RESEARCH ARTICLE

Identification of aminoreductones as active components in Maillard reaction mixtures inducing nuclear NF-κB translocation in macrophages

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Coffee, a highly processed food, and Maillard mixtures are able to activate nuclear factor κB translocation in macrophages *via* generation of hydrogen peroxide. In this study, a substructure library was prepared and used to identify Maillard products that are responsible for this effect. Three different Maillard reaction products with aminoreductone substructure (C_6-aminoreductone, C_4-aminoreductone, and aminohexose reductone) strongly induce nuclear factor κB translocation in macrophages. The effect was almost completely blocked by co-incubation with catalase, indicating that cellular activation was mediated by the ability of the test compounds to generate hydrogen peroxide. The cellular effect of a Maillard mixture, which was produced under conditions favoring aminoreductone formation, could be almost completely related to the presence of C_6-aminoreductone.

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1 Introduction

During food processing, reducing sugars react with amines, mainly amino acids or proteins, leading to complex mixtures of reaction products (Maillard reaction). So far, it is still controversially discussed if the consumption of Maillard products may have beneficial or adverse effects on the consumer's health [1, 2]. For example, it is well established that Maillard reaction generates products with antioxidative activity in model mixtures as well as in processed food [3, 4]. *In vitro*, it was shown that high molecular weight Maillard products (melanoidins) from coffee could protect cells from oxidative stress [5]. *In vivo*, the consumption of highly processed food products led to an increased oxidative resistance, which may be related to Maillard products [6, 7].

The genotoxic effect of Maillard products has also been studied extensively. On the one hand, it is well known that mutagenic and possibly carcinogenic compounds, such as acrylamide and heterocyclic amines are formed, when amino acids and carbonyl compounds react during food processing [8, 9]. On the other hand, antimutagenic effects of Maillard products *in vitro* have also been reported [10]. Furthermore, it has been hypothesized that Maillard products resorbed from nutrition increase the systemic pool of advanced glycation end-products (AGEs) [11]. AGEs are protein modifications formed *in vivo* by Maillard-type reactions, for which pro-inflammatory and fibrogenic effects were proposed [12, 13]. Thus, a high intake of dietary AGEs may enhance the adverse effect of endogenously formed AGEs [14]. Finally, several other cellular effects of Maillard products and melanoidins have been observed *in vitro*, which may be linked to the physiological activity of processed food. Among those is the inhibition of

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Abbreviations: 3-DG, 3-deoxy-D-erythro-hexos-2-ulose; **AGE,** advanced glycation end-product; **AHR,** aminohexose reductone; **AR,** aminoreductone; **C_4-AR,** 3-hydroxy-4-(morpholino)-3-buten-2-one; **C_6-AR,** 1-(butylamino)-1,2-dehydro-4-deoxy-3-hexulose; **CML,** N^{ε}-carboxymethyllysine; **DDMP,** 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one; **DFLys,** N^{ε}-(1-deoxy-D-fructos-1-yl)-L-lysine; **FMOC,** fluoren-9-ylmethoxycarbonyl-; **MM,** Maillard reaction mixture; **NF-κB,** nuclear factor κB

angiotensin-I converting enzyme [15], influence on calcium bioavailability [16], or anti-bacterial activity [17]. The effects, however, were rarely related to defined structures, but rather to crude reaction mixtures or melanoidins, that are difficult to characterize.

Recently, it was shown that coffee and Maillard reaction mixtures (MMs) strongly trigger the nuclear factor κB (NF-κB) translocation in macrophages [18]. The transcription factor NF-κB has a central position in cellular signaling. It is activated by a wide range of stimuli, such as bacteria as well as oxidative, physiological, chemical, or physical stress. As a response, more than 150 proteins can be expressed, which are involved – among others – in immune reaction, apoptosis, or stress response [19]. The effector proteins, which are expressed in response to NF-κB activation by coffee and MMs, have not been elucidated yet, but it was shown that Maillard mixtures of similar composition lead to cell death upon prolonged incubation [20]. Further, hydrogen peroxide, which is formed in the heated amino acid/sugar mixture and in coffee, was directly responsible for both effects – activation of nuclear NF-κB transformation and cytotoxicity [18, 20].

The purpose of this study was to identify components of the MMs, which may be responsible for hydrogen peroxide mediated NF-κB activation in macrophages, by the use of a substructure library.

