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Demonstration of Antioxidant and Anti-inflammatory Bioactivities from Sugar−Amino Acid Maillard Reaction Products

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ABSTRACT: Maillard reaction products (MRPs), both crude and fractionated, were assessed for antioxidant potential using cell-free, in vitro 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, Fenton reaction induced deoxyribose degradation and oxygen radical absorbance capacity−fluorescein ($ORAC_{FL}$) chemical assays. All MRPs displayed various affinities to scavenge free radicals generated in different reaction media and using different reactive oxygen species (ROS) substrates. High molecular weight MRPs consistently showed the greatest ($P < 0.05$) antioxidant potential in chemical assays. Repeating these tests in Caco-2 cells with both reactive oxygen and nitrogen (RNS) intracellular assays revealed that the low molecular weight components (LMW) were most effective at inhibiting oxidation and inflammation. In particular, a glucose−lysine (Glu-Lys) mixture heated for 60 min had marked intracellular antioxidant activity and nitric oxide (NO) and interleukin-8 (IL-8) inhibitory activities compared to other MRPs ($P < 0.05$). Further studies employing ultrafiltration, ethyl acetate extraction, and semipreparative high-performance liquid chromatography (HPLC) produced a bioactive fraction, termed F3, from heated Glu-Lys MRP. F3 inhibited NO, inducible nitric oxide synthetase (iNOS), and IL-8 in interferon γ (IFN-γ)- and phorbol ester (PMA)-induced Caco-2 cells. F3 modified several gene expressions involved in the NF-κB signaling pathway. Two components, namely, 5-hydroxymethyl-2-furfural (HMF) and 5-hydroxymethyl-2-furoic acid (HMFA), were identified in the F3 fraction, with an unidentified third component comprising a major portion of the bioactivity. The results show that MRP components have bioactive potential, especially in regard to suppressing oxidative stress and inflammation in IFN-γ- and PMA-induced Caco-2 cells.

KEYWORDS: *antioxidant, anti-inflammation, nitric oxide, Caco-2, Maillard reaction products, cell signaling*

INTRODUCTION

Generation of reactive oxygen (ROS; e.g., hydrogen peroxide, hydroxyl, superoxide, and singlet oxygen radicals) and reactive nitrogen (RNS; nitric oxide, peroxynitrate) species occurs as natural byproducts of metabolism and can initiate a shift in redox homeostasis to oxidative stress,[1] a common etiological factor of many chronic diseases such as inflammatory bowel disease (IBD).[2] Moreover, both ROS and RNS when produced under physiological conditions represent important cell signaling biomolecules that can regulate the expression of specific genes involved in oxidative stress homeostasis and the antioxidant defense system. Nuclear factor kappa B (NF-κB) is an important component of the intestinal immune system, which on nuclear activation regulates numerous cytokines, such as interleukin 8 (IL-8). Elevated levels of NF-κB are typical in subjects with IBD.[3] Increases in IL-8 along with inducible nitric oxide synthetase (iNOS) and nitric oxide (NO) produce a pro-inflammatory network both in cultured Caco-2 cells[4] and in patients with active ulcerative colitis.[5] Because RNS together with ROS are implicated in intestinal immune response to IBD, antioxidants that can scavenge ROS/RNS can also provide potential indirect anti-inflammatory activity.[6]

The antioxidant activity of MRPs was first reported in the early 1950s,[7] with much later studies showing comparable activity between synthetic butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) antioxidants and melanoidins derived from model xylose−glucose solutions.[8] Antioxidant activities have also been shown for heated advanced glycation end-products (AGEs), which are produced in similar fashion to the MR, such as casein−glucose mixtures; these products increase the shelf life of full-cream products[9] and decrease oxidation of deoxyribose in a Fenton reaction system.[10] AGEs also represent a concern as a potential source of dietary pro-inflammatory agents that may promote inflammatory responses leading to injury in diabetics.[11] Underlying physicochemical mechanisms for MRP antioxidant activity range from having a notable reducing power,[12] a capacity to scavenge free radicals and ROS,[13−18] a proficiency to chelate potential metal prooxidants,[19−22] and the capacity to inhibit hexanal oxidation and lipid peroxidation reaction.[23] Pro-oxidant and genotoxic activity of MRPs, when in the presence of transition metals, such as iron[24] or copper,[25] has also been reported in vitro. A possible extension of this has been the observed cytotoxicity effects of model sugar−amino acid MRPs.[15,26] On examination of the extensive literature that has helped to identify specific MRPs with antioxidant activity, both early to midstage[17,27] and later stage MRPs[17,28] have reported antioxidant activity, thus strongly indicating that different antioxidant mechanisms may indeed exist for different chemical constituents present in the complex MR mixture. Furans, pyrroles, pyridines, and imidazoles are examples of MRPs shown to contribute to

