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Antioxidant and Anti-Inflammatory Activities of Maillard Reaction Products Isolated from Sugar−Amino Acid Model Systems

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ABSTRACT: We investigated the antioxidant and anti-inflammatory activities of both crude and ultrafiltrated Maillard reaction (MR) products (MRPs) derived from sugar−amino acid MR models, comprising fructose (Fru), glucose (Glu) or ribose (Rib) reacted with glycine (Gly) or lysine (Lys), respectively. Crude MRPs derived from Glu-Lys showed the greatest capacity ($P < 0.05$) to inhibit nitric oxide (NO) and interleukin 8 (IL-8) production in interferon γ and phorbol ester-induced Caco-2 cells. Moreover, one ultrafiltrated fraction (MW < 1 kDa) recovered from Glu-Lys exhibited the greatest ($P < 0.05$) affinity to inhibit NO. This effect also corresponded to an inhibition of both iNOS transcription and translation. The NO and IL-8 inhibitory activities of crude MRPs were positively correlated with intracellular oxidation inhibitory activity. In conclusion, we have demonstrated an anti-inflammatory capacity of MRPs in inflamed Caco-2 cells that is specific to low molecular weight (MW < 1 kDa) Glu-Lys MRPs.

KEYWORDS: anti-inflammation, Caco-2, inducible nitric oxide synthase, interleukin 8, intestinal inflammation, Maillard reaction

INTRODUCTION

Maillard reaction products (MRPs) are generated during cooking or baking when reducing sugars react with amino acids, peptides or proteins. They are found in heat-processed foods such as coffee brews, bread crust and roasted meats. There is controversy concerning whether dietary MRPs represent potential harmful or beneficial effects.[1,2] Former studies have shown that MRPs contribute significantly to the accumulation of advanced glycation end products (AGEs) *in vivo*[3] and are recognized as endogenous inflammatory mediators that have key roles in the initiation and development of inflammatory diseases.[4] Some AGEs can induce pro-inflammatory cytokines/chemokines such as interleukin 6 (IL-6) and interleukin 8 (IL-8).[5] An increase in inducible nitric oxide synthase (iNOS) resulting in accumulation of nitric oxide (NO) in different cell lines has been reported for AGEs derived from bovine serum albumin.[6−9] Early stage MRPs, or Amadori products, also increase iNOS and NO production in human peritoneal mesothelial cells.[10] Intermediate dicarbonyl compounds, such as methylglyoxal (MGO) or glyoxal (GO), can induce IL-8 secretion in Caco-2 cells,[11] whereas GO and glycoaldehyde have been shown to attenuate the tumor necrosis factor α (TNF-α)-induced transcription factor, nuclear factor κB (NF-κB), in the HT29c34 reporter cell line.[12] Notwithstanding these findings, it is also noteworthy that the alicyclic volatile MRP, 3-methyl-1,2-cyclopentanedione (3-MCP), produced both in sugar−amino acid Maillard reaction (MR) models[13,14] and in roasted coffee,[15] can decrease pro-inflammatory gene expression, including cyclooxygenase (COX-2) and iNOS in the kidneys of older rats.[15] These findings collectively suggest that the complex mixture of MRPs can potentially produce both pro- and anti-inflammatory activities.

Inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis, affect 0.5−1% of the population in Western countries.[16] The intestinal epithelial barrier has an important role in the initiation and propagation of IBD,[17] functioning as a site for extracellular signal conversion with the induced secretion of cytokines such as IL-8, TNF-α and interleukin 1β (IL-1β).[18] Reactive oxygen species (ROS), including superoxide anion radicals and hydrogen peroxide, can promote the inflammatory process through injury of intestinal extracellular matrices. In response, intestinal epithelial cells produce various inflammatory mediators such as iNOS and IL-8. Both of these are transcriptionally regulated by NF-κB[19] and have important roles in the development of intestinal inflammation and in the pathogenesis of IBD.[20]

Nitric oxide is involved in vasodilation and host defense mechanisms,[21] as well as contributing to the etiology of mucosal inflammation, IBD, or intestinal cancer when produced in excess amounts.[22] The human colon adenocarcinoma cell line, Caco-2, can be used to study intestinal inflammation,[11] and we have shown in Caco-2 cells that increased levels of NO will follow with the upregulation of iNOS, when cells are treated with a mixture of interferon γ (IFN-γ) and phorbol ester (PMA).[23] IL-8 is a CXC chemotactic cytokine that is involved in the activation and recruitment of circulating neutrophils and mononuclear cells to areas of infection.[24] It is also secreted by epithelial cells in the inflamed IBD mucosa,[25,26] and levels are enhanced in cultured intestinal epithelial cells exposed to pro-inflammatory cytokines, such as IL-1β, TNF-α or IFN-γ[27−29] and PMA.[30] In addition, abnormally high levels of reactive oxygen species are also produced in IBD, implying again that oxidative stress is a causative factor in the initiation and/or propagation of IBD.[31,32] Dietary antioxidants or antioxidant enzymatic defense systems *in vivo* modulate the balance of redox status by removing ROS produced by oxygen metabolism, and thus can mitigate propagation of IBD.[33,34] Known dietary antioxidants, such as tea

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catechins[35] and bioactive phenolics present in oregano extracts,[36] have been shown to contribute to anti-inflammatory activities. MRPs also exhibit antioxidant activity; albeit, conflicting results exist in terms of which compounds based on molecular weight (MW) contribute to the antioxidant capacity of MRPs.[37−39] There is, however, little information available concerning the potential relationship between antioxidant and anti-inflammatory activities of MRPs in Caco-2 cells.

The present study was aimed at investigating the potential antioxidant and anti-inflammatory activities of MRPs generated from six sugar−amino acid model systems. Antioxidant activity was determined using a cell-based intracellular oxidation assay, and anti-inflammatory activity was determined using NO, iNOS and IL-8 as inflammatory indicators in IFN-γ + PMA-induced Caco-2 cells.

MATERIALS AND METHODS

Preparation of MRPs. Glucose (Glu), fructose (Fru) and ribose (Rib) (0.8 mol/L) were mixed 1:1 (molar ratio) with 0.8 mol/L glycine (Gly) or L-lysine (Lys) (Sigma, St. Louis, MO, USA) in 100 mL of distilled deionized water and adjusted to pH 7.0. The mixtures were put in screw capped glass bottles and autoclaved (Laboratory VMP, Clinton, NJ, USA) at 121 °C for 60 min, followed by rapid chill on ice. A supernatant recovered after centrifugation at 4068*g* was lyophilized.

