Extract of Oregano, Coffee, Thyme, Clove, and Walnuts Inhibits NF-κB in Monocytes and in Transgenic Reporter Mice

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Abstract

The transcription factor NF-κB is a promising target for chemoprevention. Several dietary plants are efficient inhibitors of NF-κB activation *in vitro* and could act synergistically on the NF-κB signaling pathway. In this study, we tested whether dietary plant extracts could inhibit NF-κB activation in a synergistic manner *in vitro*. Second, we investigated the potency of the same dietary plant extracts in the inhibition of NF-κB activation *in vivo*. A combined extract of clove, oregano, thyme, walnuts, and coffee synergistically inhibited lipopolysaccaride (LPS)-induced NF-κB activation in a monocytic cell line, compared with the sum of effects from the single extracts. Transgenic NF-κB luciferase reporter mice were given a single dose of the combined extract and subsequently challenged with LPS. NF-κB activation was monitored by *in vivo* imaging for 6 hours. In addition, NF-κB activity in organs and the expression of immune-related genes in liver were investigated. Based on the area under the curve, the extract decreased whole body LPS-induced NF-κB activity the first 6 hours by 35% compared with control mice. Organ-specific NF-κB activation was inhibited in intestine, liver, testis, and epididymis of the mice receiving the combination extract. In addition, dietary plants reduced the expression of genes related to inflammation, cell migration, and proliferation in liver. This study shows that dietary plants may be potent modulators of NF-κB signaling both *in vitro* and *in vivo*, and thus support further investigation of consumption of these plant foods as part of a healthy diet or as a mode of chemoprevention. *Cancer Prev Res; 3(5); 653–63. ©2010 AACR.*

Introduction

Recent studies have identified NF-κB as a direct link between inflammation and cancer (1–3) and thus renders this family of transcription factors as a key molecular target for both prevention and treatments of cancers. The NF-κB family of transcription factors are crucial mediators of cellular stress, immune, and inflammatory responses (4). Although NF-κB is essential in normal physiology, several human disorders involve inappropriate regulation of NF-κB. Several chronic degenerative diseases (5–7) and various human cancers (8) have been associated with an aberrant upregulation of NF-κB activity. Both the NF-κB activation and cytokine profile of tumor-associated macrophages are closely linked to tumor growth (9, 10).

In vertebrates, the NF-κB family is comprised of five structurally related proteins [p65 (REL A), p50, p52, c-REL, REL B] that form heterodimers and homodimers. Normally, the NF-κB dimers are sequestered in the cytoplasm by binding to inhibitory factors. Two pathways lead to the delocalization of NF-κB dimers to the nucleus and consequent transcriptional regulation of target genes (11). The classic NF-κB signaling pathway is activated by proinflammatory stimuli, bacterial or viral infection, and various forms of stress such as UV radiation and environmental toxins (12) and is essential for the innate immune system and antiapoptotic signaling. The alternative NF-κB pathway plays a critical role for both the development and maintenance of the adaptive immune system and is activated by members of the tumor necrosis factor (TNF) family other than TNF-α (11). NF-κB target genes code for proteins that are central players in inflammation, activation of the immune system, and antiapoptotic signaling, which can be involved in both the promotion and progression of cancers (13). With the central role of NF-κB activation in inflammation, cancer, and antiapoptosis, substances with the ability to inhibit NF-κB activation may be valuable in a generally healthy diet, in chemoprevention (14), and in combination with chemotherapy (15).

Plants and plant-based diets contain thousands of phytochemicals (16) in addition to well-known macronutrients and micronutrients. In epidemiologic studies, diets rich in plant-based foods reduce the risk of several cancers

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(17) and several chronic diseases (18). Clinical trials with single compounds have, however, not been able to reproduce these risk reductions (17, 19). For these reasons, it is likely that several of these compounds work in additive, synergistic, or antagonistic fashions to create the observed health effects and thus foods or combinations of foods may be better suited as the basic unit for research on these preventive effects. The preventive properties of such diets are likely to be complex combinations of numerous mechanisms, including reduced inflammation, cell proliferation and migration, and growth factor activity, and increased phase II detoxification and endogenous antioxidant defense, of which the latter two processes are orchestrated through the regulation of the transcription factor nuclear factor erythoid 2–related factor 2 (Nrf2; ref. 14). Aberrant cellular signaling is a hallmark of carcinogenesis and targeting crucial signaling pathways, such as the NF-κB and Nrf2 signaling pathways, yield valuable progress toward the elucidation of the mechanisms underlying chemoprevention. We previously screened a wide range of dietary plants and phytochemicals for their effect on NF-κB activation and found clove, thyme, oregano, coffee, and walnuts to be the most potent inhibitors (20). In this study, we show that an extract of these five dietary plants synergistically inhibit NF-κB activation *in vitro* and can also inhibit NF-κB activation and induce Nrf2 activity *in vivo* in transgenic mice. Interestingly, lowered NF-κB activation was detected in the liver and intestine, as well as in testis and epididymis of male mice, indicating organ-specific effects of the dietary plants. In addition, we found the dietary plants to reduce the expression of genes related to inflammation and cell migration and proliferation in the livers of these mice.

Materials and Methods

Cell culture

U937-κB cells are previously described (21). Cell culture conditions and experimental set up is previously described (20). Briefly, NF-κB activity was induced by lipopolysaccaride (LPS; 1 μg/mL) 30 minutes before addition of extracts and cells were coincubated with LPS and extract for an additional 6 hours. Cell viability was determined by trypan blue exclusion. A cutoff value of 10% nonviable cells was used.

HepG2 cells (hepatocellular carcinoma cell line) were cultured and transiently transfected with an construct containing two EpRE sequences coupled to the luciferase gene as previously described (22). The cell culture medium was replaced before the addition of extract and the cells were incubated with the indicated concentrations for 17 hours. Luciferase activity was measured in cell lysates as previously described (22).

Dietary plant extract

Coffee (Arabica, Medium roast), walnuts, oregano (dried), clove (dried), and thyme (dried) were purchased from a grocery store in Oslo, Norway. All samples were pulverized and extracted and concentrated by use of water/methanol (50:50,v/v) as previously described (20). The final extract was diluted in corn oil (extract used for mice) or a 50:50 mix of PBS/DMSO (v/v; for cells). The extract to be used for cell culture was thereafter sterile filtered. When measured as redox-active compounds by the ferric reducing ability of plasma (FRAP) assay, the extract administered to the mice corresponds to ~10 times the average daily intake in the Norwegian diet (data not shown).

Transgenic mice

The mice were housed in accordance with the guidelines of the Federation of European Laboratory Animal Science Associations and animal experiments were done according to national guidelines for animal welfare. The mice had *ad libitum* access to water and chow (except fasting 3 h before per os feeding). Transgenic mice with B6 background carrying a transgene with binding sites for either NF-κB (with three κB sites; ref. 21) or Nrf2 [with two electrophile response elements (EpRE); ref. 22] coupled to the luciferase gene were used. A single dose of an extract corresponding to 60 mg each of thyme, oregano, and clove, and 600 mg each of walnuts and coffee or vehicle control (corn oil) was administered by oral gavage to the mice. NF-κB transgenic mice for both groups were n = 16 (10 female and 6 male) and 11 to 24 weeks of age. EpRE transgenic mice for both groups were n = 5 female and 12 to 17 weeks of age. No differences in NF-κB activity were found between the female and male NF-κB mice and thus the results from both sexes were pooled. For the NF-κB mice, LPS (2.5 mg/kg) in 100 μL PBS was injected s.c. on the back near the tail 3 hours after oral gavage. *In vivo* imaging was done at the given time points. Directly following imaging at 6 hours, the NF-κB mice were euthanized; *ex vivo* imaging of the intestine was done; and all other organs were frozen in liquid nitrogen for further analysis. Luciferase activity in homogenates of organs was measured as previously described (21).