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antioxidant activity when derived from simple carbonyl−amino acid models[29] as well as intact browned food systems.[30]

MRPs also affect the antioxidant defense mechanism and thus the cellular redox status in cultured intestinal epithelial Caco-2 cells[31−34] and when administered in vivo to rodents.[35,36] Bioactive Maillard components identified as pyrrolinone reductonyl-lysine (pronyl-lysine) from bread crust significantly increase phase II chemopreventive enzymes, such as glutathione *S*-transferase (GST), activity by 34%, thus demonstrating for the first time that MR components had potential chemoprotective activity in Caco-2 cells.[31] In other studies, the administration of a Maillard-rich bread crust to rodents also produced marked systemic enhancement of plasma tocopherol levels and kidney and hepatic antioxidant capacity that was particularly associated with elevations in chemopreventative enzymes, such as GST and uridine diphosphate−glucuronyl-transferase (UDP-GT).[36] The generation of H_2O_2 by MRPs has also been proposed for the notable changes in catalase activity and glutathione content observed in human lymphocytes exposed to food component MRPs[37,38] and different sugar−lysine model systems.[39]

The redox status of the cell is an important regulator of inflammatory responses that result from the up-regulation of several genes and the activation of transcription factors, such as noted above with NF-κB. The affinity to stimulate transcription by MRP antioxidant activity will be based on how these compounds influence the critical cellular levels of ROS and RNS. The fact that some studies have reported that dietary intake of MRP may potentially promote the generation of pro-inflammatory mediators in diabetics,[11] whereas others show potential anti-inflammatory activity of certain MRPs,[40] indicates that more work is required to determine which MR components produce these effects and what are the underlying mechanisms for which MRPs trigger pro- or anti-inflammatory events. Exploring the potential for MRPs to be involved in both the antioxidant and anti-inflammatory responses of intestinal cells has occurred only recently.[37,38] This paper reports on the antioxidant activity of MRPs with a concentration on the bioactive components of LMW MRPs, derived from sugar−amino acid model MRPs, that have an affinity to modify the expression of genes involved in anti-inflammation.

MATERIALS AND METHODS

Preparation and Separation of MRPs. Glucose (Glu), fructose (Fru), and ribose (Rib) (0.8 mol/L) were mixed in a 1:1 molar ratio with 0.8 mol/L L-lysine (Lys) or glycine (Gly) (Sigma, St. Louis, MO, USA) in six separate reactions and heat processed at 121 °C for various time periods of 5, 15, 30, 60, and 90 min before being rapidly chilled on ice to generate substrate-specific model MRPs.[17] MRPs were fractionated into LMW and high molecular weight (HMW) MRPs according to previously published methods.[17] LMW fractions (<1 kDa) were extracted using ethyl acetate and separated into 15 fractions by semipreparative reverse phase HPLC using an Agilent 1100 LC system (Agilent, Santa Clara, CA, USA) equipped with a Zorbax Eclipse-C18 column (9.4 × 250 mm, 5 μm, 80 Å, Agilent). The samples were eluted with a linear gradient of 10−60% methanol/H_2O for 40 min at a flow rate of 2.5 mL/min and detected using a DAD at 350 nm and a fluorescent detector with excitation at 350 nm and emission at 440 nm. A specific fraction, referred to as fraction 3 (F3), was recovered using semipreparative HPLC and further purified into three subfractions (F3-A, F3-B, F3-C) by elution with 20−40% methanol in 0.2% formic acid for 10 min, at a flow rate of 1 mL/min through two connected columns, a Zorbax Eclipse-XDB-C18 (4.6 × 150 mm, 5 μm, 80 Å, Agilent) and a Zorbax SB-C18 (4.6 × 150 mm, 3.5 μm, Agilent). Standards 5-hydroxymethyl-2-furfural (HMF) and 5-hydroxymethyl-2-furoic acid (HMFA) were obtained from Sigma and Toronto Research Chemicals (ON, Canada), respectively.

