which are resistant to metabolic clearance and can penetrate better the site of inflammation may provide new means to such an approach.

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