Luciferase activity

Luciferase activity was measured by use of an IVIS 100 Imaging System (Caliper Life Sciences). D-luciferin (0.2 mg/mL medium) was added to the cell culture medium and cells were incubated at 37°C for 4 minutes. Mice were anesthetized using 2.5% isoflurane and were shaved on the abdomen. D-luciferin (Biosynth AG; 160 mg/kg) in PBS was injected i.p. and mice were imaged 7 minutes after injection of D-luciferin. Cell culture plates or mice were placed in a light proof chamber and the luminescence emitted from the cells or mice was detected for 1 minute. *Ex vivo* imaging of the intestine was done 20 minutes after the injection of D-luciferin. The number of photons emitted was calculated by use of the Living Image Software (Caliper Life Sciences). Grayscale images were used for reference of position.

Quantitative mRNA analysis

Quantitative real-time PCR was done on liver tissue from NF-κB mice included in the experiment (n = 5 for both LPS and LPS + extract), plus two nontreated NF-κB mice for reference to the basal expression of mRNA. Total RNA was isolated by use of the RNeasy Mini kit (Qiagen) and cDNA synthesis was done using the High capacity cDNA reverse transcription kit (Applied Biosystems, Inc.). Quantitative real-time PCR was done in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Inc.) using the Taqman Mouse Immune Array (Applied Biosystems, Inc.). RQ Manager v.1.2 (Applied Biosystems, Inc.) was used for data analysis. The amount of target gene relative to reference gene was quantified using the cycle threshold

Fig. 1. Dietary plant extracts inhibit NF-κB activation *in vitro*. U937-κB cells were incubated with the indicated extract for 6.5 h and LPS was added for the last 6 h. Luciferase activity was measured by imaging. Columns, mean of two or three experiments each done in triplicate; bars, SEM. *, $P < 0.05$. The image of one representative experiment is shown in the lower half of A. Luminescence was measured in photons/s/cm²/steradian and presented with the color bar. Grayscale images were used for reference of position. A, columns, fold change in luminescence at the indicated dosage of the combined extract. B, columns, fold change in luminescence at the indicated dosage of LPS or of LPS plus the single extract.
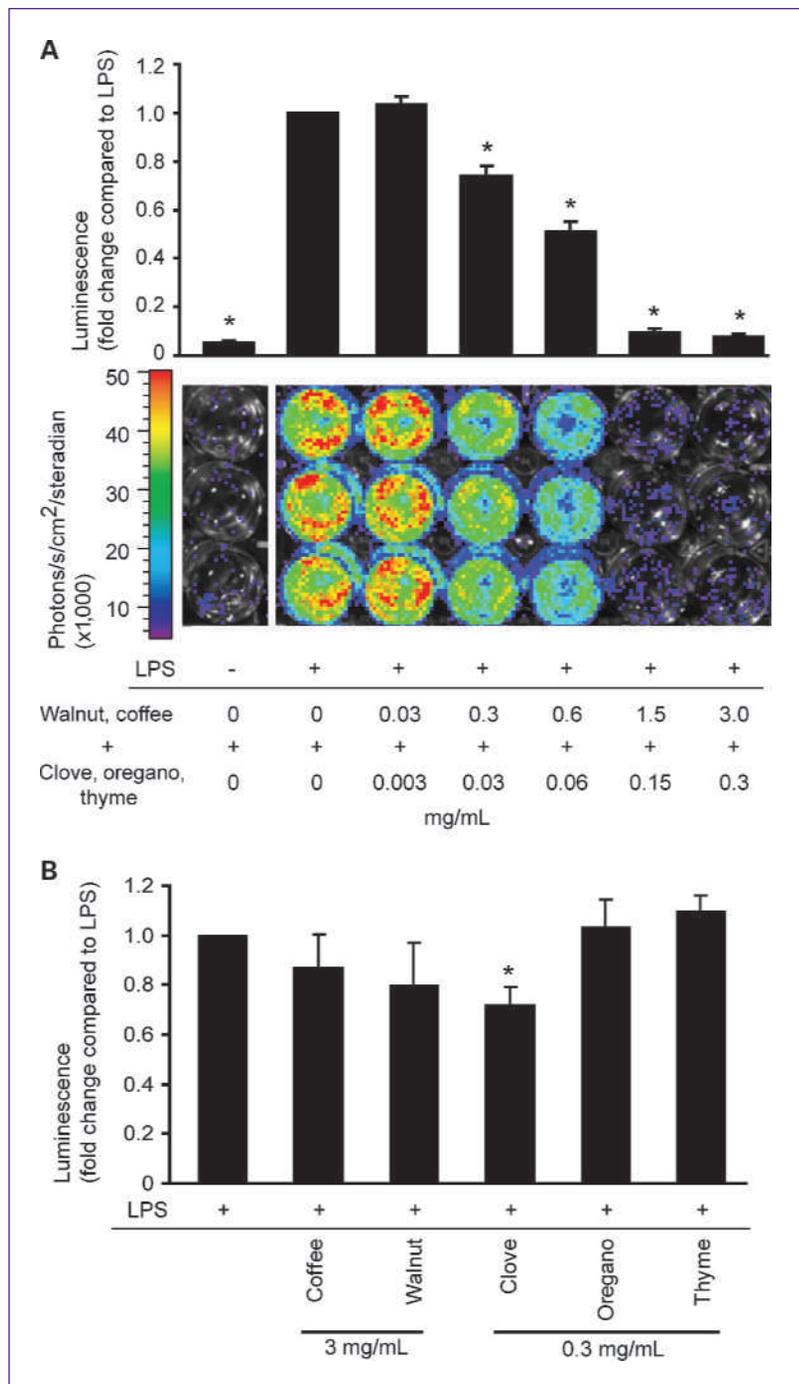
(Ct) with glucuronidase β as the endogenous control gene. Thus, $-\Delta Ct$ represents $Ct_{target\ gene} - Ct_{glucuronidase\ \beta}$ and was used to calculate $2^{-\Delta Ct}$.

Statistical analysis for *in vitro* experiments

The U937-κB cell cultures may vary in their NF-κB response to LPS depending on the passage of the cells. Therefore, the control (no extract) response to LPS was idiosyncratic for each experiment and the most stable set of experimental conditions is within a particular experiment. For these reasons, we set the control LPS level as 1, meaning 100% of NF-κB activity, and computed the responses to the LPS plus extract treatments as fold changes compared with controls.

One-way ANOVA was used to examine effects of extracts on luciferase activity in U937-κB and HepG2 cells. Differences were identified using Dunnett's comparisons.

For the calculations of synergy, we used the natural logarithm of the NF-κB activity as the response metric, which takes a value of 0 for the control situation (response to LPS alone), a negative number for inhibition of the NF-κB activity to LPS plus extract, and a positive number for accentuation of the activity. We ran a linear regression model with dependent variable ln(NF-κB), with no intercept, which was included as independent variables all five main effects of extracts, and a single term representing the combined extract. As the main test of synergy, we then estimated the contrast for the combined extract effect minus the sum of the five corresponding main effects.