Measurement of Fluorescence, UV−Vis Absorbance. Fluorescence emission and UV−vis absorbance (A_{294} and A_{420}) were measured on all crude MRPs.[17,41]

Antioxidant Assay Methods. Chemical-based antioxidant activity was determined using DPPH quenching and Fenton radical induced deoxyribose degradation assays[15,34] and the oxygen radical absorbance capacity−fluorescein ($ORAC_{FL}$) method.[17,41] Unlike the other assays, $ORAC_{FL}$ measures the peroxyl radical reaction to completion by combining both inhibition percentage and inhibition time of the free radical quenching process, expressing the final value as micromoles of Trolox per gram of dry matter.

Cell Culture. Caco-2 cells (HTB-37, ATCC) were cultured in minimum essential medium (MEM) with Earle's salts (Sigma). Medium contained 10% FBS, 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Invitrogen, Burlington, ON, Canada). Cells were cultured at 37 °C under a 5% CO_2 atmosphere. The media were changed every 2−3 days, and the cells were subcultured weekly.[4]

Intracellular Oxidation. The intracellular oxidation assay for MRPs was done according to previously published methods[41,42] Briefly, Caco-2 cells were seeded in 96-well plates (Sarstedt, Nümbrecht, Germany) at a density of 10^5 cells/cm^2 in MEM and cultured for 3 weeks followed by incubation with or without MRPs for 24 h. 2′,7′-Dichlorofluorescein diacetate (Sigma) in Hank's buffered salt solution was added to the cells and incubated for 30 min, followed by 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) for 1 h. Fluorescence intensity (FI) was measured using a fluorescence microplate reader (Fluoroskan Ascent FL, Thermo Labsystems, Helsinki, Finland) with the excitation wavelength at 485 nm and emission at 527 nm. The percent inhibition was calculated as

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

where FI_{st} is the 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. Cell viability was confirmed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays.[3] Only cells with viability >95% were used in experiments.

Nitric Oxide Measurement in Caco-2 Cells. NO was measured as reported previously.[4] Caco-2 cells seeded in 96-well plates (Sarstedt) at a density of 10^5 cells/cm^2 in MEM (100 μL) were cultured for 3 weeks, followed by incubation with, or without, MRPs for 24 h. Cells were then stimulated with 8000 U/mL IFN-γ + 0.1 μg/mL PMA for 48 h. NO levels in the culture medium were determined using the Griess reaction, after the reduction of nitrate to nitrite with nitrate reductase (Sigma). The percent inhibition was calculated using 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 MRPs and inducers, and NO_{sample} is the NO concentration of cells incubated with MRPs for 24 h and then stimulated with IFN-γ + PMA for 48 h. Cell viability was tested using MTT assays.[3] Only cells with viability >95% were used in experiments.

Interleukin-8 Measurement in Caco-2 Cells. Caco-2 cells seeded in 6-well plates (Sarstedt) at a density of 10^5 cells/cm^2 in MEM were cultured for 3 weeks followed by incubation with, or without, MRPs for 24 h. After stimulation with 8000 U/mL IFN-γ + 0.1 μg/mL PMA for 2−48 h, IL-8 in the culture medium was determined using ELISA assay kits (SABioscience, Frederick, MD, USA) according to the manufacturer's instructions. The cells were also used for iNOS expression analysis as described below. The percent IL-8 inhibition was calculated using equation[41]

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

where $IL\text{-}8_{st}$ is the IL-8 concentration of the cell supernatant incubated with IFN-γ + PMA for 24 h, $IL\text{-}8_{blank}$ is the IL-8 concentration of the

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

Reverse Transcription (RT)-PCR and Western Blotting. iNOS mRNA and protein expression in Caco-2 cells were measured using RT-PCR and Western blotting.[4] Briefly, total RNA was extracted from Caco-2 cells seeded in 6-well plates using GStract RNA isolation kits (Maxim Biotech Inc., Rockville, MD, USA). RT and PCR amplification were performed using the RTeasy reverse transcription kit and human iNOS primer set kit (Maxim Biotech Inc.). β-Atin was the housekeeping gene (Maxim Biotech Inc.). 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.