2 Materials and methods

2.1 Apparatus and analytical HPLC

Analytical HPLC was performed with a Jasco PU-1580 Intelligent HPLC pump, Jasco DG-980-50 three-line degasser, Jasco LG-980-02S tenery gradient unit, Jasco AS-1555 Intelligent sampler, and Jasco MD-1510 multiwavelength diode array detector that included Jasco Chrompass software (Jasco GmbH, Gross-Umstadt, Germany). UV spectra were directly taken from this system and are given in nanometers. ^{1}H NMR (360.1/600 MHz) and ^{13}C NMR (90.5/150.9 Hz) spectra were recorded with a Bruker Avance spectrometer with tetramethylsilane or aceton as internal standard (Bruker Daltonik GmbH, Bremen).

2.2 Chemicals and reagents

Deionized water for HPLC was distilled before use. All chemicals were of analytical reagent grade quality. Triethylamine, benzoylhydrazine, benzaldehyde, trichloroacetic acid, and butylamine were obtained from Acros (Geel, Belgium). Ammonium formate, PMSF, DTT, nonidet P40 substitute, Tris-HCl, skim milk powder, N^{α}-carbobenzoxylysine, iodoacetic acid, palladium black, diacetyl, bromine, morpholine, 2.6-dichlorphenolindophenol sodium salt hydrate, N^{α}-fluoren-9-ylmethoxycarbonyl lysine hydrochloride (N^{α}-FMOC-lysine hydrochloride), amberlit IR 120 (H^{+}), formaldehyde, glycerol and SDS, phosphoric acid, alanine, lactose, catalase (from bovine liver), mouse anti-β-actin antibody and EGTA, poly(oxyethylen)$_{20}$-sorbitan-monolaurate, BSA, glycine, ponceau red S, glucose, and IRN-78 anion-exchange resin were from Fluka-Sigma-Aldrich (Buchs, Switzerland). HAM's F12 (1176 g/L $NaHCO_3$, stable glutamine), PBS Dulbecco, fetal calf serum, penicillin/streptomycin, and cell culture material were purchased from Biochrom AG (Berlin, Germany). D_c-Protein assay, *N,N,N′,N′*-tetramethylethylendiamine, 30% acrylamide/bissolution, and ammoniumpersulfate were obtained from BioRad Laboratories GmbH (Munich, Germany). 4-(2-Hydroxyethyl)-1-piperazinethan sulfonic acid, *p*-toluidine, mercaptoacetic acid, piperidine, methanol (HPLC grade), and galactose were purchased from Merck KGaA (Darmstadt, Germany). Protease inhibitor tablet complete was obtained from Roche Diagnostics GmbH (Mannheim, Germany), enhanced chemiluminescence reaction Western blotting detection reagents and Hyperfilm^{TM} ECL were from GE Healthcare Life Sciences (Munich, Germany), and rabbit polyclonal anti-p65 antibody (sc-109) was from Santa Cruz Biotechnology (Heidelberg, Germany).

2.3 Preparation of N^{ε}-(1-deoxy-D-fructos-1-yl)-L-lysine

N^{ε}-(1-deoxy-D-fructos-1-yl)-L-lysine (DFLys) was synthesized according to Vinale *et al.* [21]. Briefly, a solution of N^{α}-FMOC-lysine x HCl and triethylamine in methanol was stirred for 20 min. Anhydrous glucose was added to the reaction mixture and stirred for 3 h at 64°C under a nitrogen atmosphere. N^{α}-FMOC-DFLys was isolated from the mixture by column chromatography. For removal of the protecting group, morpholine was added to N^{α}-FMOC-DFLys dissolved in a mixture of dimethylformamide/methanol and stirred for 2 h at 25°C. The volatiles were removed under reduced pressure and the residue was washed with diethyl ether. Product identity was confirmed by NMR.

2.4 Preparation of 3-deoxy-D-*erythro*-hexos-2-ulose

3-Deoxy-D-*erythro*-hexos-2-ulose (3-DG) was prepared according to Madson and Feather [22]. Briefly, glucose and benzoylhydrazine were reacted to give the bishydrazone. The bishydrazone was cleaved with freshly distilled benzaldehyde and soluble 3-DG was further purified using an ion exchange resin. Product identity was confirmed by NMR.

2.5 Preparation of N^{ε}-carboxymethyllysine

N^{ε}-Carboxymethyllysine (CML) was synthesized according to Huber and Pischetsrieder [23] with the exception that an RP-18 column was used for purification. Briefly,

N^{α}-carbobenzoxylysine and iodoacetic acid were dissolved in 0.2 M phosphate buffer and the pH was adjusted to ten. The solution was stirred for 48 h at room temperature. Twenty-five percent NH_3 was added and the solution was stirred overnight at room temperature. N^{α}-Carbobenzoxy-N^{ε}-(carboxymethyl)lysine was isolated by semi-preparative HPLC on a column packed with Nucleosil 100-7 C18 (250 × 21 mm id, 10 μm particle size) from Macherey-Nagel (Dueren, Germany) and protected with a guard cartridge (30 × 16 mm, 7 μm particle size) packed with the same material as the column. Ten percent methanol in 5 mM ammonium formate buffer, pH 7.0, was used as eluent with a flow rate of 8 mL/min. Carbobenzoxy-CML was detected at 217 nm, and the fractions between 13 and 15 min were collected and lyophilized. The residue was dissolved in dry ethanol, palladium black was added, and the mixture was hydrogenated overnight. The precipitated product was purified by filtration. CML was obtained as a white powder. Product identity was confirmed by NMR.