Statistical analysis for *in vivo* experiments

For *in vivo* experiments, the area under the curve (AUC) of LPS-induced luciferase activity from 0 to 6 hours was calculated for each mouse. Student's *t* tests were performed to compare the data from AUC, at each time point, from *ex vivo* imaging, and from luciferase activity in tissue homogenates. Due to a lower number of animals in the Nrf2 experiment, the two treatments were compared statistically using nonparametric Mann-Whitney Test. Principal component analysis was performed using The Uscrambler v.9.8 (Camo Software AS, http://www.camo.com) on the whole gene expression data set to identify possible intervariable relations between genes that may identify patterns caused by the treatment. Statistically significant difference was set to $P < 0.05$ for all analysis. Results are presented as mean ± SEM, unless otherwise noted.

Results

Combined extract inhibits NF-κB activation in U937-κB cells

An extract made from the combination of clove, oregano, thyme, walnuts, and coffee inhibited LPS-induced NF-κB activity in the U937-κB cells in a dose-dependent manner (Fig. 1A). In the U937-κB cells, basal NF-κB activity was 0.05-fold ± 0.009-fold (5%) of LPS- induced NF-κB activity (Fig. 1A). The extract made from 0.3 mg/mL coffee and walnut, and 0.03 mg/mL thyme, oregano, and clove reduced LPS-induced NF-κB activation to 0.74-fold ± 0.05-fold ($P < 0.001$) of LPS-controls, whereas the extract made of 0.6 mg/mL coffee and walnut, and 0.06 mg/mL thyme, oregano, and clove further reduced luciferase activity to 0.51-fold ± 0.04-fold ($P < 0.001$) of LPS controls. Increasing the concentration to 1.5/0.15 mg/mL and 3/0.3 mg/mL (coffee and walnut/thyme, oregano, clove) almost completely blocked LPS- induced NF-κB activation to, respectively, 0.09-fold ± 0.02-fold and 0.08-fold ± 0.01-fold ($P < 0.001$ for both) without cytotoxicity.

Table 1. Assessment of synergy: difference in ln(NF-κB) according to prediction from main effects of the five ingredients in the combined extract compared with the effect of the combined extract

Main effects

Parameter	Estimate	SEM	t value	Pr > |t|
A. Combined extract at 3/0.3 mg/mL* vs sum of five at 3/0.3 mg/mL				
Sum of five ingredients	−0.42	0.21	−1.95	0.075
Combined extract	−2.14	0.23	−9.17	<0.001
Difference, combined extract vs sum of five	−1.72	0.44	−3.93	0.002
B. Combined extract at 1.5/0.15 mg/mL* vs sum of five at 3/0.3 mg/mL				
Sum of five ingredients	−0.42	0.24	−1.71	0.112
Combined extract	−1.99	0.27	−7.49	<0.001
Difference, combined extract vs sum of 5	−1.57	0.50	−3.15	0.008
C. Combined extract at 0.6/0.06 mg/mL* vs sum of five at 3/0.3 mg/mL				
Sum of five ingredients	−0.42	0.20	−2.12	0.056
Combined extract	−0.26	0.21	−1.23	0.242
Difference, combined extract vs sum of five	0.15	0.40	0.38	0.711

*Concentrations: a/b mg/mL, (a) mg/mL of each of coffee and walnut and (b) mg/mL of each of clove, thyme, and oregano.

Assessment of synergy

To test for synergy, we calculated the difference between the effects of the combined extract and the sum of effects for the five single ingredients tested separately. Effects of single ingredients are shown in Fig. 1B. When comparing equal concentrations of the ingredients (3 mg/mL each of coffee and walnut, and 0.3 mg/mL each of thyme, oregano, and clove), the combined extract inhibited NF-κB significantly more than the expected additive inhibitory effects from the five ingredients tested separately (Table 1A), indicating synergistic effects by the combination extract. At combined extract concentration of 1.5 mg/mL each of coffee and walnut, and 0.15 mg/mL each of thyme, oregano, and clove, the combined extract was still significantly more potent in inhibiting NF-κB activation compared with the expected sum of inhibitions from the five ingredients at 3 mg/mL coffee and walnut, and 0.3 mg/mL thyme, oregano, and clove (Table 1B). The combined extract concentration of 0.6 mg/mL of coffee and walnut, and 0.06 mg/mL of thyme, oregano, and clove was equally potent as the expected sum of the effects for 3 mg/mL of coffee and walnut, and 0.3 mg/mL of thyme, oregano, and clove (Table 1C). A way of expressing the synergy is to note that one fifth of the total volume of food extract was needed to obtain equal NF-κB inhibition using the combined extract as was expected based on the separate effects of the individual food extracts.
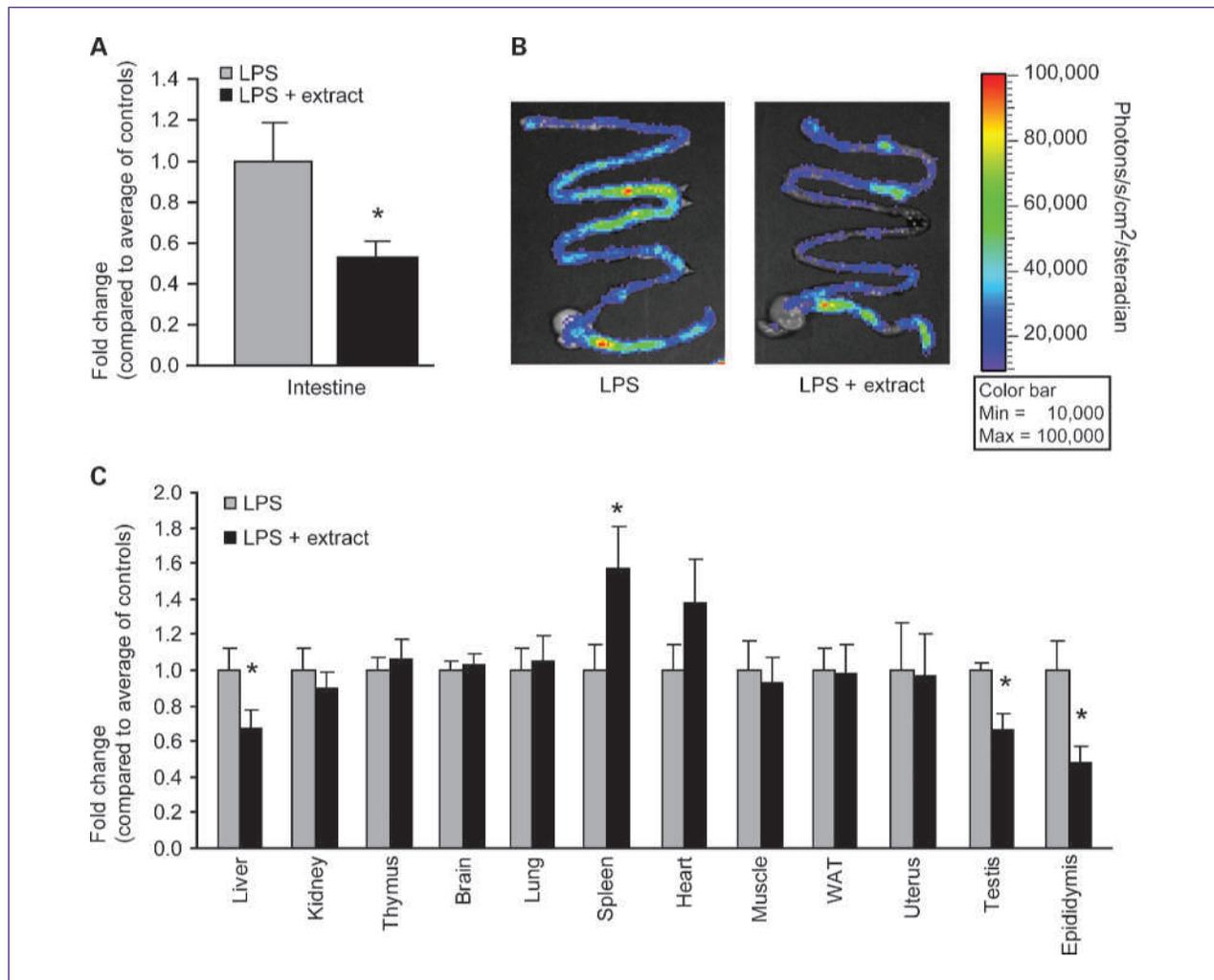
The combination extract inhibits NF-κB in transgenic mice

