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Research Article

Coffee and Maillard products activate NF-κB in macrophages via H_2O_2 production

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In this study, we investigated the immunomodulatory activity of coffee and Maillard reaction products on macrophages *in vitro*. Stimulation of macrophages with coffee, but not with raw coffee extract in PBS, led to a 13-fold increased nuclear NF-κB translocation. A Maillard reaction mixture (25 mM D-ribose/L-lysine, 30 min at 120°C) increased NF-κB translocation 18-fold (in PBS) or six-fold (in medium). MRPs also induced a two-fold increased NF-κB translocation in untransfected human embryonic kidney (HEK) cells as well as in HEK cells stably transfected with the receptor for advanced glycation endproducts (RAGE), indicating that the effect was not RAGE mediated. On the other hand, catalase totally abolished coffee- and MRP-induced NF-κB translocation. Consequently, up to 366 μM hydrogen peroxide was measured in the coffee preparation and Maillard mixtures used for cell stimulation. Stimulation of macrophages with MRPs did not lead to significantly increased IL-6 or NO release. Thus, it can be concluded that coffee and MRPs induce NF-κB translocation in macrophages *via* the generation of hydrogen peroxide.

Keywords: Coffee / Hydrogen peroxide / Immunomodulation / Maillard reaction products / NF-κB

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1 Introduction

Coffee contains many biologically active components. Caffeine, for example, stimulates the central nervous system by antagonistic binding to the adenosin-A_{2A} receptor and phosphodiesterase inhibition, leading to elevated cyclic adenosine monophosphate (cAMP) levels [1]. In addition, the diterpenes cafestol and kahweol increase cholesterol levels, presumably by downregulation of the low density lipoprotein (LDL)-receptor [2]. The antioxidative activity of coffee is well established [3, 4] and is related to phenolic compounds, such as chlorogenic acid, as well as melanoidins [5, 6]. Melanoidins are brown Maillard products formed mainly from sugars and amino acids at elevated temperatures during food processing.

Macrophages are abundant in the human intestine. Together with T and B lymphocytes, as well as dendritic cells, they are responsible for the cellular intestinal immune defense, rendering the intestine the largest immunobiological organ [7]. Thus, the intact intestine protects the organism from a wide variety of external pathogens without reacting hypersensitively against regular dietary components [8]. Apart from their physiological involvement in general intestinal immune response, macrophages play a key role in the development of inflammatory bowel diseases [9, 10]. In Crohn's disease and in ulcerative colitis, for example, the intestinal mucosa is infiltrated by a large number of macrophages which are present in an activated state and thus trigger proinflammatory cascades [11].

There are many examples that dietary components interact directly with the intestinal immune cells modulating the immune function. For example, dietary fiber changed the proportion and cell signaling of intestinal T-cells [8]. This process may be mediated by intestinal lactic acid bacteria, which preferentially grow after the consumption of dietary fiber, or by SCFAs, a fermentation product of the fiber.

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Abbreviations: AGE, advanced glycation endproduct; **ECL,** enhanced chemiluminescence reaction; **FCS,** fetal calf serum; **FOX,** ferrous oxidation xylenol orange; **HEK,** human embryotic kidney; **iNOS,** inducible nitric oxide synthase; **MRP,** Maillard reaction product; **RAGE,** receptor for advanced glycation endproducts; **ROS,** reactive oxygen species

Advanced glycation endproducts (AGEs) are formed by a Maillard-like mechanism from sugars and proteins *in vivo*. It is well established that elevated levels of AGEs, as found in diabetes or uremia, trigger cellular inflammatory reactions, for example vascular cell adhesion molecule-1 (VCAM-1) expression in endothelial cells or chemotaxis of macrophages [12]. This process is mediated by binding of AGEs to the receptor of AGEs (RAGE) and subsequent activation of the transcription factor NF-κB. Because of the structural similarities between AGEs and food derived Maillard products, this study investigates cellular reactions of macrophages after activation by coffee or Maillard products.

2 Materials and methods

2.1 Materials

D-ribose, L-lysine, xylenol orange, $Fe(NH_4)_2(SO_4)_2$, PMSF, DTT, NP-40, Tris-HCl, skim milk powder, and SDS were purchased from Fluka (Buchs, Switzerland), HAM's F12 (1176 g/L $NaHCO_3$, stable glutamine), PBS Dulbecco, FCS, Penicillin/Streptomycin, EDTA in PBS, and cell culture material were purchased from Biochrom AG (Berlin, Germany). Catalase (from bovine liver), LPS (from Escherichia coli 055:B5), mouse anti-β-actin antibody, albumin bovine, EGTA, *N*-(1-naphthyl)ethylenediaminedihydrochloride, and sulfanilamide were from Sigma-Aldrich GmbH (Buchs, Switzerland). Chelex 100 resin (100–200 mesh) and Dc-Protein assay were obtained from BioRad Laboratories GmbH (Munich, Germany). DMEM (4.5 g/L glucose, GlutaMAX™, sodiumpyruvat) and Geneticin were from Invitrogen GmbH, GIBCO® (Karlsruhe, Germany); HEPES from Merck KGaA (Darmstadt, Germany). Protease inhibitor tablet complete was obtained from Roche Diagnostics GmbH (Mannheim, Germany), ECL Western Blotting detection reagents and Hyperfilm™ ECL from Amersham Biosciences (Munich, Germany), and rabbit polyclonal anti-p65 antibody (sc-109) from Santa Cruz Biotechnology (Heidelberg, Germany). Rat IL-6 DuoSet ELISA Development kit was obtained from R&D Systems (Wiesbaden-Nordenstadt, Germany) and recombinant rat INF-γ from CytoLab Ltd. (Rehovot, Israel). The monoclonal anti-RAGE antibody was prepared as described before [13].