2.6 Preparation of 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one

2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) was synthesized according to literature [24]. Briefly, a mixture of glucose hydrate and ethanol was heated to 70–75°C under a nitrogen atmosphere. A solution of piperidine in ethanol was added to the mixture over a few minutes. After stirring for 90 min, mercaptoacetic acid in ethanol was added over a period of 30 min. The mixture was stirred for another 22 h and isolated by diethyl ether extraction. Product identity was confirmed by NMR.

2.7 Preparation of 3-hydroxy-4-(morpholino)-3-buten-2-one

3-hydroxy-4-(morpholino)-3-buten-2-one (C_4-aminoreductone, C_4-AR) was prepared according to a method described previously [3, 25] with slight modifications. Briefly, 1-bromo-2,3-butanedione was dissolved in tetrahydrofuran. Morpholine was added drop-wise. The mixture was stirred for 60 min at 50°C. The product was removed from precipitated morpholine hydrobromide by filtration. The solvent was evaporated. Product identity was confirmed by NMR.

2.8 Preparation of 1-(butylamino)-1,2-dehydro-4-deoxy-3-hexulose

1-(butylamino)-1,2-dehydro-4-deoxy-3-hexulose (C_6-AR) was synthesized according to Pischetsrieder *et al.* [26]. Briefly, lactose was heated with butylamine in phosphate buffer at pH 7.0 for 45 min at 100°C and C_6-AR was extracted with ethyl acetate. Product identity was confirmed by NMR.

2.9 Isolation of piperidinohexosereductone (aminohexose reductone)

Aminohexose reductone (AHR) was synthesized according to Hodge [27]. Briefly, anhydrous glucose was heated and stirred with piperidine in methanol at 70°C. Phosphoric acid was slowly added to the solution while stirring. Heating under reflux was continued for 18 h at 75°C. The mixture was cooled to 2°C, and the crystallized AHR was isolated by filtration. Product identity was confirmed by NMR.

2.10 Preparation of lactose-alanine, glucose-alanine and glucose-proline model mixtures

AR model mixtures of lactose-alanine, glucose-alanine, and glucose-proline were prepared by heating 0.53 mmol sugar with 2.33 mmol amino acid in 2 mL phosphate buffer at 100°C for 45 min. The mixtures were analyzed by HPLC as described below. UV spectra of the products were compared to those of C_6-AR, C_4-AR, and AHR.

2.11 Quantification of C_6-AR in a reaction mixture of lactose and butylamine

Lactose (180 mg, 0.53 mmol) and 170 mg butylamine (2.32 mmol) were dissolved in 2 mL phosphate buffer (1.28 M) and the pH was adjusted to 7.0 with phosphoric acid. The mixture was heated for 45 min at 100°C. C_6-AR was analyzed in the samples by HPLC. For HPLC analysis, the samples were diluted 1:100. Separation was performed on a column packed with Nucleosil (RP 18, 125 × 3 mm id, 5 μm particle size) from Macherey-Nagel and protected with a guard cartridge (8 × 3 mm) packed with the same material as the column. The eluents were ammonium formate buffer (5 mM, pH 6.1; A) and methanol (B). For stepwise elution, the following program was used: 98% A from 0 to 10 min (separation), 98% A to 0% A from 10–15 min, 0% A from 15–20 min (washing), 0% A to 98% A from 20–25 min and 98% A from 25–35 min (equilibration) at a flow rate of 0.4 mL/min with an injection volume of 50 μL. The substances were detected with the diode array detector recording between 200 and 640 nm. C_6-AR was identified in the reaction mixture by comparison of the retention time and UV spectra with those of the isolated reference compound. For quantification, an external calibration curve with 0.25, 0.5, 0.75, 1.0, and 1.5 mM of synthesized C_6-AR was used. The MM contained 33 mM C_6-AR. After dilution with PBS to yield a concentration of 10 mM C_6-AR, the MM was used for cell stimulation to determine the contribution of C_6-AR to the NF-κB-inducing activity of the MM (see Section 3.4) and as a positive control (see Section 3.1).