Next, we investigated whether the combination extract could modulate LPS-induced NF-κB activity *in vivo*. An s.c. injection of LPS (160 mg/kg) increased NF-κB activity to a maximum of 15.7-fold ± 2.4-fold ($n = 16$) after 4 hours as measured by whole body *in vivo* imaging of the luciferase activity. The extract or control vehicle (corn oil) was administered by oral gavage to mice 3 hours (at time −3 h) before s.c. LPS injection (at time 0 h). Whole body *in vivo* imaging was done at 0, 2, 4, and 6 hours (Fig. 2A and B). At 2 hours ($P = 0.043$) and 6 hours ($P = 0.041$) after LPS injection, the extract significantly inhibited the LPS-induced NF-κB activity, compared with mice treated with LPS only. Based on the AUC, the extract decreased LPS-induced NF-κB activity the first 6 hours by 35% ($P = 0.040$) compared with control mice (Fig. 2C).

Following *in vivo* imaging at 6 hours, the mice were euthanized and luminescence was measured in the intestine by *ex vivo* imaging and in homogenates of other tissues. There was a significant reduction in NF-κB activity to 0.53-fold ($P = 0.028$) of controls, in the intestine of mice receiving LPS plus the extract compared with mice receiving LPS only, as measured by *ex vivo* imaging (Fig. 3A). Representative intestines from the LPS only– and the LPS + extract–treated mice are shown in Fig. 3B. Furthermore, comparing luciferase activity by luminometry of tissue homogenates of organs from mice receiving the extract to mice receiving LPS only, revealed significantly decreased NF-κB activity in the liver (0.67-fold of control; $P = 0.046$),
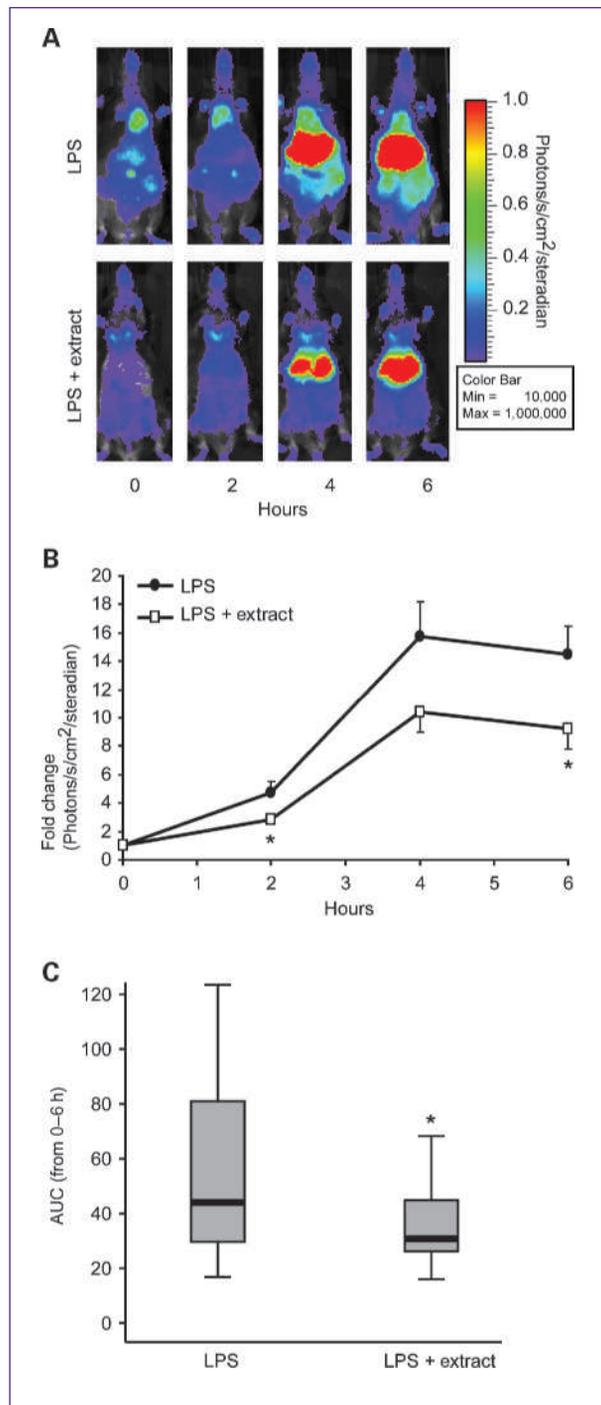
Fig. 2. Combination extract inhibits NF-κB *in vivo*. Transgenic NF-κB-luciferase reporter mice were given a single dose of extract or vehicle by oral gavage 3 h before s.c. LPS injection (at 0 hour). A, the luciferase activity was measured by *in vivo* imaging at 0, 2, 4, and 6 h (one representative mouse from each group is shown). B, the average fold change (± SEM) of emittance of photons/s/cm²/steradian (normalized to 0 h) at 0, 2, 4, and 6 h from the whole mice minus the head, limbs, and tail (□, LPS + extract; ●, LPS only; $n = 16$). C, the AUC was calculated for each mouse and compared between the two treatment groups. The average photons emitted and AUC were compared using the Student's t test. *, $P < 0.05$.
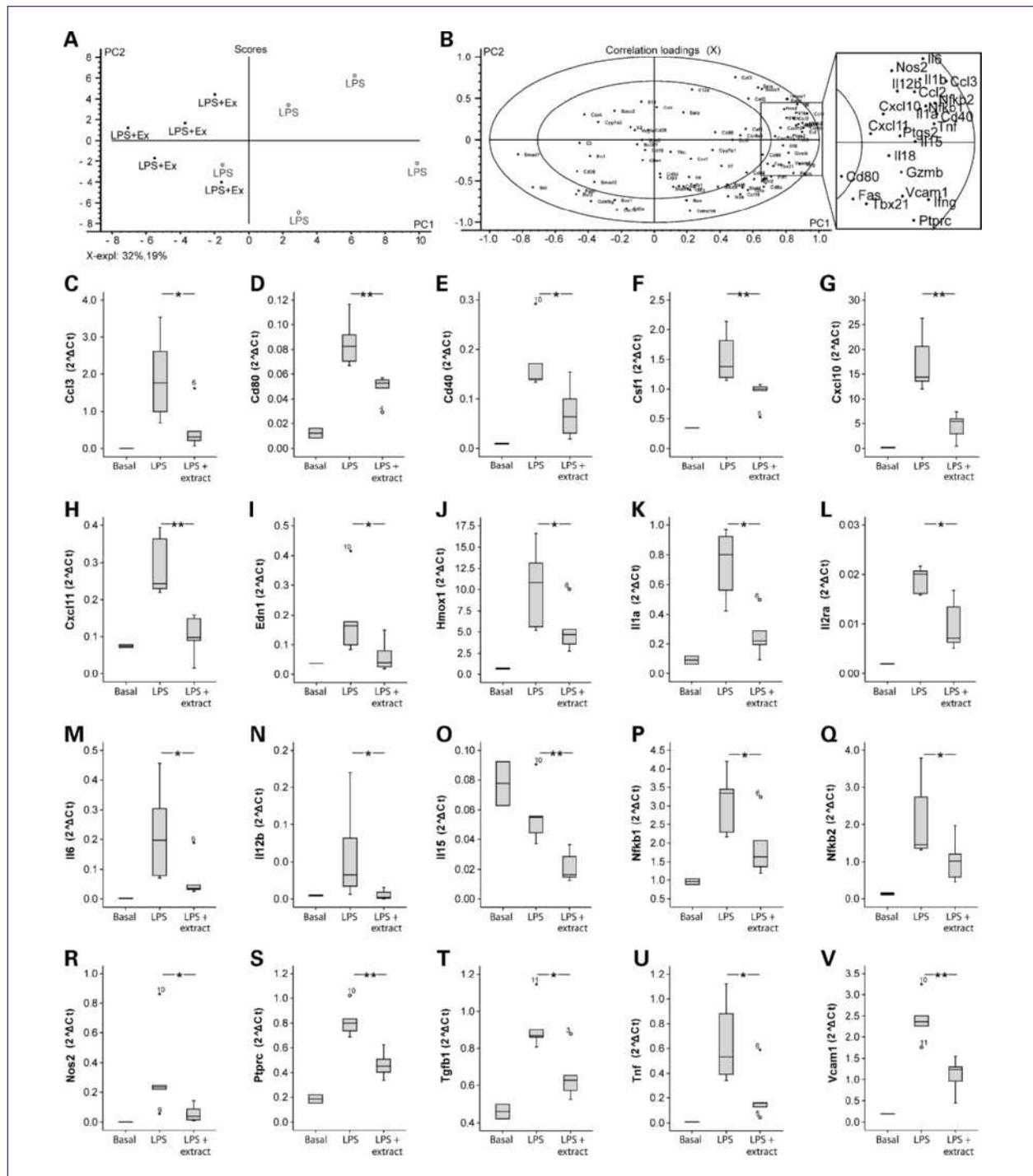
Fig. 3. Combination extract inhibits NF-κB activation in intestine, liver, testis, and epididymis. Transgenic NF-κB-luciferase reporter mice were given a single dose of extract or vehicle by oral gavage before s.c. LPS injection. Directly following imaging at 6 h, the mice were euthanized and organs were excised. A, ex vivo imaging of the intestines was done. The average fold change (± SEM) of luciferase activity from the intestines of LPS-only mice (gray columns) versus LPS + extract mice (black columns) is shown. B, one representative intestine of a mouse from each treatment group. Luminescence was measured in photons/s /cm²/steradian, and was presented with the color bar. Grayscale images were used for reference of position. C, luciferase activity was measured in tissue homogenates and normalized to the average of LPS-only mice. Columns, mean; bars, SEM. WAT, white adipose tissue. Student's t tests were used to compare the two treatments. $n = 16$ for all organs except uterus ($n = 10$) and testis and epididymis ($n = 6$). *, $P < 0.05$.