Identification and Quantification of α-Dicarbonyl Compounds. α-Dicarbonyl compounds produced in these six MR mixtures were identified and quantified after derivatization to benzoquinoxaline with 2,3-diaminonaphthalene (DAN) (Sigma, St. Louis, MO, USA) as previously described.[39] Lyophilized MR mixtures (1 mL, 10 mg/mL) or varying concentrations of standards (GO and MGO, Sigma, St. Louis, MO, USA; glucosone and 3-deoxyglucosone (3-DG), Toronto Research Chemicals Inc., Toronto, Canada) dissolved in 10 mmol/L phosphate buffer (pH 7.4) were incubated with DAN (50 μL; 2 mg/mL) in the presence of 25 μL of 0.001% 3,4-hexanedione (HD) (Sigma, St. Louis, MO, USA) overnight at 4 °C. The benzoquinoxaline derivatives were identified using a LC/ESI-MS/MS (Agilent 1100 LC/MSD-Trap-XCT system, Agilent, Santa Clara, CA, USA) equipped with a Sphereclone ODS2 column (4.6 × 150 mm, 3.5 μm, 80 Å; Phenomenex, Torrance, CA, USA). Samples were eluted using a stepwise gradient of acetonitrile (ACN) and 0.2% formic acid as follows: 0−13 min, 28−45% ACN; 13−25 min, 45−85% ACN and 25−28 min, 85% ACN at a flow rate of 0.8 mL/min. The MS operated in an electron spray ionization (ESI) positive ion mode with a scan mass-to-charge ratio (m/z) that ranged from 40 to 500, and nitrogen was the nebulizing gas. The MS parameters were as follows: ionization temperature, 350 °C; nebulizer gas flow, 12 L/min; HV capillary, 3500 V. α-Dicarbonyl benzoquinoxaline derivatives generated in MR model systems were quantified using HPLC with a fluorescent detector (excitation at 267 nm and emission at 503 nm) by comparing with the known standard benzoquinoxaline derivatives. Since standards for pentosone and 3-deoxypentosone (3-DP) were unavailable, these compounds were quantified based on standard curves generated from glucosone and 3-DG, respectively. The amount of α-dicarbonyl compounds was expressed as μg/g dry matter (dm).

Separation of MRPs. Mixtures of MRPs were passed through an ultrafiltration module (Millipore, Billerica, MA, USA) under a pressure of 40 psi and then separated based on MW of >10, 3 to <10, 1 to <3, and <1 kDa into fractions I, II, III, and IV (F-I to F-IV), respectively, using nitrocellulose membranes (Millipore, Billerica, MA, USA). Fractions were lyophilized and stored at 4 °C until further analysis.

Cell Culture. Caco-2 cells (HTB-37, ATCC) were cultured in minimum essential medium (MEM) containing Earle's salts (Sigma, St. Louis, MO, USA), as well as 10% fetal bovine serum, 100 units/mL of penicillin and 100 μg/mL of streptomycin (Invitrogen, Burlington, ON, Canada). Cells were incubated at 37 °C under a 5% CO_2 atmosphere. The medium was changed every 2−3 days, and the cells were subcultured weekly.

Cytotoxicity of MRPs. The cytotoxicity of crude and fractionated MRPs on Caco-2 cells was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, MO, USA) assays as described by Chen and Kitts.[23] Cells were incubated with various concentrations of MRPs for 24 h. The medium was removed, and the cells were then incubated with 0.5 mg/mL MTT in MEM for 4 h, followed by an overnight incubation with 10% sodium dodecyl sulfate (SDS). Absorbance was read at 570 nm using a spectrophotometer (Multiskan Ascent, Thermo Labsystems, Helsinki, Finland), and the IC_{50} of the MRPs was calculated. The viability of Caco-2 cells after intracellular oxidation and NO test was also assessed. Only cells with viability greater than 95% were accepted.

Intracellular Oxidation. The intracellular oxidation assay was investigated according to the method of Kitts and Hu[40] with minor modification. Caco-2 cells were seeded in 96-well plates (Sarstedt, Nümbrecht, Germany) at a density of 10^5 cells/cm^2 in MEM (100 μL). Three weeks later, the cells were incubated with or without MRPs for 24 h. The medium was removed, and then 10 μmol/L 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma, St. Louis, MO, USA) in 100 μL of Hanks buffered salt solution was added for 30 min, followed by 1 mmol/L 2,2′-azobis (2-amidinopropane) HCl (AAPH) for 1 h. Fluorescence was measured using a fluorescence microplate reader (Fluoroskan Ascent FL, Thermo Labsystems, Helsinki, Finland) at excitation = 485 nm and emission = 527 nm. The % inhibition was calculated as

$$(FI_{st} - FI_{sample})/(FI_{st} - FI_{blank}) \times 100\%$$

where FI_{st} is fluorescence intensity of Caco-2 cells incubated with 1 mmol/L AAPH, FI_{blank} is that of Caco-2 cells without MRPs and AAPH, and FI_{sample} is that of Caco-2 cells incubated with MRPs for 24 h followed by AAPH for 1 h.

NO Production in Caco-2 Cells. We measured the NO production in IFN-γ + PMA induced Caco-2 cells as described by Chen and Kitts.[23] Caco-2 cells were seeded in 96-well plates at a density of 10^5 cells/cm^2 in MEM (100 μL). After three weeks, cells were incubated with or without MRPs for 24 h. MRPs or medium was removed, and cells were then stimulated with 8,000 U/mL IFN-γ + 0.1 μg/mL PMA for 24 h. NO levels were determined in the culture medium using the Griess reaction after reducing nitrate to nitrite with nitrate reductase (Sigma, St. Louis, MO, USA). The % inhibition was calculated using the following equation:

$$(NO_{st} - NO_{sample})/(NO_{st} - NO_{blank}) \times 100\%$$

where NO_{st} is the NO concentration of the cell supernatant incubated with IFN-γ + PMA for 24 h; NO_{blank} is the NO concentration of the cell supernatant without sample and inducers; NO_{sample} is the NO concentration of cells incubated with MRPs for 24 h and then stimulated with IFN-γ +PMA for 24 h.