2.12 Cell culture and stimulation

A macrophage suspension cell line from rat (NR8383) was maintained in HAM's F12 medium supplemented with 15% fetal calf serum, penicillin (100 IU/mL), and streptomycin (100 μg/mL) at 37°C and 5% CO_2. Prior to stimulation, floating cells and adherent cells were washed with 5 mL PBS and cell viability was confirmed by trypan blue assay. Macrophages (2.0×10^6 cells) were stimulated with synthesized Maillard reaction products in 25 cm^2 flasks for 2 h. For this purpose, 2.5 mL of PBS and then 2.5 mL of a solution of the synthesized Maillard products in PBS were added to the cells, yielding a final concentration of 10 mM. Furthermore, 5 mL of the MM described under Section 2.11 containing 10 mM C_6-AR was used for cell stimulation. To inhibit H_2O_2-induced NF-κB activation, an aliquot of 150 U/mL catalase or heat inactivated catalase (7 min at 95°C) was added simultaneously with the test substances to the cells. As a control, the cells were incubated with 5 mL PBS.

2.13 Preparation of nuclear extract

The nuclear extract was prepared according to Muscat *et al.* [18]. DNA-binding proteins were stored at −80°C. Protein concentration was determined with the D_c-Protein assay (BioRad Laboratories GmbH), using BSA as standard.

2.14 NF-κB Western blot

NF-κB Western blots were performed according to Muscat *et al.* [18]. 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 PBS alone.

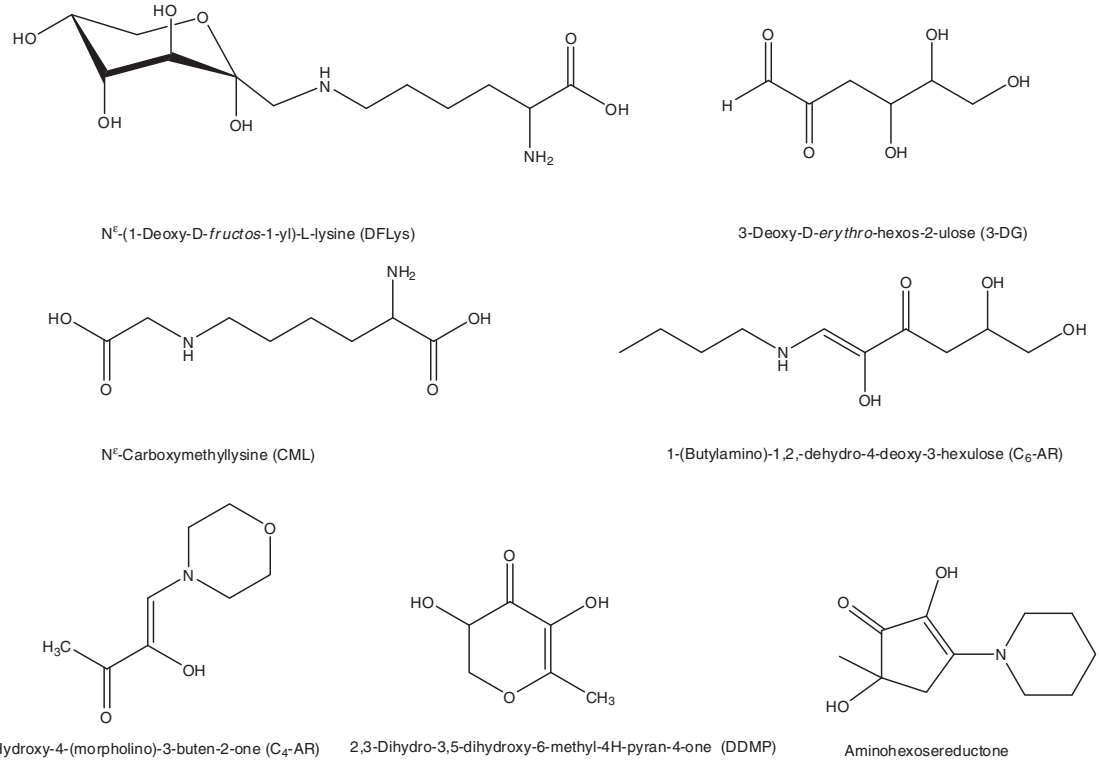
2.15 Statistical analysis

Statistical significance of the data was calculated using unpaired, two-tailed *t*-test with significance levels, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. All cell culture experiments were performed at least in triplicates and the results were expressed as the mean ± SD.