2.2 Coffee and raw coffee preparation

Coffee beans (100% arabica coffee beans) and raw coffee beans (100% arabica raw coffee beans) from the same batch were purchased from a local coffee roaster. For the coffee preparation, 50 mL hot water (100°C) was added to 3.75 g ground roasted coffee. The suspension was allowed to stand for 10 min and then filtered. The filtrate was cooled to room temperature, adjusted to pH 8.0 with KOH, and filter sterilized. The coffee was used for further experiments exactly 1 h after the preparation had been started. Raw coffee extract was obtained in the same way from raw coffee beans. The described procedure yielded 20 mg/mL dry matter from roasted coffee and 14 mg/mL from raw coffee as determined by weighing after freeze drying. The caffeine concentrations of the extracts, which were finally used for cell stimulation, were 117 μg/mL in coffee and 75 μg/mL in raw coffee extract as determined by HPLC.

2.3 Production of Maillard reaction mixtures and control reaction mixtures

Maillard reaction mixtures of lysine and ribose (rib-lys) were produced by heating 500 mM L-lysine in equimolar ratio with D-ribose in PBS at pH 9.6 and 120°C under sterile conditions for 30 min, 3 or 24 h. The unheated solution was used as a negative control (rib-lys 0 h). As further controls, lysine (lys) and ribose (rib), respectively, were heated alone under the same conditions. Before further use in cell culture experiments, mixtures were adjusted to pH 8.0. All solutions were then filter sterilized and stored at –20°C.

2.4 Chelex-treated PBS and Maillard reaction mixture

To remove metal ions, PBS and the Maillard reaction mixture were incubated with 50 mg/mL Chelex 100 resin for 1 h. Chelex 100 resin was removed by centrifugation and the solutions were filter sterilized.

2.5 Generation of HEK293 cells stably expressing RAGE

HEK293 cells stably expressing RAGE were prepared as described before [14]. Briefly, the cDNA coding for full length RAGE was amplified from a human lung cDNA (BD Clontech, Heidelberg) by PCR with primers RAN11 (5′-CAC*GAATTC*ATGGCAGCCGGAACAGCAGTTG GA-3′) and RAC11 (5′-GTG*CTCGAG*TCAAGGCCCTC-CAGTACTACTCTCG-3′), thereby introducing *Eco*RI and *Xho*I restriction sites, respectively. The PCR product was cloned into pCR2.1-TOPO (Invitrogen, Karlsruhe, Germany) and was subcloned into pcDNA4/mycHis (Invitrogen). After sequencing, HEK293 cells were transfected, stable clones were generated by Zeocin selection and were tested for expression using the monoclonal anti-RAGE IgG2a clone A11.

2.6 Cell culture and stimulation

A macrophage (NR8383) suspension cell line from rat was maintained in HAM's F12 medium supplemented with 15% fetal calf serum (FCS), penicillin (100 IU/mL) and streptomycin (100 μg/mL) at 37°C and 5% CO_2.

RAGE-transfected human embryonic kidney (HEK) cells (HEK RAGE) and untransfected HEK-cells (HEK ut) were maintained in DMEM supplemented with 10% FCS, penicillin (100 IU/mL), and streptomycin (100 μg/mL) at 37°C and 5% CO_2. For selective growth of stably RAGE-transfected cells, 0.5 mg/mL geneticin was added to the cell culture medium. For passaging, cells were detached with 1 mM EDTA in PBS.

Macrophages (2.0×10^6 cells) were stimulated with coffee, raw coffee extract, Maillard reaction mixtures, or the control reaction mixtures in 25 cm^2 flasks for 2 h. Briefly, aliquots of 500 μL coffee or 715 μL raw coffee extract were added to PBS to a total volume of 5 mL in the flasks, yielding a final concentration of 2 mg/mL.

Quantities of 100 μL (final concentration 10 mM) or 250 μL (final concentration 25 mM) of the Maillard reaction mixture or control reaction mixture were added to medium or PBS to a total volume of 5 mL prior to stimulation.

When experiments were performed in PBS, the medium was removed and floating cells were collected by centrifugation (1500 rpm, 2 min). Floating cells and remaining adherent cells in the flask were washed with 5 mL PBS. The floating cells were resuspended in 4 mL PBS and transferred back into the flask to the adherent cells. In some experiments, Chelex-treated PBS (Chelex-PBS) was used instead of PBS. When stimulation was performed in medium, the stimulants were added directly to the medium.

For determination of NF-κB activation in HEK-cells, 6×10^6 cells were starved for 24 h with medium containing 0.2% FCS. The cells were then stimulated with the Maillard reaction mixture or control reaction mixtures for 2 h in 75 cm^2 flasks containing 10 mL medium (final concentration 25 mM).

To inhibit H_2O_2-induced NF-κB activation, 150 U/mL catalase or heat inactivated catalase (5 min at 95°C) was added simultaneously with the test substances to the cells.

2.7 Preparation of nuclear extract

Adherent cells were washed with PBS and mechanically detached by scraping. Cells were suspended in ice cold PBS and collected by centrifugation (4°C, 1500 rpm, 4 min). Floating cells of the macrophage NR8383 suspension cell line were collected by centrifugation, washed with PBS, and suspended in ice cold PBS. The suspended cells were combined with the corresponding adherent cell pellet and washed in ice cold PBS. For nuclear extraction, a previously described method was used with some modifications [15]. Briefly, NR8383 macrophages were resuspended in 500 μL buffer A (10 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, 5% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1% protease inhibitor solution, 2 mM PMSF and 0.5 mM DTT). HEK-cells were resuspended in 1 mL buffer A. After 15 min incubation, 0.65% of NP-40 was added and the cells were vortexed for 10 s. Cell nuclei, collected by centrifugation, were washed with the hypotonic lysis buffer A to ensure a complete removal of cytoplasmatic proteins. Nuclear proteins were extracted for 1 h with 30 μL (NR8383) or 100 μL (HEK-cells) buffer B (20 mM HEPES, 1% NP-40, 400 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1% protease inhibitor solution, 2 mM PMSF and 0.5 mM DTT). DNA binding proteins were stored at −80°C. Protein concentration was determined with the Dc-Protein assay (BioRad), using BSA as standard.