IL-8 Production in Caco-2 Cells. Caco-2 cells were seeded in 6-well plates at a density of 10^5 cells/cm^2 in MEM (2 mL). Three weeks later, the cells were incubated with or without MRPs for 24 h. MRPs or medium was removed, and cells were then stimulated with 8,000 U/mL IFN-γ + 0.1 μg/mL PMA for 2 to 24 h. Levels of IL-8 in the culture medium were determined using ELISA assay kits from SABioscience (Frederick, MD, USA) according to the manufacturer's instructions. These cells were used for iNOS expression analysis as described below. The % inhibition was calculated using the following equation:

$$(IL\text{-}8_{st} - IL\text{-}8_{sample})/(IL\text{-}8_{st} - IL\text{-}8_{blank}) \times 100\%$$

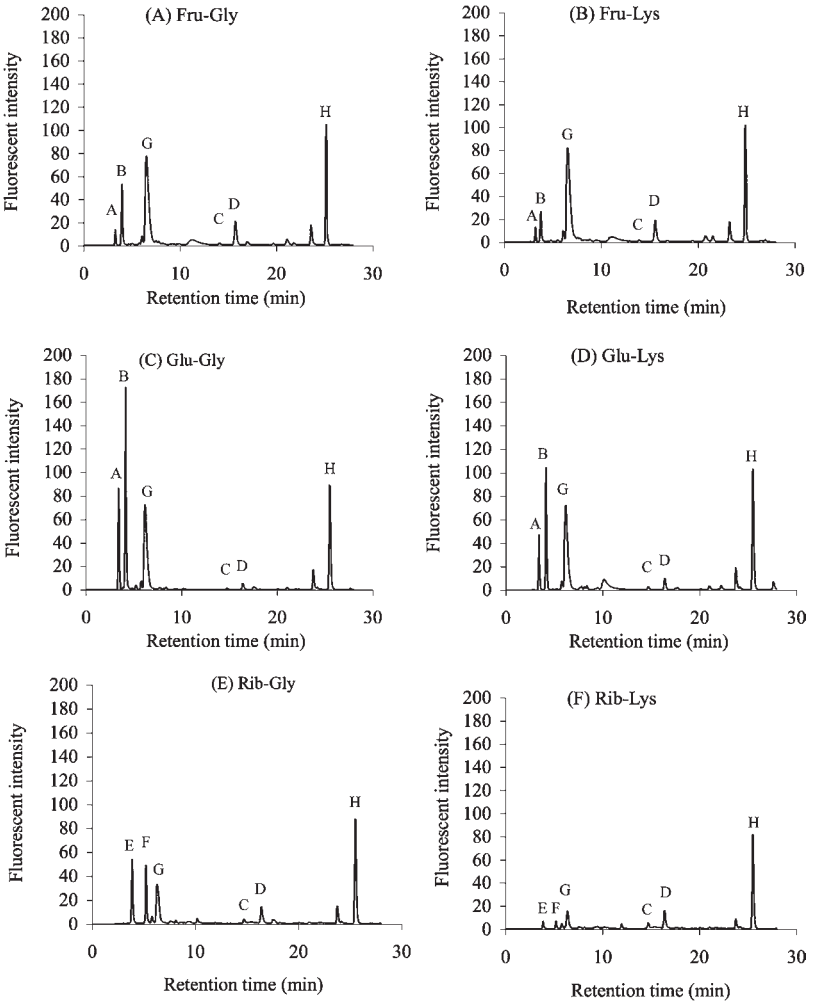

Figure 1. HPLC (fluorescence, excitation at 267 nm, emission at 503 nm) chromatographs of α-dicarbonyl benzoquinoxaline derivatives from sugar−amino acid model systems. (A) Fru-Gly; (B) Fru-Lys; (C) Glc-Gly; (D) Glc-Lys; (E) Rib-Gly; (F) Rib-Lys. Peak A, glucosone; peak B, 3-DG; peak C, GO; peak D, MGO; peak E, pentosone; peak F, 3-DP; peak G, DAN; peak H, HD.

where IL-8_{st} is the IL-8 concentration of the cell supernatant incubated with IFN-γ + PMA for 24 h; IL-8_{blank} is the IL-8 concentration of the cell supernatant without sample and inducers; IL-8_{sample} is the IL-8 concentration of cell supernatant incubated with MRPs for 24 h and then stimulated with IFN-γ + PMA for 24 h.

RT-PCR. Levels of iNOS mRNA expression in Caco-2 cells were measured using RT-PCR as described by Chen and Kitts.[23] Total RNA in Caco-2 cells seeded in 6-well plates was extracted using GStract RNA isolation kits (Maxim Biotech Inc., Rockville, MD, USA). The quality and quantity of RNA were determined using a NanoDrop spectrophotometer (NanoDrop Technology, Wilmington, DE, USA). Reverse transcription and PCR amplification proceeded according to the instructions provided with the products (RTeasy reverse transcription kit and human iNOS primer set kit, Maxim Biotech Inc., Rockville, MD, USA). The housekeeping gene was β-actin (Maxim Biotech Inc., Rockville, MD, USA). The primer sequences for iNOS were 5′ forward, CTT CAA CCC CAA GGT TGT CTG CAT, and 3′ reverse, ATG TCA TGA GCA AAG GCG CAG AAC, leading to a 231 bp fragment. The primer sequences of β-actin were 5′ forward, ACG GCC GAG CGG GAA ATC GT, and 3′ reverse, CTG CTT GCT GAT CCA CAT CT, leading to a 474 bp fragment.

Western Blotting. Levels of iNOS protein expression in Caco-2 cells were measured using Western blotting as described by Chen and Kitts (*23*). Total protein of Caco-2 cells seeded in 6-well plates was extracted using a lysis buffer, and the protein concentration in the lysate supernatant was measured using the Bradford reagent (Sigma, St. Louis, MO, USA) and adjusted to the same concentration. The denatured protein was resolved by electrophoresis (Mini-PROTEIN II, Bio-Rad) on 8% SDS−polyacrylamide minigels and subsequently transferred to nitrocellulose membranes (0.2 μm, 7 × 8.4 cm, Bio-Rad Laboratories, Hercules, CA, USA). Nonspecific binding was blocked with 5% nonfat milk, and then the membranes were incubated with anti-human iNOS polyclonal primary antibody (Zymed, Invitrogen, Carlsbad, CA, USA) or rabbit anti-actin primary antibody (Sigma, St. Louis, MO, USA), followed by the secondary antibody, horse radish peroxidase-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA). Proteins (iNOS, 130 kDa; β-actin, 43 kDa) were visualized using enhanced chemiluminescence by exposing the blots to the film (Amersham Biosciences UK Ltd., Bucks., U.K).

Statistics. Data were analyzed by a one-way or two-way ANOVA using MINITAB software (Version 14, Minitab Inc., State College, PA, USA). Significant differences were compared using Tukey's test with $P < 0.05$ representing a statistically significant difference.