Total protein was extracted and adjusted to the same concentration. The denatured protein was resolved by electrophoresis (Mini-PROTEIN II, Bio-Rad, Hercules, CA) on 8% sodium dodecyl sulfate (SDS)−polyacrylamide mini-gels and subsequently transferred to nitrocellulose membranes (0.2 μm, 7 × 8.4 cm, Bio-Rad Laboratories, Hercules, CA, USA). Nonspecific binding was blocked with nonfat milk powder, and membranes were incubated with anti-human iNOS polyclonal primary antibody (Zymed, Invitrogen, Carlsbad, CA, USA) or rabbit anti-actin primary antibody (Sigma), followed by the secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (Invitrogen). 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.).

Real-Time Quantitative Reverse Transcription PCR (RQ-RT-PCR) Array. Total RNA in Caco-2 cells seeded in 6-well plates was extracted using RT2 qPCR-Grade RNA Isolation Kits (SABioscience) according to the manufacturer's instructions. The quality and quantity of RNA were determined using a NanoDrop spectrophotometer (NanoDrop Technology, Wilmington, DE, USA). Only RNA with a 260/280 ratio of ≥2.0 and a 260/230 ratio of ≥1.7 was accepted. Total RNA (1.0 μg/mL) was reverse-transcripted into first-strand cDNA using the RT2 First Strand Kit (SABioscience). Real-time PCR proceeded using the RT2Profiler PCR Array system (SABioscience) on a Bio-Rad iQ5. Two sets of 84 genes involved in the NF-κB and NO signaling pathways were quantified in triplicate. The data were analyzed according to the manufacturer's instructions.

LC-ESI-MS/MS. F3 was analyzed by LC-ESI-MS/MS using an Agilent 1100 LC/MSD-Trap-XCT system using a Zorbax SB-C18 column (4.6 × 50 mm, 1.8 μm, Agilent, USA) and eluted with 10% acetonitrile (ACN) in 0.2% formic acid at a flow rate of 1.2 mL/min. The MS operated with an ionization temperature set at 350 °C. The electron spray ionization was in positive ion mode. A scan mass-to-charge ratio (m/z) ranged from 40 to 750, and nitrogen gas was the nebulizer (12 L/min).

High-Resolution Mass Spectrometry. High-resolution electrospray mass spectra were obtained on a Micromass LCT time-of-flight (TOF) mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ion source. Samples were dissolved in methanol to a final concentration of 20 μmol/L. The mass parameters were as follows: flow rate, 20 μL/min; sample cone, 90 V; source and desolvation temperatures, both 120 °C. Tryptophan, phenylalanine, and methionine were the reference compounds for accurate mass measurement of recovered fractions F3-A, F3-B, and F3-C, respectively.

Statistics. Data were analyzed using a one-way ANOVA using MINITAB software (version 14, Minitab Inc., State College, PA, USA). Correlations for chemical properties of MRPs and bioactive activities were made using the same MINITAB software. Significant differences were determined using Tukey's test at $P < 0.05$.