2.8 NF-κB Western blot

Nuclear proteins (10 μg) were denaturated with lysis buffer (60 mM Tris-HCl, 70 mM SDS, 1.6 M urea, 65 mM DTT, and 7% glycerol) and electrophoretically separated, using 12% SDS- polyacrylamide gels. The proteins were separated for 20 min at 80 V (stacking gel) followed by 75 min at 120 V (separation gel). The transfer of proteins onto a nitrocellulose membrane was performed in transfer buffer (25 mM Tris base, 0.2 M glycine, and 20% methanol) at 150 mA for 1 h. The membrane was cut between 41 and 65 kDa. To block nonspecific binding sites, the membranes were incubated with blocking buffer (5% skim milk solution in PBS containing 0.1% Tween) for 1 h, followed by primary antibody incubation overnight at 4°C. The NF-κB subunit p65 was detected on membrane (≥64 kDa) with a rabbit anti-p65 antibody (1:500 in blocking buffer). On the other membrane (≤41 kDa), β-actin was detected with a mouse anti-β-actin antibody (1:20 000 in blocking buffer). The proteins were visualized using horseradish peroxidase labeled secondary antibodies by enhanced chemiluminescence reaction (ECL). The membranes were incubated for 1 min with the ECL detection solution according to the instructions of the manufacturer. The light emission was detected on a film, and the relative intensity of the bands was determined by densitometric analysis. The intensity of the p65 signal was related to the β-actin band. NF-κB translocation is expressed as fold increase of p65 to β-actin in comparison to the control, which was maintained in the solvent (medium or PBS) alone.

2.9 RAGE Western blot

Cell lysates of HEK RAGE and HEK ut cells were prepared from 1.6×10^5 cells with 20 μL of five-fold concentrated lysis buffer (300 mM Tris, 350 mM SDS, 8 M urea, 325 mM DTT, and 35% glycerol) and 80 μL protease inhibitor solution (Protease inhibitor tablet complete, Roche). For complete protein denaturation, the samples were heated for 5 min at 95°C. An aliquot of 5 μL of the protein solution was electrophoretically separated using 12% SDS-polyacrylamide gels. RAGE was detected by Western blot using a mouse monoclonal anti-RAGE antibody. The membrane was incubated with the primary antibody (1:100 diluted in

blocking buffer) overnight at 4°C, followed by the incubation with a horseradish peroxidase labeled secondary antibody. RAGE was visualized by enhanced chemiluminescence as described above and the emitted light was detected on a film.

2.10 NO determination

The Griess reaction was used to measure nitrite spontaneously formed from NO [16]. A quantity of 3×10^4 NR8383 cells/well was cultivated in a 96-well plate for 4 h. The cells were stimulated with the Maillard reaction mixture in medium (rib-lys 30 min) for 3 h. As a positive control, INF-γ was added to the cells in medium in the indicated concentrations. Cells were washed twice with PBS and cultivated in the medium for additional 48 h. In some experiments, the medium was removed after MRP-stimulation and INF-γ was added to the cells in the indicated concentrations for 48 h. For NO determination, 50 µL of the cell supernatant was mixed with 50 µL Griess reagent (0.1% N-(1-naphthyl)ethylenediaminedihydrochloride in water; 1% sulfanilamide in 5% HCl, 1:1) and the absorbance was read at 540 nm. NO concentrations were determined as NO_2^- using $NaNO_2$ as standard.

2.11 Ferrous oxidation xylenol orange (FOX)-assay

Coffee, raw coffee extract, Maillard reaction mixtures, and control mixtures were diluted in PBS to a total volume of 0.5 mL. H_2O_2 was determined either directly or after incubation for 2 h at 37°C, 5% CO_2 with the perchloric acid (PCA)-FOX assay [17]. A blank was substracted from each sample, which was treated in the same way except for the addition of catalase 15 min prior to the FOX-reagent. Briefly, an aliquot of 10 µL PBS or -for the blank- 10 µL catalase (880 U/mL) was added to 60 µL sample and diluted 1:10 with FOX-reagent (0.45 mM xylenol orange, 0.45 mM $Fe(NH_4)_2(SO_4)_2$ in 0.11 M $HClO_4$). After 30 min the absorbance was read at 550 nm. H_2O_2 content was quantified with an external calibration graph. The use of a catalase blank excludes interference of the FOX assay with other components of the test solutions, such as metal ions.

2.12 IL-6 determination

A quantity of 1×10^6 NR8383 cells was cultivated for 4 h and stimulated with the Maillard reaction mixture (rib-lys 30 min) in 2 mL medium in a 12-well plate for 17 h. As a positive control, LPS in medium was used. The supernatant was lyophilized, stored at −80°C and resuspended in 200 µL medium. IL-6 was determined with a rat IL-6 ELISA (R&D Systems, Wiesbaden-Nordenstadt) according to the instruction manual.

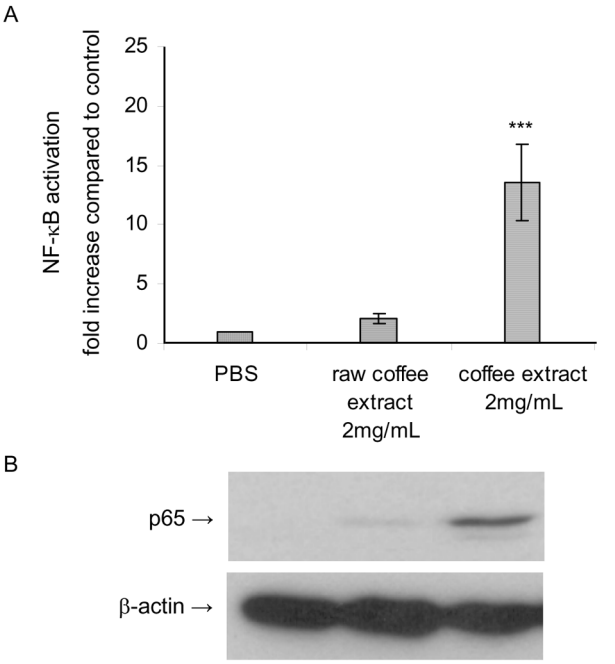

Figure 1. (A) NF-κB activation in NR8383 macrophages compared to the PBS treated control ($n = 4$). Cells were stimulated in PBS for 2 h with 2 mg/mL of raw coffee extract and 2 mg/mL of coffee extract. Nuclear NF-κB subunit p65 was detected by Western blot. The intensity of p65 signal was related to the β-actin; *** $p < 0.001$, (B) Representative Western blot of p65 and β-actin.