RESULTS

α-Dicarbonyl Compounds in MR Mixtures. We identified six α-dicarbonyl benzoquinoxaline derivatives in hexose and pentose

Table 1. α-Dicarbonyl Compounds Derived in Different MR Mixtures[a]

	MR mixtures					
dicarbonyl compds (μg/g dm)	Fru-Gly	Fru-Lys	Glu-Gly	Glu-Lys	Rib-Gly	Rib-Lys
GO	11.0 ± 1.1 A[b]	20.4 ± 2.1 B	27.4 ± 2.8 C	43.1 ± 1.3 D	9.6 ± 0.7 A	4.8 ± 0.7 A
MGO	90.1 ± 0.4 BC	97.0 ± 13.1 C	83.1 ± 5.6 B	80.8 ± 0.9 B	78.3 ± 5.3 B	40.6 ± 0.4 A
glucosone	185.1 ± 9.3 A	157.0 ± 23.5 A	1005.5 ± 17.3 C	546.7 ± 4.4 B	–	–
3-DG	718.8 ± 39.9 B	352.1 ± 21.0 A	2200.3 ± 47.4 D	1335.9 ± 43.5 C	–	–
pentosone	–	–	–	–	1368.5 ± 114.7 B	307.7 ± 13.1 A
3-DP	–	–	–	–	800.06 ± 47.7 B	128.49 ± 14.0 A

[a] The experiments were performed in triplicate. Data were expressed as mean ± SD. "–" indicates not determined. [b] A–D: Means in same row with different letters are significantly different ($P < 0.05$).

Table 2. Cytotoxicity of Crude and Ultrafiltrated MRPs Derived from Different MR Mixtures[a]

	IC_{50} (mg/mL)					
MRPs	Fru-Gly	Fru-Lys	Glu-Gly	Glu-Lys	Rib-Gly	Rib-Lys
crude[b]	49.81 ± 0.90 E[c]	40.43 ± 0.78 D	38.96 ± 0.17 D	22.81 ± 0.09 C	15.27 ± 0.18 B	11.63 ± 0.12 A
fractions[d]						
F-I	7.71 ± 0.28 CX	9.05 ± 0.20 CY	6.33 ± 0.60 BCX	7.27 ± 0.11 CX	5.49 ± 0.10 AX	5.62 ± 0.25 ABX
F-II	8.17 ± 0.15 CX	7.22 ± 0.23 BCX	6.35 ± 0.43 ABX	7.03 ± 0.39 BCX	4.78 ± 0.02 AX	4.85 ± 0.09 AX
F-III	7.30 ± 0.23 BX	7.38 ± 0.06 BXY	8.57 ± 0.10 BY	7.63 ± 0.37 BX	5.14 ± 0.03 AX	4.93 ± 0.47 AX
F-IV	51.60 ± 2.07 EY	49.80 ± 0.26 EZ	45.06 ± 0.91 DZ	30.96 ± 0.48 CY	16.04 ± 0.48 BY	14.25 ± 0.79 AY

[a] Three independent experiments were performed in triplicate. Data were expressed as mean ± SEM. [b] IC_{50} of crude MRPs heated for 60 min. [c] A–E: Means in same row with different letters are significantly different ($P < 0.05$). X–Z: Means of ultrafiltrated MRP fractions in same column with different letters are significantly different ($P < 0.05$). [d] IC_{50} of ultrafiltrated MRPs: F-I, >10 kDa; F-II, 3 to <10 kDa; F-III, 1 to <3 kDa; F-IV, <1 kDa.

Table 3. Yield of Ultrafiltrated MRPs Derived from Different MR Mixtures[a]

	yield[b](%)					
fraction[c]	Fru-Gly	Fru-Lys	Glu-Gly	Glu-Lys	Rib-Gly	Rib-Lys
F-I	0.19 ± 0.05 AX[d]	0.30 ± 0.07 AX	0.78 ± 0.01 AX	3.34 ± 0.84 BX	9.44 ± 0.09 CY	15.29 ± 0.11 DY
F-II	0.78 ± 0.01 AX	0.94 ± 0.41 AX	1.72 ± 0.09 ABX	3.36 ± 0.84 BX	1.54 ± 0.53 ABX	5.88 ± 1.36 CX
F-III	0.31 ± 0.01 AX	1.05 ± 0.05 AX	1.01 ± 0.40 AX	1.24 ± 0.56 AX	1.14 ± 0.10 AX	1.83 ± 0.03 AW
F-IV	98.72 ± 0.04 DY	97.70 ± 0.44 DY	96.50 ± 0.31 DY	92.06 ± 0.57 CY	87.88 ± 0.55 BZ	77.01 ± 1.44 AZ

[a] The experiments were performed in triplicate. Data were expressed as mean ± SD. [b] Yield of each fraction = weight of each fraction/sum of weight of all four fractions × 100%. [c] Ultrafiltration fractions: F-I, >10 kDa; F-II, 3 to <10 kDa; F-III, 1 to <3 kDa; F-IV, <1 kDa. [d] A–D: Means in same row with different letters are significantly different ($P < 0.05$). W–Z: Means in same column with different letters are significantly different ($P < 0.05$).

MR models (Figure 1). GO (peak C) and MGO (peak D) were detected in all six model systems. Glucosone (peak A) and 3-DG (peak B) were identified only in hexose MR systems, whereas pentosone (peak E) and 3-deoxypentosone (peak F) were predominant α-dicarbonyl compounds in both ribose MR systems (Table 1). Peaks G and H were DAN and HD benzoquinoxaline derivatives, respectively. The yields of total α-dicarbonyl compounds were relatively higher in the glucose MR mixtures (0.32%, 0.21%, respectively for Glu-Gly and Glu-Lys) compared to Fru-Gly, Fru-Lys (0.1% and 0.06%) and Rib-Gly and Rib-Lys (0.23% and 0.05%).

MRPs Yield and Cytotoxicity. We first determined the appropriate concentrations for use in cell-based experiments by testing the cytotoxicity of crude and fractionated MRPs (Table 2). The order of cytotoxicity among crude MRPs was Fru-Gly < Fru-Lys ≈ Glu-Gly < Glu-Lys < Rib-Gly < Rib-Lys. The IC_{50} of Fru-Gly was 4.3-fold that of Rib-Lys, indicating that MRPs were relatively more toxic when produced in Rib-Lys than in Fru-Gly models. Among fractions with the same MW, MRPs from ribose model systems had lower ($P < 0.05$) IC_{50} values than those from fructose and glucose models, respectively. Of interest was the finding that the IC_{50} value of low MW F-IV was significantly ($P < 0.05$) higher than those of the other three fractions in all six models. The finding that MRPs with a MW > 1 kDa were more toxic than those with MW < 1 kDa was related to the unreacted sugar or amino acid in F-IV. F-IV accounted for 98.72% (Table 3) of crude Fru-Gly, whereas significantly lesser ($P < 0.05$) amounts of F-IV were recovered in crude Rib-Lys MRPs. This finding indicated that low MW MRPs produced in relatively fast reaction systems, such as Rib-Lys, are also likely to rapidly polymerize into higher MW compounds.