RESULTS AND DISCUSSION

Physical−Chemical and Antioxidant Properties of MRPs. Changes in temporal fluorescent intensity (FI) and absorbance patterns at A_{294} and A_{420} for all MRPs were negatively correlated with changes in pH, whereas the production of intermediate MRPs (A_{294}) was positively correlated with browning products (A_{420}) (Table 1). The appearance of fluorescence did not correlate with antioxidant capacity derived from the $ORAC_{FL}$ assay, although $ORAC_{FL}$ did correlate well with both A_{294} and A_{420} (Table 1). Other workers have associated antioxidant activity of Maillard products with pyrrole and furan content, which have UV absorbance in the

Table 1. Correlations between Fluorescent Intensity, UV−Vis Absorbance, and $ORAC_{FL}$ of Glu-Lys MRPs[a]

model		pH	FI	A_{294}	A_{420}
Fru-Lys	FI	−0.952*			
	A_{294}	−0.736	0.778		
	A_{420}	−0.672	0.712	0.995*	
	$ORAC_{FL}$	−0.732	0.77	0.999*	0.996*
Glu-Lys	FI	−0.488			
	A_{294}	−0.709	−0.265		
	A_{420}	−0.65	−0.344	0.995*	
	$ORAC_{FL}$	−0.776	−0.159	0.991*	0.973*
Rib-Lys	FI	−0.375			
	A294	−0.97*	0.408		
	A_{420}	−0.953*	0.289	0.99*	
	$ORAC_{FL}$	−0.919*	0.138	0.948*	0.968*

[a]Data are correlation coefficients (r) shown for only sugar−lysine MRPs. FI, fluorescent intensity; A_{294}, UV absorbance at 294 nm; A_{420}, visible absorbance at 420 nm; Fru-Lys, fructose−lysine; Glu-Lys, glucose−lysine; Rib-Lys, ribose−lysine. *, significant correlation at $P < 0.05$.

Table 2. Antioxidant Scavenging Activities of MRPs Derived from Different Reactants[a]

	free radical source			
MRP	DPPH[b]	TEAC[c]	hydroxyl[d]	peroxyl[e]
crude mixtures				
Glc-Lys	52.6 ± 4.4*	0.18	87 ± 2*	270*
Fru-Lys	39.9 ± 1.9*,**	0.14	81 ± 1*	123*,**
Rib-Lys	60.5 ± 1.6*	0.20	85 ± 6*	562*
LMW MRP fractions				
Glc-Lys	31.7 ± 0.7*	0.11	83 ± 7*	2002*
Fru-Lys	10.4 ± 0.2*,**	0.04	80 ± 8*	1480*,**
Rib-Lys	39.5 ± 1.2*	0.13	90 ± 3*	2212*
HMW MRP fractions				
Glc-Lys	72.0 ± 2.7*	0.24	56 ± 6*	2240*
Fru-Lys	48.8 ± 1.0*,**	0.17	56 ± 9*	2534*
Rib-Lys	73.6 ± 0.6*	0.25	66 ± 7*	2558*

[a]The values represent enzyme activity, expressed as the mean ± SD control = PBS only; Glc-Lys, Fru-Lys, Rib-Lys = glucose-, fructose-, and ribose-lysine model MRPs, respectively; LMW = <1 to <3 kDa; HMW = 3 to >10 kDa. *, significantly different from control (PBS); **, significantly different within group ($P < 0.05$). [b]Values are DPPH scavenging activity (%) = ($Abs_{control}$ − Abs_{sample})/$Abs_{control}$ × 100%. [c]TEAC = trolox equivalent antioxidant capacity (1 unit or TEAC is defined as the concentration (mg/mL) of Trolox having equivalent antioxidant capacity to a 1.0 mg/mL solution of sample. [d]Fenton reagent produced hydroxyl radicals that oxidize deoxyribose. Values represent % deoxyribose oxidized. [e]$ORAC_{FL}$ antioxidant assay using AAPH production of peroxyl radical. Values are expressed as (μmol Trolox/g dm).

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Table 3. Pearson Correlation Coefficients (r) of Antioxidant and Anti-inflammatory Activities of Crude MRPs Isolated from MR Models[a]

	$ORAC_{FL}$	intracellular oxidation	NO inhibition
intracellular oxidation	0.104 (0.844)		
NO inhibition	−0.023 (0.965)	0.943 (0.005)	
IL-8 inhibition	−0.155 (0.77)	0.886 (0.019)	0.954 (0.003)

[a]Numbers in parentheses are P values.