testis (0.66-fold of controls; $P = 0.007$), and epididymis (0.48-fold of control; $P = 0.021$; Fig. 4C). In the spleen, the NF-κB activity was further increased in the mice receiving the extract (1.57-fold of control; $P = 0.047$). Although there was a suggestive but not significant increase in heart NF-κB activity, no other differences in luciferase activity were found in brain, thymus, lung, kidney, fat, muscle, or uterus.

The combination extract inhibits gene expression of immune-related genes

We proceeded to investigate the expression levels of genes related to immune responses, cancer development, and NF-κB activity in livers of mice receiving LPS + extract versus LPS only. Two nontreated mice were included for

reference to non–LPS-induced expression levels. The principal component analysis score plot (Fig. 4A) shows that the LPS and LPS + extract treatments are separated by PC1 and PC2, which totally explain 51% of the variation in the gene expression data set. The correlation loading (Fig. 4B) indicate that a set of genes are important in terms of explaining the variance in the data and thus the effect of the treatment. This group of genes important for the PC1 and PC2 (Fig. 4B, enhanced area) include chemokine ligand 3 (Fig. 4C, Ccl3), Cd80 antigen (Fig. 4D, CD80), Cd40 antigen (Fig. 4E, CD40), chemokine ligand 10 (Fig. 4G, Cxcl10), chemokine ligand 11 (Fig. 4H, Cxcl11), interleukin 1 α (Fig. 4K, Il1α), interleukin 6 (Fig. 4M, Il6), interleukin 12b (Fig. 4N, Il12b), interleukin 15 (Fig. 4O, Il15), nuclear factor κB 1 (Fig. 4P, Nfkb1;

Fig. 4. Combination extract inhibits immune responses *in vivo*. Transgenic NF-κB-luciferase reporter mice were given a single dose of extract or vehicle by oral gavage before s.c. LPS injection. Directly following imaging at 6 h, the mice were euthanized and liver tissue was taken out for quantitative reverse transcription-PCR analysis. A and B, the results of the principal component analysis done on the whole gene expression data set. A, the Score plot of PC1 versus PC2. B, the correlation loading plot for all the gene expression data for PC1 versus PC2. C to V, for determination of relative mRNA expression, the expression level was normalized against glucuronidase β. Box plots, $2^{\Delta Ct}$ values (see Materials and Methods). Between the LPS and LPS + extract animals, expression levels were compared using Mann-Whitney tests. Basal, nontreated mice included for reference to non–LPS-induced expression levels ($n = 2$). *, $P < 0.05$; **, $P < 0.01$. Ccl3, chemokine ligand 3; Cd80, CD80 antigen; Cd40, CD40 antigen; Csf1, colony stimulating factor 1 (macrophage); Cxcl10, chemokine ligand 10; Cxcl11, chemokine ligand 11; Edn1, endothelin 1; Hmox1, heme oxygenase 1; Il1a, interleukin 1 α; Il2ra, interleukin 2 receptor α chain; Il6, interleukin 6; Il12b, interleukin 12b; Il15, interleukin 15; Nfkb1, nuclear factor of κ B 1/p50/p105; Nfkb2, nuclear factor of κ B 2/p52/p100; Nos2, nitric oxide synthase 2 inducible; Ptprc, protein tyrosine phosphatase receptor type C; Tgfb1, transforming growth factor, β 1; Tnf, tumor necrosis factor; Vcam1, vascular cell adhesion molecule 1.

p50/p105), nuclear factor κB 2 (Fig. 4Q, Nfkb2; p52/p100), nitric oxide synthase 2 inducible (Fig. 4R, Nos2), protein tyrosine phosphatase receptor type C (Fig. 4S, Ptprc), TNF α (Fig. 4U, Tnf), and vascular cell adhesion molecule 1 (Fig. 4V, Vcam1), which are also all significantly differently expressed between the two treatments. In addition, colony stimulating factor 1 (macrophage) (Fig. 4F, Csf1), endothelin 1 (Fig. 4I, Edn1), heme oxygenase 1 (Fig. 4J, Hmox1), interleukin 2 receptor α chain (Fig. 4L, Il2ra), and transforming growth factor β 1 (Fig. 4T, Tgfb1) were all differentially expressed in the livers from the LPS + extract–treated mice compared with in the livers of mice treated with LPS only. Of the 20 above-mentioned genes, all except interleukin 15 show a significantly lower mRNA expression in the livers of LPS + extract mice.