Antioxidant Activity of MRPs. Crude MRPs from all six model systems were effective at lowering the intracellular oxidative stress induced in Caco-2 cells exposed to the peroxyl radical generator, AAPH (Table 4). The activity of MRPs to lower

Table 4. Intracellular Oxidation Inhibitory Effects of Crude and Fractionated MRPs Derived from Different MR Mixtures[a]

	% inhibition					
MRPs	Fru-Gly	Fru-Lys	Glu-Gly	Glu-Lys	Rib-Gly	Rib-Lys
crude[b]	17.95 ± 2.15 A[c]	28.75 ± 5.19 AB	22.40 ± 2.16 A	37.98 ± 3.39 B	21.57 ± 1.79 A	31.03 ± 1.73 AB
fractions[d]						
F-I	14.25 ± 1.40 AX	21.81 ± 4.50 AXY	17.93 ± 1.41 AX	29.29 ± 2.37 AX	23.39 ± 2.86 AX	21.07 ± 4.06 AX
F-II	23.19 ± 2.26 AXY	27.03 ± 1.17 ABXY	28.67 ± 3.52 ABXY	40.69 ± 1.75 CY	26.57 ± 1.83 ABX	36.09 ± 2.33BCY
F-III	28.45 ± 1.69 AY	36.21 ± 3.95 AY	32.80 ± 2.63 AY	41.49 ± 2.71 AY	30.49 ± 2.80 AX	34.64 ± 2.52 AY
F-IV	19.15 ± 3.83 AXY	20.29 ± 3.32 AX	27.74 ± 3.74 AXY	29.92 ± 2.19 AX	20.61 ± 3.04 AX	20.83 ± 0.49 AX

[a] Three independent experiments were performed in triplicate. Data were expressed as mean ± SEM. [b] Inhibition (%) of crude MRPs heated for 60 min at a concentration of 1.25 mg/mL. [c] ABC: Means in same row with different letters are significantly different ($P < 0.05$). XY: Means of ultrafiltrated MRP fractions in same column with different letters are significantly different ($P < 0.05$). [d] Inhibition (%) of ultrafiltrated MRPs (1.25 mg/mL): F-I, MW > 10 kDa; F-II, MW 3 to <10 kDa; F-III, MW 1 to <3 kDa; F-IV, MW < 1 kDa.

intracellular oxidation was greater with lysine−MR mixtures compared to corresponding glycine−MR mixtures. Fractions II and III recovered from Glu-Lys MR model exhibited the highest intracellular oxidation inhibitory activity ($P < 0.05$).

Effects of Inducers on iNOS and IL-8 Expression. We previously[23] showed that NO as well as iNOS mRNA and protein expression are increased in Caco-2 cells after stimulation with IFN-γ and PMA. Here, we report the time-dependent effects of inducers on iNOS expression. We found that iNOS mRNA was induced at 6 h and persisted during stimulation for 24 h (Figure 2A). The expression of iNOS protein was also induced after incubation with IFN-γ and PMA for 6 h and thereafter increased in a time-dependent manner up to 24 h (Figure 2B).

To understand which cytokines responded to our IFN-γ + PMA-induced model of intestinal inflammation, we tested the effects of inducers on the production of 12 cytokines related to inflammation. Only IL-8 increased after IFN-γ + PMA stimulation, whereas IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17A, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) were undetectable. Unstimulated, differentiated Caco-2 cells did not produce detectable IL-8. The production of IL-8 in Caco-2 cells stimulated with IFN-γ + PMA increased in a time-dependent manner, reaching maximum concentration (1177.3 pg/mL) at 24 h (Figure 2C). We therefore stimulated cells for 24 h in the following experiments for iNOS and IL-8 inhibitory assays.

Anti-Inflammatory Effects of MRPs. Crude MRPs alone did not induce or inhibit NO compared to the cells without treatment (data not show). Crude MRPs derived from all models, except for Fru-Gly, inhibited NO production in a dose-dependent manner in IFN-γ + PMA-stimulated Caco-2 cells (Table 5). When Caco-2 cells were incubated with Glu-Lys MRPs at 1.25 mg/mL, the NO production was decreased by 52.4%, which was significantly ($P < 0.05$) more effective than the other five model systems. Only crude Glu-Lys MRPs showed transcriptional and translational inhibition of iNOS expression in inflamed Caco-2 cells (Figures 3A and 3B). The MRPs derived from Fru-Lys and Glu-Lys models significantly ($P < 0.05$) decreased IL-8 production in IFN-γ + PMA-stimulated Caco-2 cells, and a prior incubation of Caco-2 cells with Glu-Lys MRPs at 1.25 mg/mL for 24 h resulted in 35.0% inhibition of IL-8 (Figure 3C).

The affinity of MRPs to inhibit NO production was dependent on the type of reactants used in the MR model systems and was distinct to the molecular weight fractions tested (Table 6).

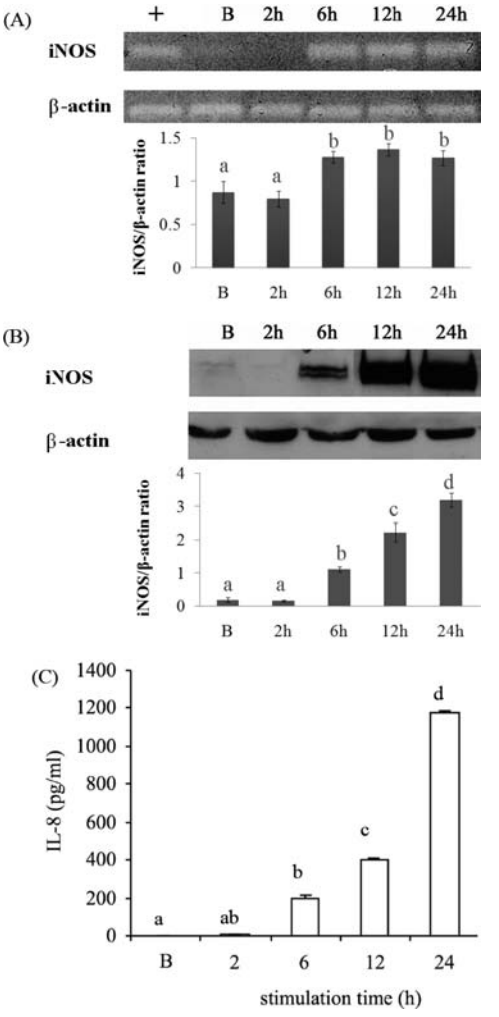

Figure 2. Expression of iNOS mRNA (A) and protein (B), and IL-8 secretion (C) in Caco-2 cells after 2−24 h of stimulation with IFN-γ + PMA. B, unstimulated cells; +, positive control. Expression of iNOS mRNA and protein is representative of two independent experiments performed in duplicate. iNOS/β-actin ratio and IL-8 data are expressed as means ± SEM from two independent experiments performed in duplicate. a−e: Bars with different letters are significantly different ($P < 0.05$).