294 nm range.[43] Common to all MRPs studied was the initial decrease in pH, which influenced the subsequent rates of the MRs and most likely the final composition of each reaction, which would explain the range of antioxidant activities observed for different sugar−amino acid model MRPs (Table 2).

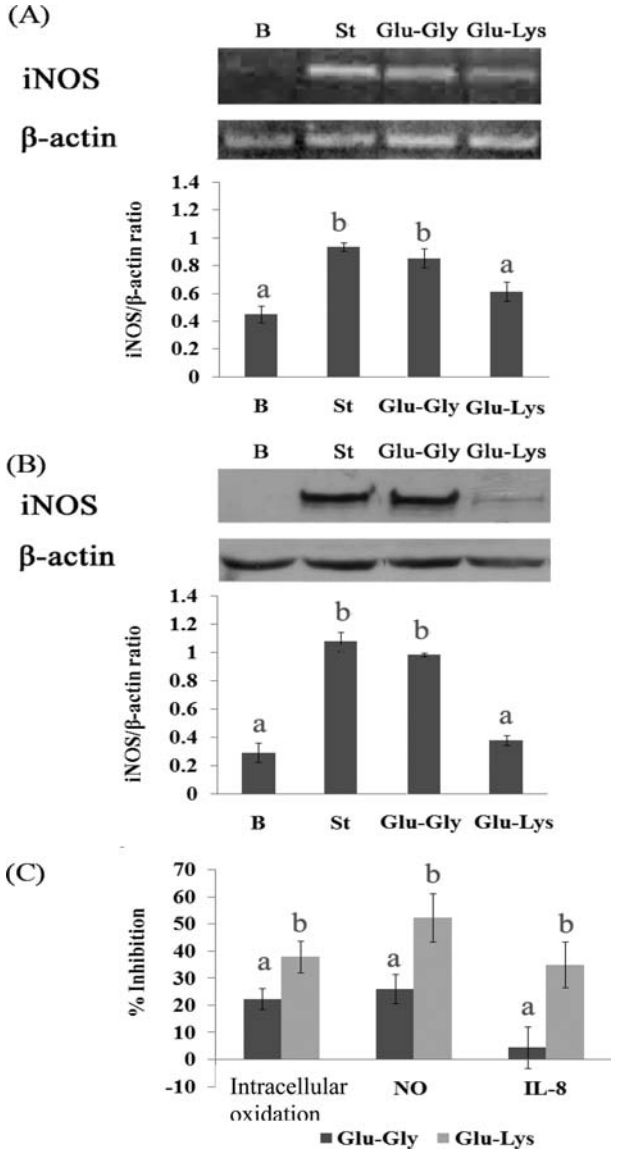

Figure 1. Intracellular oxidation and NO, iNOS, and IL-8 inhibitory effects of crude Glu-Gly and Glu-Lys: (A) iNOS mRNA; (B) iNOS protein; (C) intracellular oxidation, NO and IL-8 inhibition. Glu-Gly, glucose−glycine; Glu-Lys, glucose−lysine; B, Caco-2 cells without crude MRPs and IFN-γ + PMA stimulation; St, Caco-2 cells incubated only with IFN-γ + PMA; Glu-Gly, Glu-Lys, Caco-2 cells incubated with 1.25 mg/mL Glu-Gly or Glu-Lys for 24 h followed by stimulation with IFN-γ + PMA for 24 h. Different lower case letters over bars denote significant difference ($P < 0.05$).