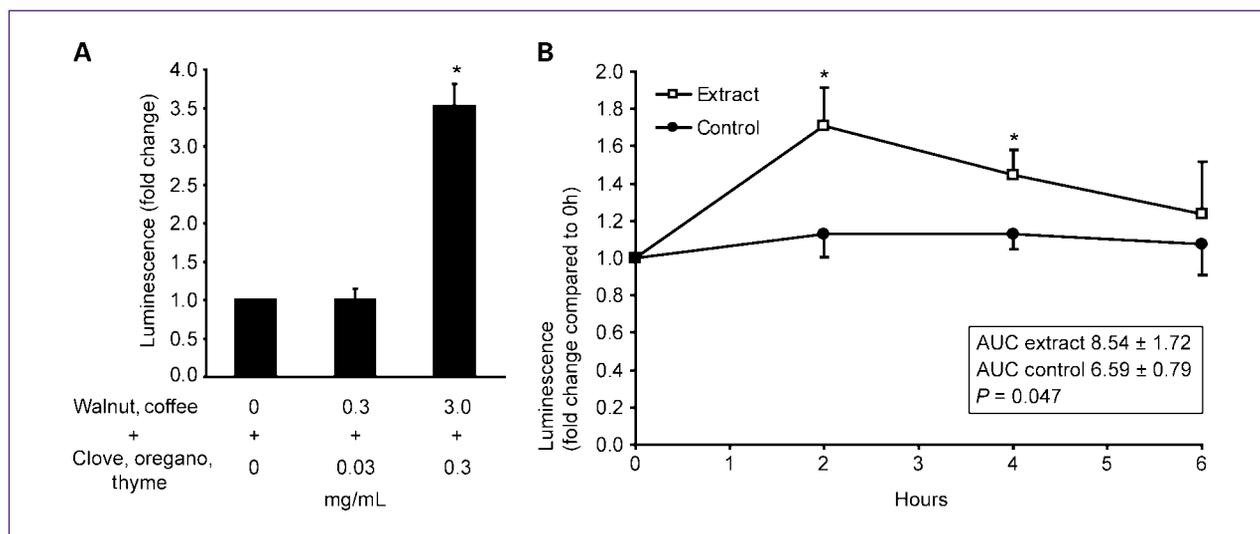### Induction of Nrf2 by combination extract

Previous research has found correlations between the ability to induce Nrf2 and anti-inflammatory properties, and a cross-talk between NF-κB and Nrf2 has been suggested (23–25). Thus, we tested the ability of the combination extract to induce Nrf2 activity through EpRE sites. In HepG2 cells, the combination extract potently induced Nfr2 activity at a concentration of 3 mg/mL coffee and walnut plus 0.3 mg/mL thyme, oregano, and clove, whereas one tenth of this concentration did not induce Nrf2 activity (Fig. 5A). Furthermore, we monitored the Nrf2 activity in EpRE-luciferase mice over the same time span as for the NF-κB mice. A single dose of the combination extract or control vehicle was administered by oral gavage to mice at time 0 hour and luminescence was measured at 0, 2, 4, and 6 hours. The mice receiving the extract had sig-

nificantly increased Nrf2 activity at 2 and 4 hours, and higher total activity (calculated by AUC), compared with the mice receiving control vehicle only (Fig. 5B).

Discussion

Inhibitors of NF-κB carry great promise for both prevention and therapy in cancers and chronic inflammation. An extract made from coffee, walnut, thyme, oregano, and clove inhibited NF-κB activation in U937-κB cells in a synergistic fashion compared with the effects of the single extracts. By using noninvasive molecular imaging of transgenic NF-κB luciferase reporter mice, we showed that the same combined extract efficiently inhibit LPS-induced NF-κB activation in vivo. Furthermore, a large set of downstream target genes were found to have significantly lower expression levels in the livers of mice receiving the extract, compared with in the LPS-treated mice. NF-κB may also be influenced by the activation of other transcription factors, such as Nrf2, which is orchestrating the endogenous antioxidant defense and phase II enzymes through binding to EpRE sites. In this study, we also found induction of Nrf2 activity by the combination extract both in vitro and in vivo.

As recently shown, NF-κB is a direct link between inflammation and cancer (1–3), and aberrant activation of NF-κB has been found in several types of human cancers (8). Inflammation coordinated through NF-κB activation will most often promote tumor growth (13) and inflammation-promoted tumor growth can be turned to tumor regression by inhibition of NF-κB in cancerous cells (2). NF-κB is essential for survival and cytokine production of monocytes and macrophages (26) and further NF-κB activation in macrophages is associated with cancer

Fig. 5. Combination extract induces Nrf2 activity in HepG2 cells and transgenic mice. A, HepG2 cells transiently transfected with EpRE-luciferase construct. Cells were incubated with the indicated concentration of extract for 17 h, at which point luciferase activity was measured. Columns, mean of four experiments each done in triplicate; bars, SEM. *, $P < 0.05$. B, transgenic EpRE-luciferase mice were given a single dose of extract or vehicle by oral gavage and luciferase activity was monitored by in vivo imaging for 6 h. $n = 5$ for each treatment. *, $P < 0.05$ between the treatment groups at the indicated time point.

development. The cytokine profiles of monocytes and macrophages are also closely linked to tumor growth (9, 10). In the liver, the connections between hepatitis infection, inflammation or fibrosis, and development of hepatocellular carcinoma is well established (27, 28). Kupffer cells (resident liver macrophages) display increased NF-κB activation in response to liver injury, resulting in production and secretion of proinflammatory cytokines such as TNF-α and IL-6 (29). These cytokines may in turn promote fibrosis, tumor growth, and metastasis (27, 30); however, knocking out NF-κB activation in myeloid cells has been shown to reduce both cytokine production and hepatocellular carcinoma (10). In this study, 18 of 20 genes displaying a significantly different liver expression in the LPS + extract–treated mice compared with mice receiving LPS only are known NF-κB target genes (ref. 12 and references herein). These genes include a range of inflammatory cytokines/chemokines (including TNF-α and IL-6), immune receptors (Cd80, Cd40, Il2ra, and Ptprc), growth factor (Csf1), cell adhesion molecule (Vcam1), enzymes (Hmox1 and Nos2), and mitogen (Edn1), plus two NF-κB proteins (Nfkb1 and Nfkb2). The combination of chemokines, Vcam1, Nos2, and Csf1 are important in leukocyte recruitment, infiltration, and differentiation in tissues (reviewed in refs. 31, 32). Thus, the reduced expression of genes coding for these molecules, in combination with the observed decrease in LPS-induced NF-κB activation in the monocytic cell line as well as in the liver (and intestine) following administration of plant extracts, suggest that these plant extracts may contribute to chemoprevention by altering the microenvironment to a less inflammatory, less tumor-promoting milieu. In addition, to the extent that inflammation is an important factor in disease conditions other than cancer, the consumption of these foods should be regarded as part of a generally healthy diet.