Fractions with a MW < 3 kDa inhibited IFN-γ + PMA-induced NO production, whereas fractions with a MW > 3 kDa could

Table 5. NO inhibitory effects of crude MRPs derived from different MRP mixtures[a]

	NO inhibition (%)					
concn (mg/mL)	Fru-Gly	Fru-Lys	Glu-Gly	Glu-Lys	Rib-Gly	Rib-Lys
0.31	15.54 ± 1.90 ABX[b]	15.13 ± 2.13 ABX	14.60 ± 2.3 AX	25.66 ± 2.43 BX	18.55 ± 1.24 ABX	19.38 ± 3.30 ABX
0.63	22.19 ± 1.65 ABX	21.12 ± 1.28 AX	24.31 ± 2.61 ABXY	34.53 ± 4.18 BXY	19.65 ± 3.50 ABXY	25.38 ± 1.65 ABXY
1.25	22.21 ± 2.67 AX	29.96 ± 0.61 AY	26.11 ± 3.12 AXY	52.40 ± 5.19 BYZ	22.37 ± 4.42 AXY	33.92 ± 2.97 AY
2.50	24.48 ± 1.81 AX	48.47 ± 0.97 BZ	31.42 ± 2.47 AY	64.78 ± 4.10 CZ	34.93 ± 5.24 ABY	52.59 ± 3.13 BCZ

[a] Three independent experiments were performed in triplicate. Incubation time = 24 h. Data were expressed as mean ± SEM. [b] A−C: Means in same row with different letters are significantly different ($P < 0.05$). X−Z: Means in same column with different letters are significantly different ($P < 0.05$).

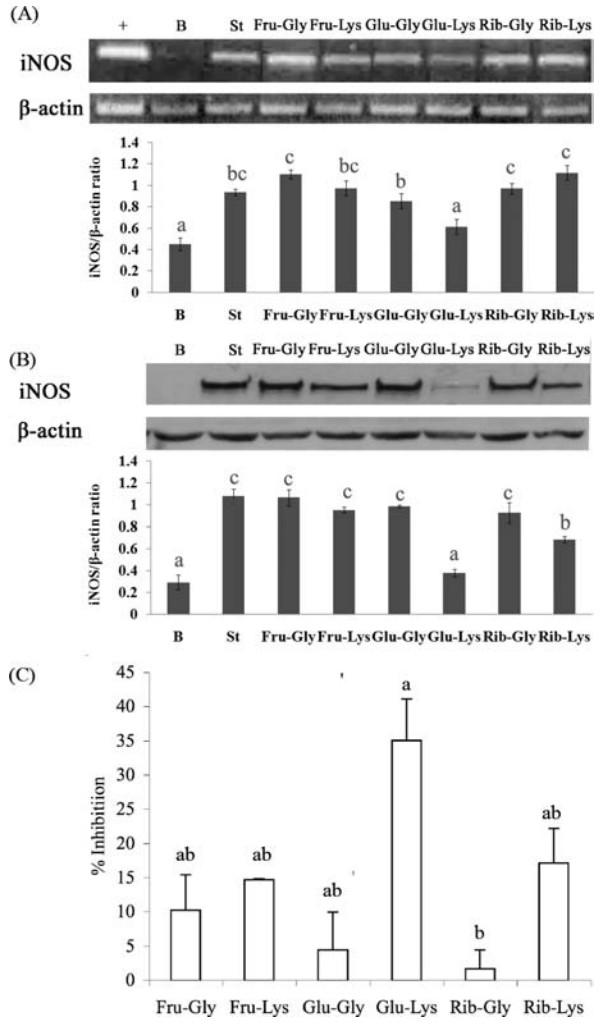

Figure 3. Expression of iNOS mRNA (A) and protein (B), and IL-8 secretion (C) in Caco-2 cells after incubation with crude MRPs (1.25 mg/mL) for 24 h followed by stimulation with IFN-γ + PMA for 24 h. B, unstimulated cells; +, positive control; St, cells stimulated with IFN-γ + PMA for 24 h without prior incubation with MRPs. Results of iNOS mRNA and protein expression are representative of two independent experiments performed in duplicate. iNOS/β-actin ratio and IL-8 inhibition data are expressed as means ± SEM from two independent experiments performed in duplicate. a, b: Bars with different letters are significantly different ($P < 0.05$).

increase NO production in induced Caco-2 cells with the exception of Glu-Lys. F-IV from lysine MR model systems produced significantly ($P < 0.05$) greater inhibition toward NO than that from other glycine MR models containing the same type of sugar (Table 6). F-IV recovered from Glu-Lys had the highest NO inhibitory effect among all MR models and related fractions tested. F-IV MR mixtures recovered from heated Glu-Lys also inhibited iNOS expression at both the transcriptional and translational levels (Figures 4A and 4B). In addition, F-IV from the heated Glu-Lys model system at a concentration of 1.25 mg/mL also inhibited 18.4% of the IL-8 generated in IFN-γ + PMA-induced Caco-2 cells (Figure 4C).

DISCUSSION

Caco-2 cells express an array of pro-inflammatory cytokines/chemokines when signaling mucosal inflammation.[25,41] The generation of NO, another important modulator of the mucosal inflammatory response, is elevated through iNOS upregulation.[22] IL-8 upregulates iNOS mRNA in cultured human keratinocytes,[42] and NO induces IL-8 production in LPS-stimulated human whole blood,[43] in LPS-stimulated human THP-1 cells and monocytes,[44] and in melanoma cells.[45] When iNOS expression is upregulated and NO is overproduced, gastric mucosal inflammation will follow and lead to cell damage and even carcinogenesis.[46] The upregulated NO can further activate NF-κB, a transcription factor which has an important role in inducing the production of more inflammatory cytokines, including IL-8, IL-6, TNF-α, and IL-1β. Therefore, IL-8, iNOS, and NO are most likely involved in a "pro-inflammatory network" in the human intestinal mucosa. In our study, we found that, among 12 cytokines tested, only IL-8 was elevated in IFN-γ and PMA induced Caco-2 cells, thus showing that IL-8 has a key role in intestinal inflammation.