3 Results

3.1 Development of a substructure library of Maillard reaction products

The goal of this study was to identify components which could be responsible for the hydrogen peroxide mediated effect of Maillard mixtures and coffee to activate nuclear translocation of NF-κB in macrophages. For this purpose, a substructure library of products was synthesized, whose



Scheme 1: Structures of the test compounds

major structural elements are often found in Maillard products. Under these aspects, DFLys, an Amadori product derived from glucose and lysine, 3-DG, an α-dicarbonyl intermediate, CML, one of the best studied AGEs with a carboxylate group, DDMP, a Maillard product with reductone structure, and 3-hydroxy-4-morpholino-3-buten-2-one (C_4-AR), a Maillard product with amino reductone structure, were chosen as potential lead structures and used as test compounds (Scheme 1).

Macrophages were then incubated for 2 h with the test compounds and nuclear NF-κB translocation was analyzed by Western blot. In the tested concentration of 10 mM, DFLys, 3-DG, CML, and DDMP did not show significantly higher NF-κB activation than the PBS-treated control. In contrast, C_4-AR showed an eightfold increased activation compared to the PBS control ($p < 0.001$; Fig. 1). The effect was in a similar range as that of an MM of butylamine and lactose (MM), that served as a positive control.

3.2 Influence of different Maillard products with AR structure on nuclear NF-κB translocation in macrophages

In order to investigate if the observed effect is caused by the AR structure, or if it is specific for C_4-AR, two other Maillard products with AR structure were synthesized: C_6-AR, which contains the entire hexose backbone; and AHR, a cyclic AR. Macrophages were incubated for 2 h with the test compounds and nuclear translocation of NF-κB was analyzed as before. All the three ARs led to a highly significant increase of nuclear NF-κB concentration compared to the PBS-treated control (Fig. 2). The differences among the three ARs, however, were not significant. Thus, it was concluded that the observed effect was not specific for C_4-AR, but that the AR structure is the bioactive functional group, responsible for nuclear NF-κB translocation in macrophages.

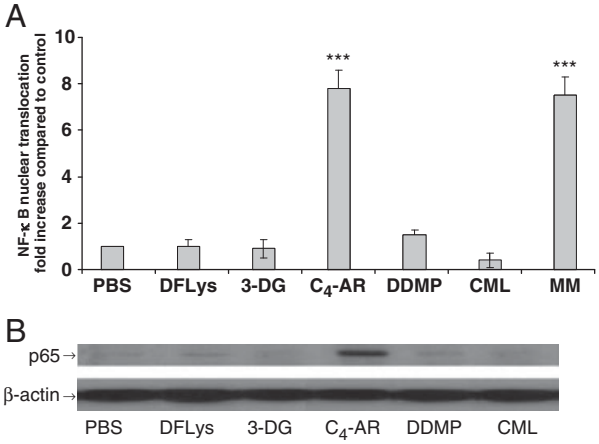


Figure 1. (A) Nuclear NF-κB translocation in NR8383 macrophages compared with PBS-treated control. Cells were stimulated for 2 h with PBS, 10 mM of DFLys, 3-DG, C_4-AR, DDMP, CML, or an MM. The intensity of the NF-κB subunit p65 was related to β-actin; $^{***}p < 0.001$, significant differences are related to the PBS control (mean ± SD, $n = 4$). (B) Representative Western blot of p65 and β-actin.

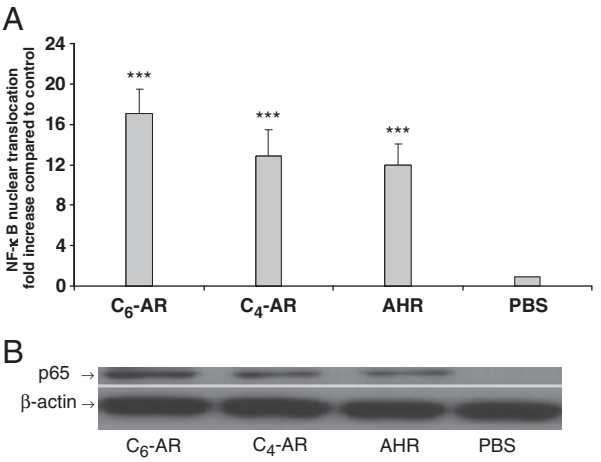


Figure 2. (A) Nuclear NF-κB translocation in NR8383 macrophages compared with PBS-treated control. Cells were stimulated for 2 h with PBS, 10 mM of C_6-AR, C_4-AR, or AHR. The intensity of the NF-κB subunit p65 was related to β-actin; $^{***}p < 0.001$; significant differences are related to the PBS control; the differences between C_6-AR, C_4-AR, and AHR were not significant (mean ± SD, $n = 3$). (B) Representative Western blot of p65 and β-actin.