2.13 Statistical analysis

Statistical significance of the data was calculated using unpaired, two-tailed *t*-test with significance levels, *$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$. All results are expressed as the mean ± SD of independent experiments (number of independent experiments is indicated in the legends).

3 Results

3.1 Influence of coffee on NF-κB activation

In the first set of experiments, the influence of coffee on NF-κB activation in macrophages was investigated. Filtered coffee was prepared and diluted 1:10 in PBS to a final extract concentration of 2 mg/mL. Raw coffee was extracted and diluted to the same extract concentration (1:7).The cells were incubated with the coffee extracts for 2 h and NF-κB levels were determined by Western blot in the isolated nuclei.

Whereas raw coffee extract did not change nuclear NF-κB levels, freshly brewed coffee led to a significant 13-fold increase compared to the cells incubated only with PBS (Fig. 1).

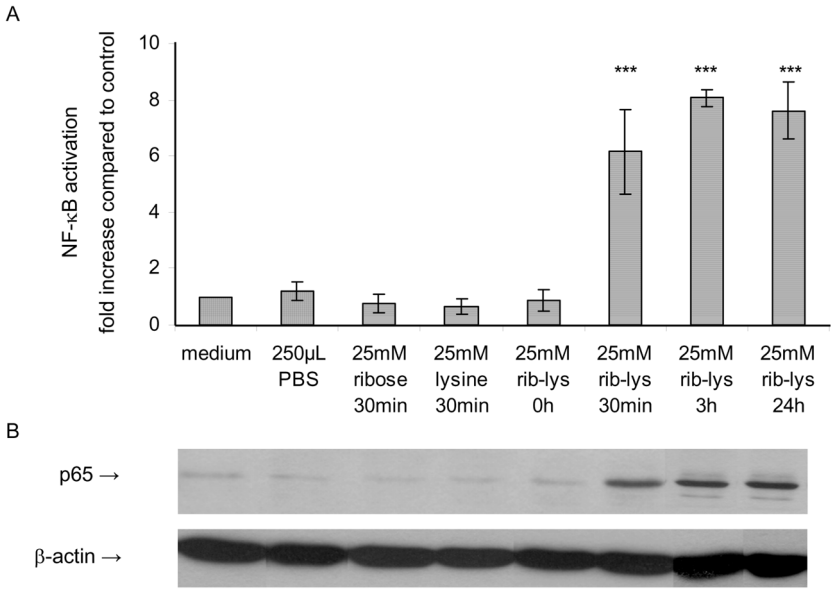

Figure 2. (A) NF-κB activation in NR8383 macrophages compared to the control, which was maintained in medium alone ($n \geq 2$). Cells were stimulated in medium for 2 h with PBS, 25 mM of heated ribose (ribose 30 min), 25 mM of heated lysine (lysine 30 min), 25 mM of unheated ribose–lysine mixture (rib-lys 0 h), 25 mM ribose and lysine heated for 30 min, 3, and 24 h at 120°C (rib-lys 30 min, rib-lys 3 h, rib-lys 24 h); *** $p < 0.001$. The differences between rib-lys 30 min, rib-lys 3 h, and rib-lys 24 h were not significant, (B) Representative Western blot of p65 and β-actin.

3.2 Influence of Maillard reaction products (MRPs) on NF-κB activation

Thus, it was hypothesized that MRPs formed during the roasting procedure are responsible for macrophage activation. To test this hypothesis, a model Maillard mixture, prepared by heating D-ribose and L-lysine for 30 min at 120°C (rib-lys 30 min), was added in a final concentration of 25 mM (D-ribose and L-lysine concentration prior to heating) in medium to the cells and incubated for 2 h. After preparation of nuclear extracts, NF-κB concentration was determined by Western blot.

A significant six-fold increased NF-κB translocation was detected compared to the medium treated controls. When the Maillard mixture was heated for 3 or 24 h, an eight-fold increase was noted. The observed effect was due to MRPs, because stimulation of the macrophages with the same concentration of the unheated reaction mixture (rib-lys 0 h), or ribose and lysine heated alone (ribose 30 min, lysine 30 min), did not lead to NF-κB translocation (Fig. 2).

3.3 Involvement of RAGE in MRP-induced NF-κB activation

It is well established that AGEs, which are Maillard products formed from sugars and proteins *in vivo*, lead to NF-κB activation in several cell types by interaction with the RAGE [18]. Therefore, it was investigated, if the MRPs used in this study also exert NF-κB translocation *via* RAGE. Thus, HEK cells were stably transfected with RAGE, resulting in high levels of RAGE expression. RAGE expression in the untransfected cells was below detection limit (Fig. 3). In medium, a Maillard reaction mixture, which had been heated for 24 h, induced a two-fold NF-κB activation in the RAGE-transfected HEK-cells (Fig. 4A). However, stimulation of the untransfected HEK-cells by the MRPs also led to a two-fold NF-κB activation (Fig. 4B). Thus it can be concluded that NF-κB activation by the MRPs takes place in HEK-cells similar to macrophages, but is not RAGE mediated. These results suggest that a second mechanism of NF-κB activation by MRPs must exist, which does not involve RAGE.

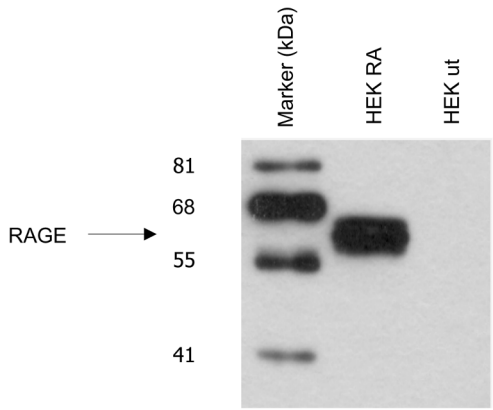

Figure 3. Protein lysates of RAGE-transfected HEK-cells (HEK RA) or untransfected HEK-cells (HEK ut) were prepared and RAGE was immunochemically detected by Western blot using a monoclonal anti-RAGE antibody.