The inhibition of NF-κB activity in liver, testis, and epididymis indicate that compounds from the extract have been absorbed and are bioactive after absorption. In the intestine, the extract may produce its effect both by acting in the intestinal lumen and after absorption into intestinal cells. Further work should be done to identify the bioactive compounds as well as the mechanisms of action, in all four organs. The intestine and liver are the main organs for metabolism of phytochemicals. There is limited knowledge on the tissue distribution of phytochemicals present in our extract. However, quercetin, resveratrol, and epigallocatechin gallate have previously been detected in the intestine, liver, and testes of mice and rats (33–35). Consistent with these results, we would expect the highest concentrations of phytochemicals from our extract in these organs and thus also a greater chance of detectible effects on a major signaling pathway such as NF-κB.

Induction of Nrf2 may affect the organ specificity of NF-κB responses as there are lines of evidence suggesting a cross-talk between the NF-κB and Nrf2 signaling pathways (23–25), and this cross-talk is most likely to be evident in organs with the highest Nrf2-activity. A wide range of phytochemicals are known to activate Nrf2, leading to induced expression of phase 2 enzymes and endogenous antioxidants (collectively called cytoprotective proteins; ref. 36), and many of these phytochemicals are also known to inhibit NF-κB (25). We also found our combination extract to induce Nrf2 activity, both in vitro and in vivo in transgenic EpRE reporter mice. Like NF-κB, Nrf2 has been implicated in carcinogenesis and inflammation both in liver and the gastrointestinal tract (37–39), and an induction of cytoprotective proteins is an alternative approach to reducing the tissue damage caused by carcinogens or excessive inflammatory response.

Phytochemicals are mainly metabolized in the intestine and liver. Thus, other organs will be exposed to metabolites or conjugates of the ingested compounds, possibly resulting in organ-specific NF-κB responses. In the spleen and possibly in the heart, the LPS-induced NF-κB activation was further increased in the mice receiving extract. It should be noted, however, that the absolute NF-κB activity (adjusted for protein contents of the samples) in the liver is 8- and 4-fold higher than in spleen and heart, respectively. In combination with the large size of the liver, this implies that the liver is contributing considerably more than any other organs to the overall NF-κB activity in the mice. We speculate that it would be an advantage to maintain a high NF-κB activity in the spleen during an infection to ensure the activity of adaptive immune responses and that this activation of NF-κB might be through the alternative NF-κB pathway, which is important in both development and maintenance of the spleen (11).

In the search for mechanisms behind the protective effects of plant-based diets, reductionistic approaches studying isolated compounds have dominated. Such an approach might fit well into pharmacologic research, but in doing so, we might overlook additive, synergistic, or antagonistic effects that arise from combinations of compounds found in plant foods and diets. Thus, we have chosen to make extracts of foods the focus of this study. In the present work, we found synergistic inhibitory effects on LPS-induced NF-κB activation with the combination of five dietary plant extracts, compared with the sum of effects of the five individual extracts. Keeping the concentration for the single extracts constant, the inhibitory synergistic effect on NF-κB activation by the combined extract persisted even at half the concentration. At a level of one fifth of the concentration, the added concentration of extracted foods in the combined extract is comparable with each individual extract tested alone, meaning that the effect of the combined extract is not simply an effect of a higher dose of food. This underlines the potential of synergistic effects being created by a variety of foods. Such effects would not be detectable in a more reductionistic model.

The most recent report from the World Cancer Research Fond/American Institute for Cancer Research (17) concludes that coffee is unlikely to have an effect on cancers of the pancreas or kidneys. At present, there is limited evidence on the effects of coffee intake on other cancer sites; however, some epidemiologic studies suggest preventive

effects of coffee in the liver (40, 41). In a large epidemiologic study, we observed that intake of coffee was inversely associated with reduced death attributed to oxidative stress and inflammatory diseases (42). Oxidative stress and inflammation are both closely linked to NF-κB activation and are involved in cancer initiation and development (43, 44). In this perspective, inhibition of NF-κB might contribute to the inverse relationship between coffee intake and disease, with the liver as a main target organ.

Walnuts, clove, thyme, and oregano are all rich in redox-active phytochemicals (45, 46); however, none of these dietary plants have been extensively studied with respect to chemopreventive properties. The observed NF-κB inhibition by our combination extract may, at least partly, be attributed to rosmarinic acid (47) found in thyme and oregano, or eugenol (48) found in clove, which are all identified as inhibitors of NF-κB; however, thymol, a major phytochemical in thyme, did not modulate NF-κB activity (20). Thyme and oregano essential oils in combination decreased the levels of IL-1β and IL-6, as well as inflammation-related tissue damage in a model of colitis (49), both of which may be a result of NF-κB inhibition in the colon. In addition, thyme may induce the level of endogenous cytoprotective proteins in the liver (50). Walnuts are also a rich source of essential fatty acids with known anti-inflammatory properties (51).

Approaches that focus on foods rather than single compounds should be valuable in future research to unravel the chemopreventive effects of foods. To our knowledge, this is the first report to indicate synergistic effects of foods on NF-κB activation; however, more research is warranted to confirm and to expand the yet limited knowledge about these synergistic effects. Our experiments show that ordinary dietary plants may be potent modulators of the NF-κB signaling pathway both *in vitro* and *in vivo*, and thus support a role of chemoprevention through dietary means.