Furthermore, the dependence of the effect on the concentration of the test compounds was studied. For all the three ARs, a concentration-dependent increase of activity was observed (Figs. 3A–C). Whereas the results of 1 mM solutions of the test compounds did not differ significantly from the PBS control, 5 mM solutions led to an increase of nuclear NF-κB concentration, which was significant for C_4-AR and AHR. For 10 mM solutions, a further increase of activation was recorded.

3.3 Involvement of reactive oxygen species in AR-induced nuclear NF-κB translocation

It has been clearly demonstrated that MMs activate nuclear NF-κB translocation *via* formation of hydrogen peroxide [18]. Therefore, it was investigated if ARs activate macrophages by a similar mechanism. For this purpose, incubation of the cells with all the three ARs was repeated without any addition and in the presence of catalase or heat inactivated catalase. The addition of catalase almost completely abolished NF-κB activation that was induced by the ARs, leading only to non-significant differences compared to the PBS-treated control (Fig. 4). In contrast, heat inactivated catalase did not influence the activity of the ARs. These results indicate that AR-induced NF-κB translocation in macrophages is mediated by H_2O_2.

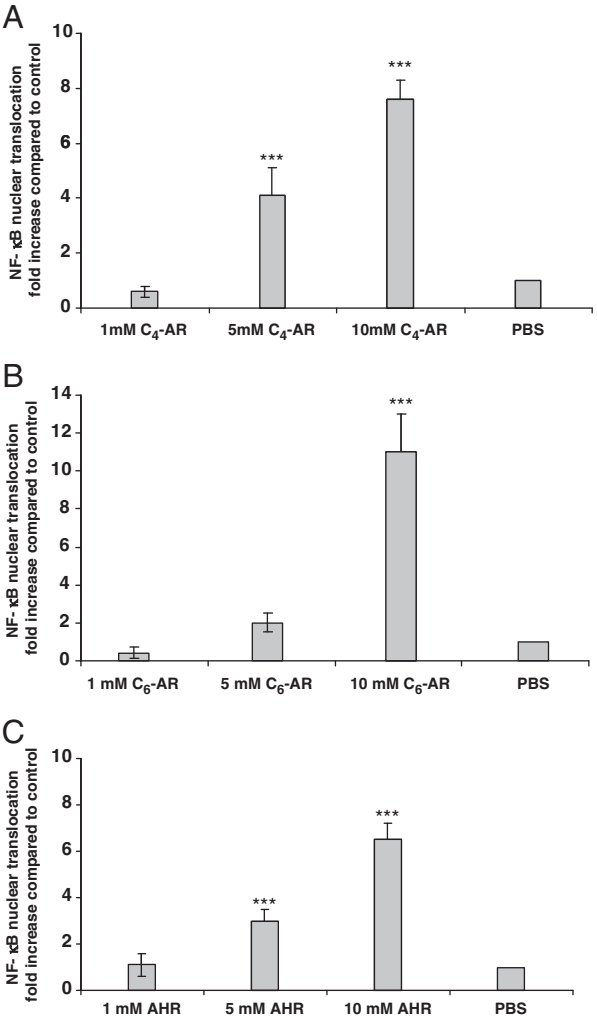


Figure 3. Nuclear NF-κB translocation in NR8383 macrophages compared with PBS-treated control. Cells were stimulated for 2 h with PBS, 1 mM, 5 mM, and 10 mM of C_4-AR (A; mean ± SD, $n = 3$), C_6-AR (B; mean ± SD, $n = 4$), or AHR (C; mean ± SD, $n = 4$). The intensity of the NF-κB subunit p65 was related to β-actin; $^{***}p < 0.001$, significant differences are related to the PBS control.

3.4 Contribution of C_6-AR to the activity of a Maillard model mixture in inducing nuclear NF-κB translocation in macrophages

In the last part of the study, it was investigated to which extent ARs may contribute to the activity of MMs in triggering nuclear NF-κB translocation in macrophages. For this purpose, a lactose and butylamine mixture was heated under conditions that favor the formation of C_6-AR. The reaction mixture contained 33 mM C_6-AR as quantified by HPLC/UV using the synthesized C_6-AR standard as external standard. For cell stimulation, the mixture was diluted to a final concentration of 10 mM C_6-AR. Figure 5 shows that both, the model mixture containing 10 mM C_6-AR and 10 mM pure C_6-AR led to similar nuclear NF-κB translocation in macrophages.