3.4 Involvement of reactive oxygen species (ROS) in lysine-ribose MRP-induced NF-κB activation

To test the role of ROS in macrophage activation by Maillard products, stimulation was repeated in the presence of

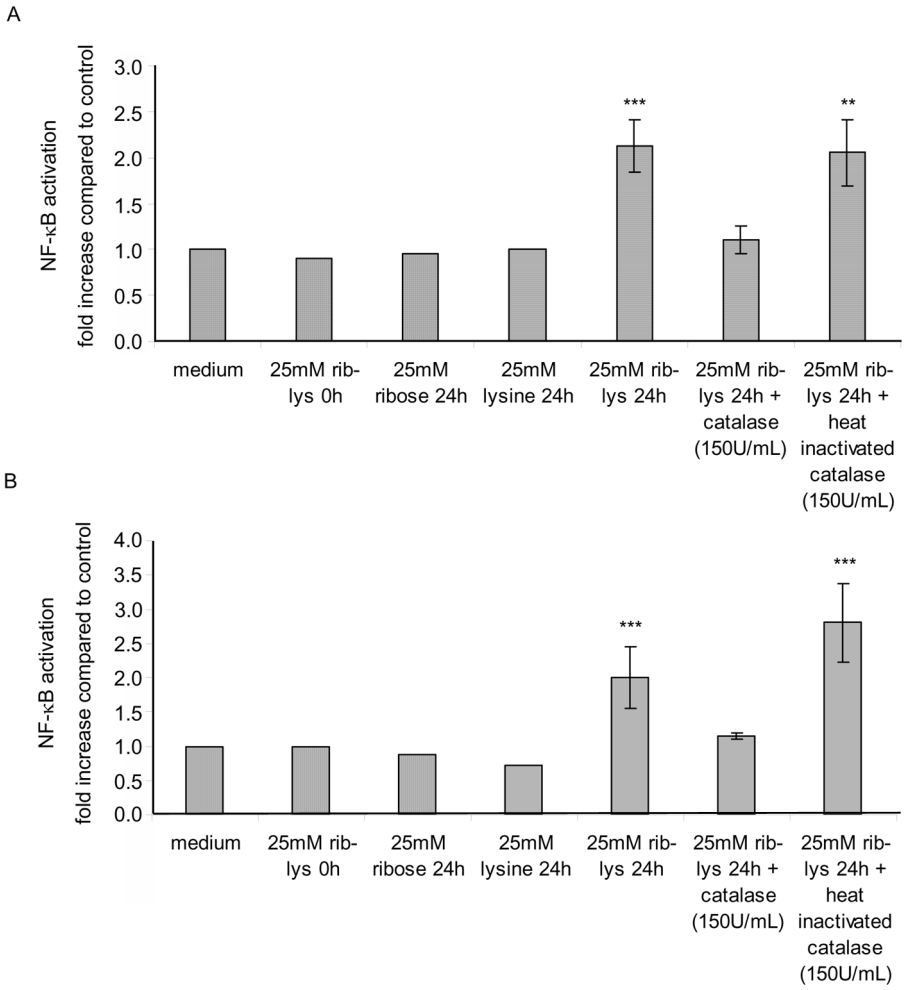

Figure 4. (A) NF-κB activation in RAGE-transfected HEK-cells (HEK RA) compared to the cells maintained in medium alone. Cells were stimulated in medium for 2 h with 25 mM of unheated control- reaction mixture (rib-lys 0 h; $n = 2$), 25 mM of 24 h heated ribose (ribose 24 h, $n = 2$), 25 mM of 24 h heated lysine (lysine 24 h; $n = 2$), 25 mM of Maillard- reaction mixtures (rib-lys 24 h; $n = 8$), in the absence and presence of catalase or heat inactivated catalase ($n = 4$). (B) NF-κB activation in untransfected HEK-cells (HEK ut) compared to the untreated control ($n = 4$). Cells were treated and stimulated according to the HEK RA experiment; ***$p < 0.001$, ** $p < 0.01$.

catalase, which converts H_2O_2 into water and oxygen. Catalase fully abolished MRP-induced NF-κB activation, whereas heat inactivated catalase did not show an effect (Fig. 5A). Likewise, catalase was also able to inhibit MRP-induced cell activation in RAGE-transfected as well as in untransfected HEK-cells (Figs. 4A and B).

3.5 Involvement of ROS in coffee-induced NF-κB activation

A similar effect was also observed when macrophages were stimulated with coffee. Catalase, but not heat inactivated catalase, efficiently reversed coffee-induced NF-κB activation in macrophages (Fig. 5B). These results indicate that NF-κB activation in macrophages by the Maillard reaction mixtures, as well as by coffee, is mediated by H_2O_2. H_2O_2 concentrations were then measured in the Maillard reaction mixtures, in the control mixtures, in coffee, and in raw coffee extracts immediately after preparation. Additionally, H_2O_2 was determined after incubation of the test solutions at 37°C under analogous conditions as applied in the cell culture experiments, but in absence of the cells. The results are summarized in Table 1. Maillard products and coffee produced -independent from the presence of cells- up to 366 μM H_2O_2.