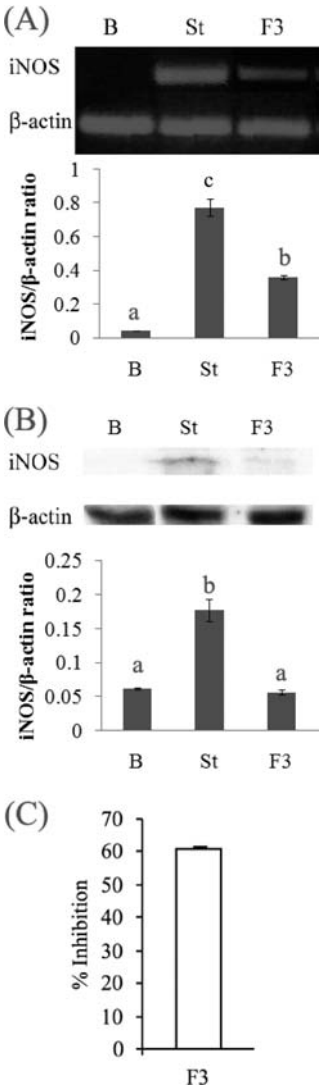

Figure 2. iNOS and IL-8 inhibition effects of F3: (A) iNOS mRNA; (B) iNOS protein; (C) IL-8 inhibition. B, Caco-2 cells without F3 and IFN-γ + PMA; St, Caco-2 cells incubated only with IFN-γ + PMA; F3, Caco-2 cells preincubated with 50 μg/mL of F3 for 24 h followed by stimulation with IFN-γ + PMA for 24 h. Different lower case letters over bars denote significant difference ($P < 0.05$). Two independent experiments were performed in duplicate. Data are expressed as the mean ± SEM.

Antioxidant measurements employing different cell-free assays systems, including stable free-DPPH scavenging, Fenton reaction-induced hydroxyl radical degradation of deoxyribose, and the AAPH-induced peroxyl radical ($ORAC_{FL}$) assay, all revealed various degrees of antioxidant activity from both crude MRPs and respective LMW and HMW fractions, respectively (Table 2). These results agree with other studies that used high temperature (e.g., 120 °C) to heat histidine and glucose and produce MRPs that exhibited peroxyl radical scavenging activity generated by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH).[44] Common to all assay results was the finding that a relatively greater ($P < 0.05$) free radical scavenging power was observed for the HMW MRP fractions (e.g., >1 kDa) compared to both crude and LMW MRP fractions, respectively. Other

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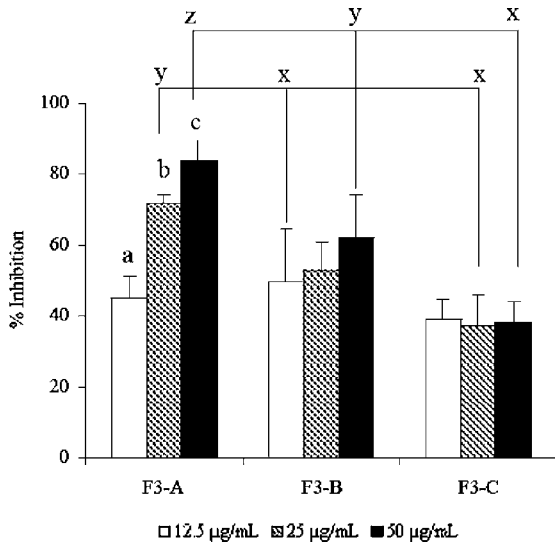

Figure 3. NO inhibitory effect of F3 components Caco-2 cells preincubated with F3-A, F3-B, and F3-C for 24 h and then stimulated with IFNγ + PMA for 24 h. F3-A, F3-B, and F3-C are compounds A, B and C isolated from F3. Different lower case letters a−c over bars denote significant differences ($P < 0.05$) for different concentrations of the same sample. Different lower case letters x−z over bars denote significant differences ($P < 0.05$) for different samples (F3-A, F3-B, and F3-C) at the same concentration. IC_{50} of F3-A is 0.076 mmol/L.

studies using similar cell-free chemical antioxidant assays have attributed antioxidant activity to late-stage MRPs[28,45,46] Concern, however, has also been expressed that the presence of nonantioxidant components in the food extract may interfere with absolute measurements of antioxidant capacity, particularly for the $ORAC_{FL}$, which may explain this apparent discrepancy.[47]