4 Discussion

In previous studies, it was shown that MMs are able to induce nuclear NF-κB translocation in several cell types by their ability to generate hydrogen peroxide [18]. The purpose of this study was to identify structures in the mixtures which could be responsible for this effect. Therefore, a library was synthesized, whose substructures are characteristic for a wide range of Maillard reaction products and have been related to the formation of reactive oxygen species. The substructure library contained DFLys, 3-DG, CML, DDMP, and C_4-AR. DFLys was chosen to represent Amadori products, which are the first relatively stable reaction products of sugars with amines. In model mixtures of proteins and sugars, as well as in processed protein rich food, the Amadori products are usually the adducts which can be detected in highest concentrations [28–30]. Furthermore, Amadori products are already present in lightly processed diet, so that their consumption seems to be inevitable [31]. Amadori products can be formed from various sugar and amine moieties, leading to various different compounds. However, all Amadori products have the α-amino-ketone structure in common, which can partly tautomerize to an enediol, but also to an eneaminol. Enediols and enaminols are principally able to form reactive oxygen species, such as hydrogen peroxide [32, 33]. On the other hand, it was shown that the Amadori product is mainly present in the cyclic β-pyranose form, in which tautomerization is impaired by the hemiacetal bond [34]. Thus, DFLys was chosen as a test compound not only because of the high abundance of Amadori products in Maillard model mixtures and processed food, but also because of its potential reactivity to generate reactive oxygen species.

Apart from the Amadori products, α-dicarbonyl intermediates, which comprise a great variety of different compounds such as 3-DG, are formed in relatively high concentrations in Maillard mixtures, processed food, and heated sugar solutions [35–38]. The α-dicarbonyl group is a reactive moiety, which can generate, for example, superoxide anions, which may further disproportionate to give hydrogen peroxide [39].

Amadori products and α-dicarbonyl intermediates are further degraded to yield advanced products, the so-called AGEs. Although AGEs are formed in much lower concentration than the Amadori products, the great variety of different structures may include highly reactive products of interesting functional properties. Among the AGEs, CML was chosen, because it is the best-studied AGE in food and biomedical science, and because it is relatively abundant, compared to other protein-bound AGEs [28, 30]. Furthermore, it has been shown that protein-bound CML

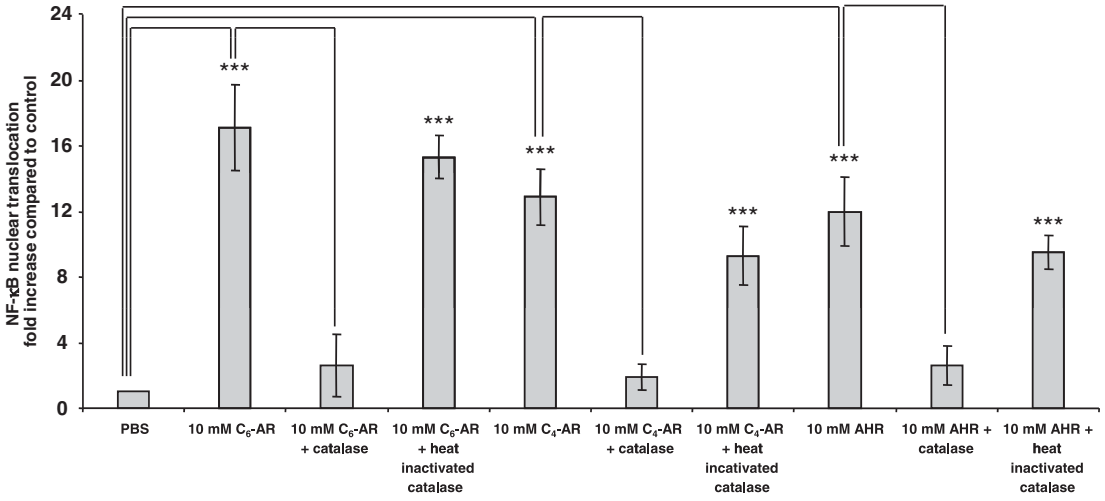


Figure 4. Nuclear NF-κB translocation in NR8383 macrophages compared with PBS control. Cells were stimulated for 2 h with PBS, 10 mM of C_6-AR, C_4-AR, or AHR, in the absence and presence of catalase or heat inactivated catalase (150 U/mL). The intensity of the NF-κB subunit p65 was related to β-actin; $^{***}p < 0.001$, significant differences are related to the PBS control unless otherwise indicated. Differences between the samples+catalase and the PBS control as well as between the samples and the samples+heat inactivated catalase were not significant (mean ± SD, $n = 3$).