Furthermore, the influence of the solvent used for stimulation of the macrophages was investigated. Thus, the Maillard reaction mixtures were dissolved in medium, PBS, or Chelex-treated PBS. In some experiments, also the Maillard reaction mixtures were treated with Chelex. Chelex is a che-

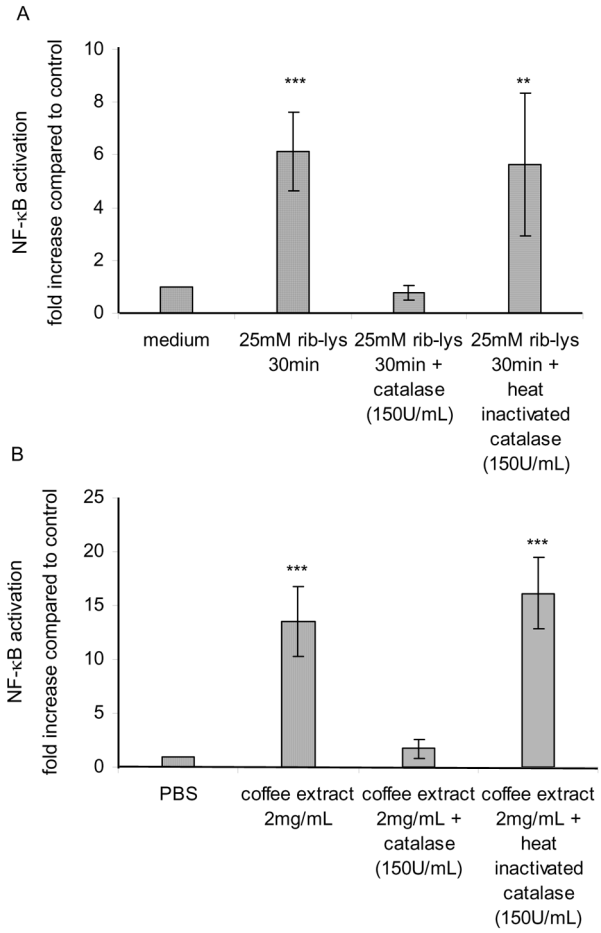

Figure 5. NF-κB activation in NR8383 macrophages compared to control, which was incubated with medium or PBS alone ($n = 3$). Cells were stimulated for 2 h in medium with 25 mM Maillard reaction mixture (rib-lys 30 min) or in PBS with 2 mg/mL of coffee extract. Catalase (150 U/mL) or heat inactivated catalase (5 min at 95°C; 150 U/mL) was added simultaneously with the test solutions to the cell culture; *** $p < 0.001$, ** $p < 0.01$.

lator efficiently removing free metal ions. Whereas the MRPs in medium led to a six-fold elevated NF-κB activation, MRPs in PBS increased nuclear NF-κB concentration 18-fold (Fig. 6). Similar to the experiments in medium, catalase inhibited the MRPs-induced NF-κB activation in PBS. Pretreatment of PBS and/or MRPs with Chelex did not attenuate NF-κB activation.

3.6 Influence of MRPs on NO and IL-6 release in macrophages

The transcription factor NF-κB is a central switch in redox-sensitive signal transduction pathways of cells and is involved in diverse cellular reactions. Therefore, it was investigated, if MRPs lead to an enhanced immune response mediated, besides others, by the release of IL-6 or reactive nitrogen species (*e.g.*, NO) [10]. Thus, macrophages were treated with the Maillard reaction mixture for 3 or 17 h. IL-6 and NO concentrations were measured in the supernatant. MRPs, however, did not induce the expression of IL-6 or the generation of NO in contrast to LPS or INF-γ, which were used as positive control stimulants (Figs. 7 and 8).

It has been shown before that AGEs did not activate cytokine expression in mononuclear cells by itself, but augmented LPS-induced inflammatory response [19]. Synergistic enhancement of inducible nitric oxide synthase (iNOS) transcription has also been described for INF-γ and NF-κB activating stimuli like LPS [20]. Therefore, it was also tested if MRPs show a similar enhancing effect to INF-γ-induced NO expression. But neither 10 nor 25 mM Maillard mixture added to 10 – 500 U/mL INF-γ lead to a significant increase in NO release (Fig. 8).

In summary, coffee and MRPs-induced activation of NF-κB by a RAGE-independent and H_2O_2-dependent mechanism. However, NF-κB activation by this mechanism is not sufficient to induce expression of the NF-κB regulated genes iNOS and IL-6 on its own.

Table 1. H_2O_2 concentrations in the Maillard mixtures, coffee, and control samples. H_2O_2 was measured in the solutions directly or after incubation for 2 h in PBS at 37°C

	H_2O_2concentration after 0 h incubation (μM, mean ± SD)	H_2O_2 concentration after 2 h incubation (μM, mean ± SD)
Coffee (2 mg/mL, $n = 4$)	81.1 ± 9.4[a)]	156.8 ± 7.9[g)]
Raw coffee (2 mg/mL, $n = 3$)	2.3 ± 1.2[b)]	4.2 ± 3.3[h)]
rib-lys (25 mM, 30 min; $n = 3$)	286.9 ± 10.9[c)]	366.4 ± 8.2[i)]
rib-lys (25 mM, unheated; $n = 3$)	13.2 ± 8.8[d)]	35.0 ± 9.5[j)]
Ribose (25 mM, 30 min; $n = 3$)	4.2 ± 5.1[e)]	2.5 ± 2.8[k)]
Lysine (25 mM, 30 min; $n = 3$)	2.5 ± 3.4[f)]	0.6 ± 1.0[l)]

a–l): Statistical significant differences ($p < 0.001$) among all groups were confirmed by Kruskal – Wallis-H-test. Differences between two groups were then determined with an unpaired, two-tailed *t*-test with the significance levels *** $p < 0.001$ (a-b, a-g, a-h, b-g, c-d, c-e, c-f, c-i, c-j, c-k, c-l, d-i, e-i, f-i, g-h, i-j, i-k, i-l), ** $p < 0.01$ (e-j, f-j, j-k, j-l), * $p < 0.05$ (d-j); $p = 0.49$ (b-h), $p = 0.20$ (d-e), $p = 0.12$ (d-f, d-k); $p = 0.07$ (d-l); $p = 0.66$ (e-f), $p = 0.65$ (e-k), $p = 0.29$ (e-l), $p = 1$ (f-k), $p = 0.38$ (f-l), $p = 0.32$ (k-l).