A typical Western diet of heat-processed foods such as bread, roast meat, cake, baked potatoes and coffee contains high levels of MRPs that are thought to influence inflammatory processes in the intestine.[47] Although MRPs or AGEs are generally considered as pro-inflammatory compounds,[4−11] other studies have shown that MRP such as 3-MCP have an anti-inflammatory capacity in the aged rat kidney.[15] Due to the complexity of MRP mixture it is possible that some MRPs provide pro-inflammatory activity, whereas others exhibit anti-inflammatory or no activity. In the present study, we investigated the potential of MRPs generated from simple sugar−amino acid model systems having anti-inflammatory capacity in IFN-γ + PMA-induced differentiated Caco-2 cells. Since the production of MRPs is influenced by the types of reactants, we compared the influence of different sugar and amino acid reactants on the anti-inflammatory capacity of products derived from six different model systems and evaluated the individual anti-inflammatory ability of these products in terms of differences in MW. The MRPs derived from Glu-Lys

Table 6. Inhibitory Effects of Ultrafiltrated MRPs Derived from Different MR Mixtues on NO Production[a]

	NO inhibition (%)					
fraction[b]	Fru-Gly	Fru-Lys	Glu-Gly	Glu-Lys	Rib-Gly	Rib-Lys
F-I	19.43 ± 2.59 CY[c]	−3.98 ± 2.56 BX	−22.36 ± 3.03 AX	38.22 ± 2.46 DY	−19.71 ± 2.56 AX	13.79 ± 1.67 CY
F-II	−9.88 ± 2.94 BX	12.07 ± 1.96 CY	−23.67 ± 2.79 ABX	9.96 ± 3.04 CX	−11.67 ± 4.10 ABX	−11.98 ± 1.78 AX
F-III	45.93 ± 2.10 BZ	23.15 ± 0.64 AY	28.30 ± 3.68 AY	46.56 ± 3.98 BY	22.54 ± 1.82 AY	21.71 ± 0.16 AY
F-IV	28.90 ± 2.47 ACY	46.69 ± 2.20 BDZ	17.88 ± 1.68 AY	56.72 ± 3.97 DY	25.58 ± 0.68 ACY	35.85 ± 3.59 BCZ

[a] MRP concentration = 1.25 mg/mL. Three independent experiments were performed in triplicate. Data were expressed as mean ± SEM. Caco-2 cells were incubated with 1.25 mg/mL MRPs for 24 h followed by stimulation with IFN-γ + PMA for 24 h. Negative values equal pro-inflammatory activity; positive values denote anti-inflammatory activity. [b] NO inhibition (%) of ultrafiltrated MRPs: F-I, >10 kDa; F-II, 3 to <10 kDa; F-III, 1 to <3 kDa; F-IV, <1 kDa. [c] A−D: Means in same row with different letters are significantly different ($P < 0.05$). X−Z: Means in same column with different letters are significantly different ($P < 0.05$).

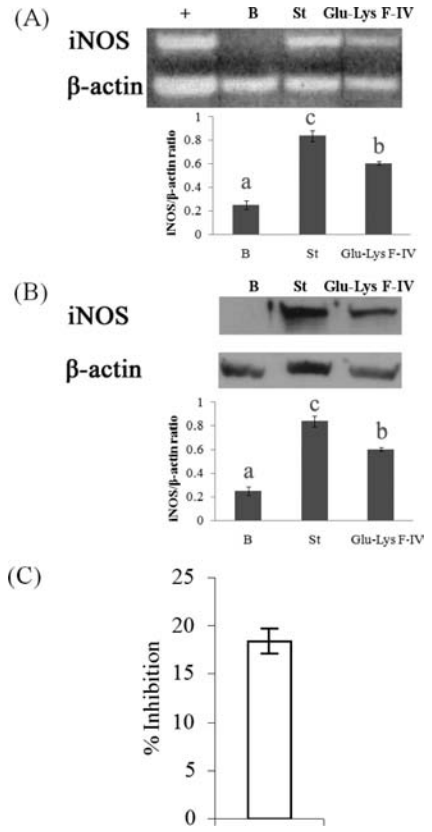

Figure 4. Expression of iNOS mRNA (A) and protein (B), and IL-8 secretion (C) in Caco-2 cells after incubation with 1.25 mg/mL of Glu-Lys F-IV for 24 h and subsequent stimulation with IFN-γ + PMA for 24 h. B, unstimulated cells; +, positive control; St, cells stimulated with IFN-γ + PMA for 24 h without prior incubation with MRPs. Results of iNOS mRNA and protein expression are representative of two independent experiments performed in duplicate. iNOS/β-actin ratio and IL-8 inhibition data are shown as means ± SEM from two independent experiments performed in duplicate.

model systems had the greatest NO, iNOS and IL-8 inhibitory activity, without showing cytotoxicity within the tested concentration range. In addition, the ability to inhibit NO and IL-8 was greater among MRPs derived from lysine than from glycine model systems, indicating that the anti-inflammatory capacities of MRPs are reactant-dependent and that the type of amino acid is relevant to the observed effect. Among the four different MW fractions, F-IV contributed to a major proportion of the anti-inflammatory activity of Glu-Lys. This effect could be attributed to a relative greater permeability in Caco-2 cells by the lower MW MRPs.

Former studies have shown that the glucose-derived intermediate product 1-alkyl-2-formyl-3,4-diglycosyl-pyrrole quenched NO, whereas 4-furanyl-2-furoyl-1*H*-imidazole-1-hexanoic acid, an end product of glycation, did not.[48] In addition, the NO quenching MRPs were not early glycosyl adducts, such as a Schiff base, or the Amadori product, but products that were formed after the Amadori product but relatively early in the MR.[48] Verbeke et al.[49] showed that early (Amadori products) and late (AGEs)-stage glycated BSA decreased iNOS activity and NO production in proximal tubular epithelial cells. Although high concentrations of dicarbonyl compounds are normally toxic to many cells, low concentrations of dicarbonyls can decrease inflammation in HT29c34 cells.[12] The inhibitory effects of GO and glycoaldehyde on inflammatory signals induced by TNF-α are possible because dicarbonyl interferes with NF-κB ubiquitination by modification at lysine and arginine residues, thus resulting in the inhibition of its activation.[12] α-Dicarbonyl compounds can also react with arginine to produce AGEs,[50] thus resulting in the reduced availability of arginine for NO biosynthesis. Others have shown that 3-MCP inhibits the generation of ROS resulting in suppression of the NF-κB cascade,[15] which could be another viable explanation for the anti-inflammatory mechanism. In the present study, the yield of α-dicarbonyls was relatively low in different MR mixtures and no correlation was found between the concentration of α-dicarbonyl compounds and the relative extent of intracellular oxidative stress and level of NO, and IL-8 inhibitory activities (Table 7). We conclude thereafter that α-dicarbonyl compounds did not contribute to the antioxidant and anti-inflammatory activities of the MR mixtures that were exposed to Caco-2 cells in our study.