Disclosure of Potential Conflicts of Interest

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References

1. Greten FR, Karin M. The IKK/NF-κB activation pathway-a target for prevention and treatment of cancer. Cancer Lett 2004;206:193–9.
2. Luo JL, Maeda S, Hsu LC, Yagita H, Karin M. Inhibition of NF-κB in cancer cells converts inflammation-induced tumor growth mediated by TNFα to TRAIL-mediated tumor regression. Cancer Cell 2004;6:297–305.
3. Pikarsky E, Porat RM, Stein I, et al. NF-κB functions as a tumour promoter in inflammation-associated cancer. Nature 2004;431:461–6.
4. Karin M, Yamamoto Y, Wang QM. The IKK NF-κB system: a treasure trove for drug development. Nat Rev Drug Discov 2004;3:17–26.
5. Valen G, Yan ZQ, Hansson GK. Nuclear factor κ-B and the heart. J Am Coll Cardiol 2001;38:307–14.
6. Mattson MP, Camandola S. NF-κB in neuronal plasticity and neurodegenerative disorders. J Clin Invest 2001;107:247–54.
7. Neurath MF, Becker C, Barbulescu K. Role of NF-κB in immune and inflammatory responses in the gut. Gut 1998;43:856–60.
8. Karin M, Cao Y, Greten FR, Li ZW. NF-κB in cancer: from innocent bystander to major culprit. Nat Rev Cancer 2002;2:301–10.
9. Colombo MP, Mantovani A. Targeting myelomonocytic cells to revert inflammation-dependent cancer promotion. Cancer Res 2005;65:9113–6.
10. Maeda S, Kamata H, Luo JL, Leffert H, Karin M. IKKβ couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. Cell 2005;121:977–90.
11. Bonizzi G, Karin M. The two NF-κB activation pathways and their role in innate and adaptive immunity. Trends Immunol 2004;25:280–8.
12. Pahl HL. Activators and target genes of Rel/NF-κB transcription factors. Oncogene 1999;18:6853–66.
13. Karin M, Greten FR. NF-κB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 2005;5:749–59.
14. Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 2003;3:768–80.
15. Nakanishi C, Toi M. Nuclear factor-κB inhibitors as sensitizers to anticancer drugs. Nat Rev Cancer 2005;5:297–309.
16. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004;79:727–47.
17. World Cancer Research Fund/American Institute for Cancer Research. Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. Washington (DC): AICR; 2007.
18. FAO/WHO. Joint FAO/WHO Expert Report on Diet, Nutrition and the Prevention of Chronic Disease. WHO Technical Report Series; 2003. Report No.: 916.
19. Bjelakovic G, Nikolova D, Ll G, Simonetti RG, Gluud C. Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. Cochrane Database Syst Rev 2008; Jul 16:CD004183.
20. Paur I, Austenaa LM, Blomhoff R. Extracts of dietary plants are efficient modulators of nuclear factor κ B. Food Chem Toxicol 2008;46:1288–97.
21. Carlsen H, Moskaug JO, Fromm SH, Blomhoff R. *In vivo* imaging of NF-κ B activity. J Immunol 2002;168:1441–6.
22. Myhrstad MC, Carlsen H, Dahl Ll, et al. Bilberry extracts induce gene expression through the electrophile response element. Nutr Cancer 2006;54:94–101.
23. Liu GH, Qu J, Shen X. NF-κB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. Biochim Biophys Acta 2008;1783:713–27.

24. Li W, Khor TO, Xu C, et al. Activation of Nrf2-antioxidant signaling attenuates NFκB-inflammatory response and elicits apoptosis. Biochem Pharmacol 2008;76:1485–9.

25. Surh YJ, Na HK. NF-κB and Nrf2 as prime molecular targets for chemoprevention and cytoprotection with anti-inflammatory and antioxidant phytochemicals. Genes Nutr 2008;2:313–7.

26. Park JM, Greten FR, Wong A, et al. Signaling pathways and genes that inhibit pathogen-induced macrophage apoptosis-CREB and NF-κB as key regulators. Immunity 2005;23:319–29.

27. Elsharkawy AM, Mann DA. Nuclear factor-κB and the hepatic inflam-mation-fibrosis-cancer axis. Hepatology 2007;46:590–7.

28. Di Bisceglie AM. Hepatitis C and hepatocellular carcinoma. Hepatol-ogy 1997;26:34–8S.

29. Racanelli V, Rehermann B. The liver as an immunological organ. Hepatology 2006;43:S54–62.

30. Maeda S, Hikiba Y, Sakamoto K, et al. Iκ B kinase/nuclear factor-B activation controls the development of liver metastasis by way of interleukin-6 expression. Hepatology 2009;50:1851–60.

31. Imhof BA, Aurrand-Lions M. Adhesion mechanisms regulating the migration of monocytes. Nat Rev Immunol 2004;4:432–44.

32. Hamilton JA. Colony-stimulating factors in inflammation and autoim-munity. Nat Rev Immunol 2008;8:533–44.

33. Suganuma M, Okabe S, Oniyama M, Tada Y, Ito H, Fujiki H. Wide distribution of [3H](-)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue. Carcinogenesis 1998;19:1771–6.

34. Mullen W, Graf BA, Caldwell ST, et al. Determination of flavonol me-tabolites in plasma and tissues of rats by HPLC-radiocounting and tandem mass spectrometry following oral ingestion of [2-(14)C]-quercetin-4'-glucoside. J Agric Food Chem 2002;50:6902–9.

35. Vitrac X, Desmouliere A, Brouillaud B, et al. Distribution of [14C]-trans-resveratrol, a cancer chemopreventive polyphenol, in mouse tissues after oral administration. Life Sci 2003;72:2219–33.

36. Dinkova-Kostova AT, Talalay P. Direct and indirect antioxidant prop-erties of inducers of cytoprotective proteins. Mol Nutr Food Res 2008;52 Suppl 1:S128–38.

37. Ramos-Gomez M, Kwak MK, Dolan PM, et al. Sensitivity to carcino-genesis is increased and chemoprotective efficacy of enzyme indu-cers is lost in nrf2 transcription factor-deficient mice. Proc Natl Acad Sci U S A 2001;98:3410–5.

38. Khor TO, Huang MT, Prawan A, et al. Increased susceptibility of Nrf2 knockout mice to colitis-associated colorectal cancer. Cancer Prev Res 2008;1:187–91.

39. Xu W, Hellerbrand C, Kohler UA, et al. The Nrf2 transcription factor protects from toxin-induced liver injury and fibrosis. Lab Invest 2008; 88:1068–78.

40. Ruhl CE, Everhart JE. Coffee and tea consumption are associated with a lower incidence of chronic liver disease in the United States. Gastroenterology 2005;129:1928–36.

41. Larsson SC, Wolk A. Coffee consumption and risk of liver cancer: a meta-analysis. Gastroenterology 2007;132:1740–5.

42. Andersen LF, Jacobs DR, Jr., Carlsen MH, Blomhoff R. Consumption of coffee is associated with reduced risk of death attributed to inflam-matory and cardiovascular diseases in the Iowa Women's Health Study. Am J Clin Nutr 2006;83:1039–46.

43. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflam-mation. Nature 2008;454:436–44.

44. Hussain SP, Hofseth LJ, Harris CC. Radical causes of cancer. Nat Rev Cancer 2003;3:276–85.

45. Dragland S, Senoo H, Wake K, Holte K, Blomhoff R. Several culinary and medicinal herbs are important sources of dietary antioxidants. J Nutr 2003;133:1286–90.

46. Halvorsen BL, Holte K, Myhrstad MC, et al. A systematic screen-ing of total antioxidants in dietary plants. J Nutr 2002;132: 461–71.

47. Lee J, Jung E, Kim Y, et al. Rosmarinic acid as a downstream inhibitor of IKK-β in TNF-α-induced upregulation of CCL11 and CCR3. Br J Pharmacol 2006;148:366–75.

48. Chainy GB, Manna SK, Chaturvedi MM, Aggarwal BB. Anethole blocks both early and late cellular responses transduced by tumor necrosis factor: effect on NF-κB, AP-1, JNK, MAPKK and apoptosis. Oncogene 2000;19:2943–50.

49. Bukovska A, Cikos S, Juhas S, Il'kova G, Rehak P, Koppel J. Effects of a combination of thyme and oregano essential oils on TNBS-induced colitis in mice. Mediators Inflamm 2007;2007:23296.

50. Sasaki K, Wada K, Tanaka Y, Yoshimura T, Matuoka K, Anno T. Thyme (Thymus vulgaris L.) leaves and its constituents increase the activities of xenobiotic-metabolizing enzymes in mouse liver. J Med Food 2005;8:184–9.

51. Ros E, Mataix J. Fatty acid composition of nuts-implications for cardiovascular health. Br J Nutr 2006;96 Suppl 2:S29–35.

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