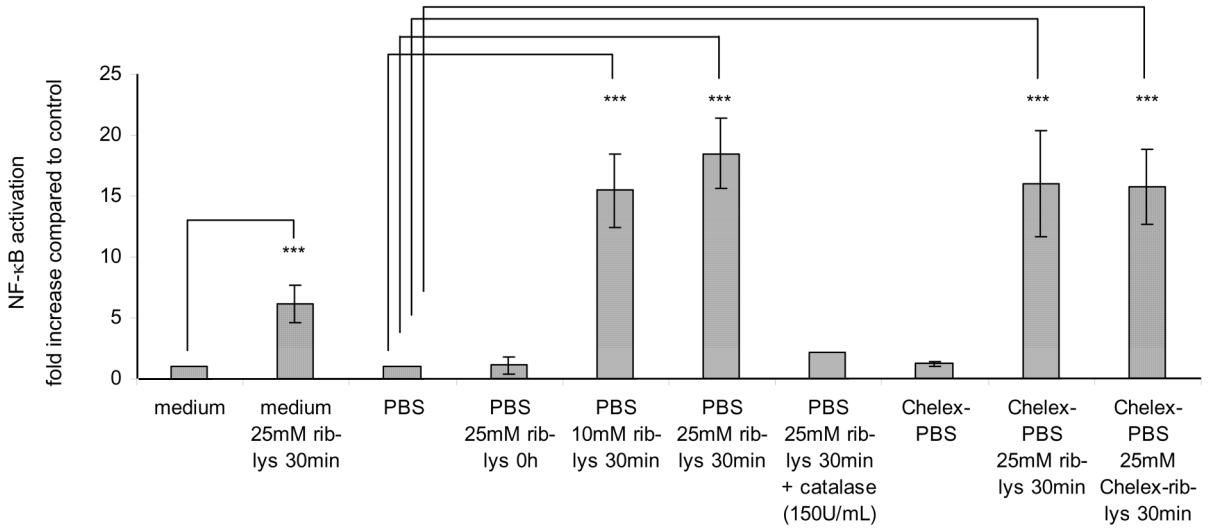

Figure 6. NF-κB activation in NR8383 macrophages compared to the control, which was maintained in medium, PBS, or Chelex-treated PBS (Chelex-PBS; $n = 3$). Cells were stimulated in medium, PBS, or Chelex-treated PBS for 2 h with 10 mM, 25 mM of Maillard reaction mixture or 25 mM Maillard mixture pretreated with Chelex. The Maillard mixtures consisted of 30 min heated ribose and lysine (rib-lys 30 min or Chelex-rib-lys 30 min). When indicated, catalase (150 U/mL) was added simultaneously with the Maillard reaction mixture to the cell culture; *** $p < 0.001$. The difference between PBS 10 mM rib-lys 30 min and PBS 25 mM rib-lys 30 min was not significant.

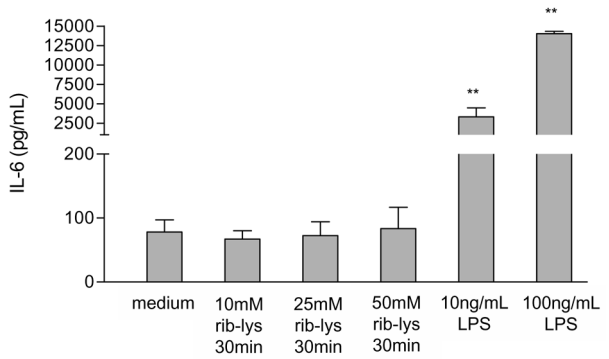

Figure 7. IL-6 expression in MRP treated NR8383 macrophages ($n \geq 5$). Cells were stimulated in medium for 17 h with 10 mM, 25 mM, and 50 mM of the Maillard reaction mixture (rib-lys 30 min) or with LPS as positive control ($n = 2$); ** $p <$ 0.01.

4 Discussion

Macrophages differentiate from circulating monocytes after migration into the gut. In this active form, they are key players in the intestinal immune response, mediate inflammatory reaction, and are also involved in tissue repair and remodeling. Thereby, macrophages maintain the inflammatory process by the release of chemoattractants, cytokines, proteases, and reactive oxidative species. In the absence of pathogenic microorganisms, however, intestinal macrophages are rather inactive. In contrast, a large number of activated macrophages are found in the mucosa under conditions of inflammatory bowel diseases, such as Crohn's disease or ulcerative colitis. They are characterized by the expression of IL-2 receptors as well as ongoing release of cytokines. [10]

NF-κB has a central position in the signal transduction of immune cells. It is activated by many bacteria, virus, or their products. As an immune response, NF-κB promotes the expression of more than 150, mostly immune related, proteins, including cytokines [21, 22]. Thus, activation of NF-κB is one of the first steps in the immune response of macrophages. Beyond its role in the immune response, NF-κB mediates stress response to a great variety of stimuli, such as oxidative, physiological, chemical, and physical stress [21]. In this context, NF-κB mediates antiapoptotic and antimicrobial survival mechanisms by induction of the iNOS, transcription of acute phase and stress response genes, and also proapoptotic effects [23]. Inflammatory bowel diseases are characterized by continuously elevated nuclear NF-κB levels; beneficial effects were observed in these patients after blockage of NF-κB activation [23]. Because of the diverse functions of NF-κB, activation of the transcription factor in macrophages appears to be an excellent target to monitor any immune modulating effects of food components.

In this study, coffee induced the activation and nuclear translocation of NF-κB 13-fold. Since extracts of raw coffee were not active, the effect was related to Maillard products formed during roasting. And indeed, Maillard mixtures of ribose and lysine exerted a similar 18-fold activation in PBS (Fig. 6). The effect was due to MRPs, because