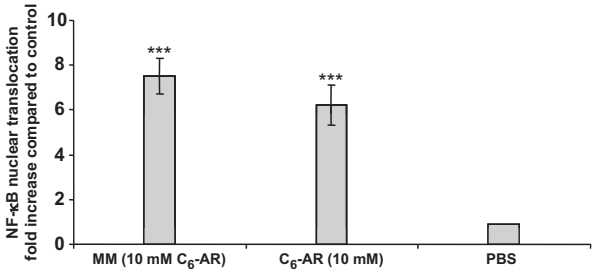


Figure 5. NF-κB nuclear translocation in NR8383 macrophages compared with PBS-treated control. Cells were stimulated for 2 h with PBS, an MM containing 10 mM C_6-AR or 10 mM pure C_6-AR. The intensity of the NF-κB subunit p65 was related to β-actin; $^{***}p < 0.001$; significant differences are related to the PBS control (mean ± SD, $n = 3$).

can evoke nuclear NF-κB translocation in several cell types [12, 40], a pathway which may be mediated by the generation of intracellular oxidative stress [41].

DDMP, which has been detected in several processed food items [42, 43], was chosen as a representative of Maillard products with reductone (ether) structure (for more Maillard products with reductone structure see Ledl and Schleicher [44]). It has been suggested that reductones and reductone ethers are at least partially responsible for reducing properties of processed food and Maillard model mixtures [44]. For ascorbic acid, probably the best characterized reductone in food, it is well established that it can generate hydrogen peroxide, particularly in the presence of copper [45]. Additionally, the potential of other reductones to generate hydrogen peroxide has been demonstrated [46].

ARs are aza analogs of reductones with even higher reducing properties [47]. Thus, C_4-AR showed a similar antioxidative and prooxidative effect in the presence of Cu^{2+} as ascorbic acid [25]. Therefore, C_4-AR was enclosed in the substructure library as an example of a Maillard product with AR structure.

The macrophages were stimulated for 2 h with the test substances, and nuclear NF-κB translocation was measured by Western blot. Among all test substances, only the C_4-AR induced a response which was significantly increased compared to the PBS-treated control. The effect was not specific for C_4-AR, but was evoked in a similar way by two other Maillard products with AR structure, C_6-AR and AHR. The AR substructure is a group that is found in several Maillard products [48–53]. Therefore, it can be assumed that the activity of complex Maillard mixtures or processed foods, such as coffee, is not necessarily related to a defined AR, but to a group of reaction products with AR substructures. It is also not unlikely that complex reaction products, for example melanoidines, contain AR substructures, which may be responsible for their functional properties.

AR-induced nuclear NF-κB translocation could be clearly inhibited by co-incubation with catalase. Since catalase specifically degrades hydrogen peroxide, it can be concluded that the cellular effect of the ARs was mediated by hydrogen peroxide. Similar results were obtained for coffee, which activated NF-κB in macrophages only in the absence of catalase [18]. The reducing properties of ARs, including C_4-AR, C_6-AR, and AHR are well established [25, 54, 55]. In line with these findings, it was recently reported that an AR can inhibit photo-degradation of riboflavin in milk [56]. Similar to other reducing compounds, also a prooxidative activity of ARs was found under certain reaction conditions

[25]. The findings of this study are most likely explained by direct reduction of oxygen by ARs yielding reactive oxygen species, such as hydrogen peroxide. The latter are then responsible for the observed prooxidative activity or cell stimulation. ARs may also be responsible for other cellular effects of Maillard mixtures, such as cytotoxicity, which are mediated by hydrogen peroxide [20]. Thus, further studies are required to investigate the cytotoxic effect of ARs and other defined Maillard reaction products. Under the tested conditions, it also cannot be fully ruled out that a strong cytotoxic effect of one of the test compounds may disguise its activity to induce NF-κB translocation. Finally, it was shown that in a Maillard mixture, which was produced under conditions favoring C_6-AR formation, the nuclear NF-κB translocation in macrophages was caused largely by its content of C_6-AR. This finding indicates that ARs may indeed be important contributors to hydrogen peroxide dependent cellular effects of MMs and foods that are rich in Maillard reaction products. However, it cannot be excluded that other products are formed under different conditions, which are also able to generate hydrogen peroxide and cause nuclear NF-κB translocation.

ARs are very labile compounds that decompose easily [57]. Therefore, the preparation of analog products derived from natural amino acids, which would require more isolation steps, has not been achieved so far. However, in reaction mixtures of glucose or lactose with the natural amino acids alanine or proline, abundant products were detected by HPLC with the characteristic UV absorbance maxima of ARs at 312 nm (C_4-AR and AHR) and 320 nm (C_6-AR). This finding indicates the formation of amino acid derived ARs (data not shown).

In conclusion, the use of a substructure library allowed the identification of Maillard products which are responsible for NF-κB activation by MMs. It was demonstrated that ARs, which are formed during the Maillard reaction, are able to strongly activate nuclear NF-κB translocation in macrophages *via* the generation of hydrogen peroxide. Further, it can be hypothesized that Maillard products or melanoidines with an AR substructure are at least partially responsible for the activity of processed food, such as coffee, in activating nuclear NF-κB translocation in macrophages.

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