Numerous chemical-based analytical methods have shown that MRPs isolated from simple sugar−amino acid models have antioxidant activity.[37,39,40,51,52] Cell-based assays have also found that nondialyzed MRPs (MW > 3.5 kDa) from Fru−, Glu− and Rib−sugars that react with Lys can inhibit H_2O_2 and AAPH induced intracellular oxidation in RAW264.7 cells.[40] While antioxidant activity has been found more so among high molecular weight MRPs, compared to low MW fractions,[37−39] other workers have indicated that the main antiradical properties of heat-processed foods are due to the presence of intermediate or low MW MRPs and that high MW, or melanoidins, contain only weak antiradical activity.[53] Different reaction conditions and

Table 7. Pearson Correlation Coefficients (r) of Antioxidant and Anti-Inflammatory Activities of Crude MRPs Isolated from Different MR Mixtures[a]

	α-dicarbonyl compounds[b]	intracellular[c] oxidation	NO inhibition[c]
intracellular oxidation	−0.162 (0.759)		
NO inhibition	−0.003 (0.996)	0.943 (0.005)	
IL-8 inhibition	−0.237 (0.651)	0.886 (0.019)	0.954 (0.003)

[a] Numbers in parentheses are P values. [b] α-Dicarbonly compounds are the sum of glucosone, 3-DG, GO, and MGO in hexose MR models and the sum of pentosone, 3-DP, GO, and MGO in pentose MR models. [c] The intracellular oxidation and NO inhibition effects of MR mixtures represent the percent inhibition at a concentration of 1.25 mg/mL.

model systems used to generate the MRPs, combined with the various methods used for antioxidant analysis, could explain the conflicting results. Our present study showed that crude MRPs derived from lysine MR systems exhibited a relatively higher capacity to inhibit intracellular oxidation, and the ability of MRPs to inhibit intracellular oxidation varied among fractions according to MW. In general, MRPs that ranged 1−10 kDa had a relatively greater affinity to inhibit intracellular oxidation compared with MRPs with MW > 10 or < 1 kDa. Gu et al.[54] also found that the antioxidant activities of MRPs with various MW generated from a glucose−casein MR model differed according to the analytical method used to assess antioxidant capacity. Low MW MRPs had more DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacity than high MW fractions, whereas the results were quite the opposite in terms of ferrous ion chelating ability.[54] The mechanisms of MRP antioxidant activities include trapping positive electrophilic species, scavenging free radicals, chelating metal ions, or reducing transition metal ions.[55] In the intracellular oxidation assay, AAPH generates peroxyl radicals that oxidize nonfluorescent 2′,7′-dichlorofluorescein (DCFH), produced via DCFH-DA hydrolysis by intercellular esterases, to fluorescent 2′,7′-dichlorofluorescein (DCF).[56] Thus, MRPs that can penetrate cells should have greater peroxyl radical scavenging activity. Our previous study showed that MRPs with MW > 3.5 kDa inhibited intracellular oxidation induced by H_2O_2 and AAPH, which might be due to directly scavenging H_2O_2 and/or peroxyl radicals in the culture media, thus reducing cellular exposure to ROS.[40] However, in the present study, the relatively greater intracellular oxidation inhibitory activity of MRPs with MW ranging from 1 to 10 kDa indicates that they either were effective at penetrating the cell in order to reduce the AAPH derived peroxyl radicals used to induce intracellular oxidation or instead formed a protective layer on the cell membrane to prevent AAPH permeating into the cells.

ROS together with NO, a reactive nitrogen species (RNS), have been considered to be implicated in the pathogenesis of the mucosal lesion in IBD.[57] Oxidative stress caused by the imbalance between generated ROS/RNS and the presence of antioxidant will increase the production of pro-inflammatory cytokines such as IL-8 and TNF-α.[28] Therefore, antioxidants with ROS/RNS scavenging activity can also function as anti-inflammatory agents. This was confirmed in the present study, where the affinity of crude MRPs to inhibit intracellular oxidation was positively correlated with NO and IL-8 inhibitory activities. To our knowledge this is the first experiment to show that MRPs with antioxidant activity also act as anti-inflammatory agents in Caco-2 cells. Bioactive phenolics present in oregano extract with measured antioxidant activity have been shown to also prevent inflammatory disease.[36] The small molecular weight MRPs in the present study might also function as both peroxyl radical scavengers and activators of cellular antioxidant defense systems, which strongly suggests that they may also provide an anti-inflammatory function. A positive and significant correlation has been identified between NO and IL-8 inhibitory capacity, indicating a close relationship between NO and IL-8 in the intestinal pro-inflammatory network.[43] However, no similar correlation for the fractionated MRPs was obtained and the low MW MRPs other than α-dicarbonyl compounds contributed to these bioactivities.

In conclusion, we report on the finding that low molecular weight (MW < 1 kDa) MRPs showed anti-inflammatory capacity as evidenced by a decrease in the production of IL-8, NO and iNOS in an IFN-γ + PMA induced inflamed Caco-2 cell model system. Greater anti-inflammatory activity was identified in Glu-Lys MRPs than the other MR models. The anti-inflammatory activity was positively correlated with the relative intracellular oxidation inhibitory activity of the different crude MRPs, but not with the presence of total α-dicarbonyl compounds in the crude MRPs. The low MW fraction of Glu-Lys that showed especially high anti-inflammatory activity requires further investigation.

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ABBREVIATIONS USED

3-DG, 3-deoxyglucosone; 3-DP, 3-deoxypentosone; 3-MCP, 3-methyl-1,2-cyclopentanedione; AAPH, 2,2′-azobis (2-amidinopropane) HCl; AGEs, advanced glycation end products; BSA, bovine serum albumin; COX-2, cyclooxygenase; DAN, 2,3-diaminonaphthalene; DCF, fluorescent 2′,7′-dichlorofluorescein; DCFH, nonfluorescent 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FI, fluorescent intensity; Fru, fructose; Glu, glucose; Gly, glycine; GM-CSF, granulocyte-macrophage colony-stimulating factor; GO, glyoxal; HD, 3,4-hexanedione; IBD, inflammatory bowel diseases; iNOS, inducible nitric oxide synthase; Lys, lysine; MEM, minimum essential medium; MGO, methylglyoxal; MR, Maillard reaction; MRPs, Maillard reaction products; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MW, molecular weight; NO, nitric oxide; PMA, phorbol ester; Rib, ribose; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate

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