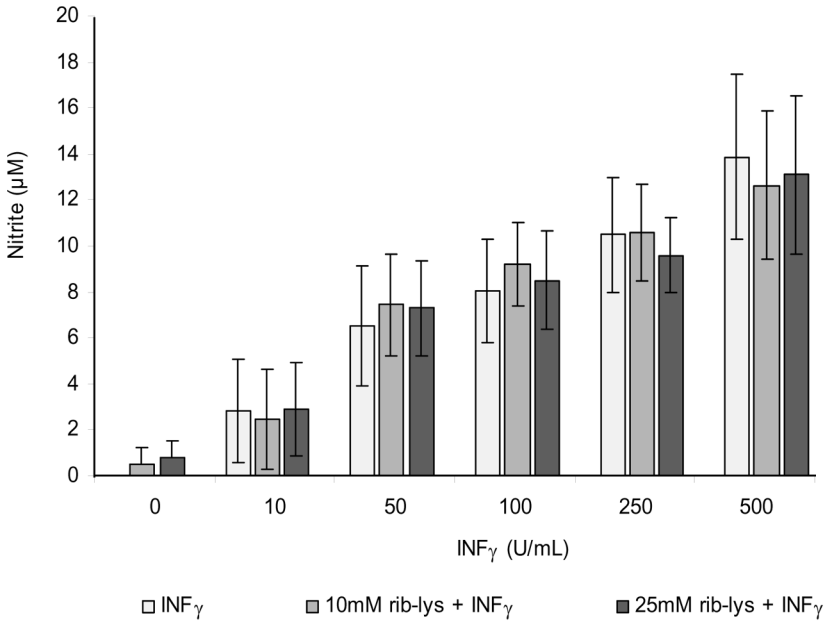

Figure 8. Mean of NO (µM) release from NR8383 macrophages ($n = 5$). The cells were stimulated for 3 h in medium with 10 or 25 mM of the Maillard reaction mixture (rib-lys 30 min). After removing the media, the cells were stimulated with indicated concentrations of INF-γ for 48 h.

ribose or lysine heated alone did not lead to NF-κB translocation. Maillard products, mainly melanoidins, are important components of coffee, accounting for about 25% of the dry matter [24]. The physiological activity of coffee melanoidins is not yet fully understood. In several previous studies, antioxidative, metal chelating, and antibacterial properties of melanoidins were described [25 – 27]. The present study suggests that MRPs in coffee further activate NF-κB translocation in macrophages.

It has been shown before that Maillard products induce translocation of NF-κB: protein bound N^{ε}-(carboxymethyl)lysine activates NF-κB cell signaling pathways in endothelial cells, vascular smooth muscle cells, and mononuclear phagocytes [12]. The effect was clearly mediated by RAGE. To test the hypothesis that NF-κB activation by MRPs in macrophages is due to RAGE engagement, stimulation was performed with HEK cells stably transfected with RAGE. However, NF-κB activation was similar in transfected and untransfected cells, so that MRPs exert their activity independently from RAGE.

On the other hand, NF-κB activation by MRPs was almost completely inhibited by coincubation with catalase, suggesting that H_2O_2 plays a key role in the process. Further investigations revealed that coffee and the Maillard mixtures used for stimulation produce up to 366 µM H_2O_2 in a cell free matrix. H_2O_2 was not generated by raw coffee extract, or when ribose or lysine was heated alone. These results are in good accordance with literature, which indicates that oxidative stress may be involved in physiological effects of Maillard products and AGEs. It has been shown, for example, that cytotoxicity of protein-bound AGEs in fibroblasts can be inhibited by antioxidants [28], and that superoxide anions and hydrogen peroxide are directly formed through the Maillard reaction [29]. In the presence of UVA light, Maillard products can serve as photosensitizers, leading to enhanced formation of reactive oxygen species [30, 31]. Furthermore, aminoreductones generated by Maillard reaction showed pronounced pro-oxidative activity in the presence of metal ions [32]. The formation of H_2O_2 in coffee beverages has been described before. In freshly prepared coffee, 0 – 100 µM H_2O_2 was measured. The levels were considerably higher when the coffee was allowed to stand at 37°C [33]. Akagawa *et al.* [34] suggested that H_2O_2 formation in coffee is due to the polyphenol fraction, but in our own studies raw coffee extract, which is rich in phenols, did not generate any H_2O_2. Under the conditions applied here, roasting products, mainly Maillard products and melanoidines, are, therefore, responsible for the observed effect. There is strong evidence that H_2O_2 formed in the coffee beverage and in other MRP containing food is of relevance *in vivo*, because increased urinary H_2O_2 levels were measured after the consumption of coffee [35, 36]. This means that either H_2O_2 is directly consumed with the coffee beverage and not fully detoxified, or that active components of the coffee continue H_2O_2 generation *in vivo*.

It has been suggested that antioxidants can exert pro-oxidative activity in cell culture medium by metal ion catalysis. This could lead to cell activation, which is, however, limited to cell culture conditions [37]. In order to investigate whether NF-κB activation in macrophages by a Maillard reaction mixture is an artifact derived from the cell culture media, the stimulation experiments were also performed in physiological PBS. A three-fold higher activation in PBS compared to medium was observed, which is probably due to the presence of FCS in the medium. FCS contains low levels of catalase and can, therefore, weaken the MRP-

induced NF-κB activation caused by H_2O_2. Buffers, used for cell stimulation or preparation of Maillard mixtures, may also be a source of metal ions, which could artificially influence the results of the cell culture experiments. Therefore, traces of metal ions in PBS and MRPs were removed with Chelex. However, the pretreatment did not influence NF-κB activation in macrophages indicating that the observed effect of the MRPs is not an artifact of the cell culture conditions.

NF-κB mediates various cellular responses in macrophages. For example, it is involved in immune and inflammatory reactions by the release of cytokines, particularly TNF-α, IL-1, and IL-6. Macrophage-released IL-6 induces growth and differentiation of lymphocytes as well as acute phase protein in the liver [9, 21]. Furthermore, iNOS, which synthesizes NO, is activated by NF-κB [38]. In the intestinal immune system, NO seems to have a protective and antiapoptotic effect on various cell types [23]. However, in our study, activation of NF-κB by MRPs was not associated with an increased expression of IL-6. Likewise, NO production was not increased after activation of NF-κB by melanoidins alone or applied as costimulants in the presence of INF-γ.

Thus, we hypothesize that besides the RAGE-mediated pathway, there is a second mechanism how Maillard products can stimulate NF-κB translocation and thus lead to cell activation: Maillard products generate reactive oxygen species such as H_2O_2 by a cell independent mechanism. Oxidative stress would then cause cellular activation. This hypothesis is supported by other studies, which showed that H_2O_2 induce NF-κB activation in several cell lines [39, 40]. On a long term, foods rich in melanoidines, such as coffee, may, therefore, influence the intestinal immune function. This effect may be particularly enhanced in patients with inflammatory bowel disease, where NF-κB activation in macrophages has an important role in pathogenesis.

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