A cell-based assay has also showed antioxidant activity of MRPs isolated from fructose, glucose, and ribose with lysine model systems that inhibit H_2O_2 and AAPH-induced intracellular oxidation in RAW264.7 cells.[42] In this study, AAPH-induced peroxyl radical oxidation in cultured Caco-2 cells confirmed that crude MRPs were effective at lowering intracellular oxidation (e.g., Fru-Lys, 28.8 ± 9% inhibition; Glu-Lys, 38 ± 6% inhibition; Rib-Lys, 31 ± 3% inhibition). Again, however, there was no correlation between the in vitro $ORAC_{FL}$ antioxidant activity of crude MRPs and the affinity to inhibit intracellular oxidation, nitric oxide production, or IL-8 production in Caco-2 cells (Table 3). The affinity of crude MRPs to inhibit intracellular oxidation was, however, significantly correlated with nitric oxide inhibition ($P = 0.05$) and IL-8 inhibition ($P = 0.019$). LMW (e.g., <1 kDa) MRP fractions (Fru-Lys, 36.2 ± 7%; Glu-Lys, 42 ± 7; Rib-Lys, 34 ± 4%) had greater affinity to inhibit intracellular oxidation than HMW MRPs. This finding is important because it signifies that LMW components of MRPs are effective intracellular antioxidant components, likely due to the fact that they are able to permeate the cell, thus influencing redox balance. It is plausible that the larger polymer-sized HMW MRPs are not as permeable to the cell and therefore display activity that is limited less to intracellular reactions.

Identification of Anti-inflammatory MRPs. Anti-inflammatory activity of crude MRPs was observed only in Caco-2 cells that were previously treated with IFN-γ and PMA, where NO, iNOS, mRNA ,and protein expression were induced at 6−24 h after cytokine treatment.[4] In cytokine-stimulated Caco-2 cells, crude Glu-Lys MRPs produced the strongest inhibition of NO production, ranging from 25 to 52% over a concentration range of 0.3−2.5 mg/mL for all MRPs tested. Examples of two MRPs, both generated with glucose but varying in respect to lysine and glycine content as the amine sources, displaying inhibition to both transcriptional and translational iNOS expression in inflamed Caco-2 cells are presented in Figure 1, panels A and B, respectively. The relatively stronger affinity of crude Glu-Lys MRPs to produce inhibition of both iNOS mRNA and iNOS protein expression corresponded with significantly higher ($P < 0.05$) affinities to inhibit NO and IL-8, as well to protect against intracellular oxidation (Figure 1C). LMW MRPs derived from Glu-Lys and Glu-Gly MRPs exhibited marked affinities to inhibit both intracellular oxidation (39.9 ± 4 and 27.7 ± 6%) and nitric oxide (56.7 ± 7 and 17.9 ± 3%), respectively, at the concentration of 1.25 mg/mL.

The finding that LMW (<1 kDa) MRPs derived from Glu-Lys reactants evoked significant inhibition of iNOS, NO, and IL-8 synthesis in IFN-γ + PMA-induced Caco-2 cells led to further studies to identify the bioactive components involved in the anti-inflammatory activities of LMW MRPs, as well as to elucidate the molecular mechanisms of action. LMW MRPs recovered from an ethyl acetate extraction had a concentration-dependent (0.1−1.25 mg/mL) inhibition in both iNOS mRNA and iNOS protein expression. A final isolation and characterization step was therefore performed to identify the specific components present in the hydrophobic LMW fraction, using semipreparative HPLC. A total of 15 different fractions were recovered according to UV−vis and fluorescent spectra. One particular fraction, referred to herein as F3, was concentrated and shown to have significant affinities to inhibit both iNOS mRNA and iNOS protein (Figure 2). This fraction was chosen for further characterization analysis.

Chemical Composition of F3. The F3 fraction was further purified using analytical HPLC into three major subfractions (F3-A, F3-B, and F3-C) (Figure 3). F3-A, a dark brown pigment, had a maximum UV absorbance (λ_{max}) at 370 nm. LC-ESI-MS revealed that this fraction possessed a positive ion $[M + 1]^+$ at m/z 192, with an odd number of nitrogen atoms. The LC-ESI-MS/MS spectrum showed m/z 174, 147, 107, and 81 fragments. The fragment at m/z 174 was due to the loss of one H_2O molecule from the molecular ion, indicating the presence of a hydroxyl group. The accurate mass of $[M + 1]^+$ was 192.0999 Da, so the most likely empirical formula for F3-A was either $C_6H_{13}N_3O_4$ (deviation, 1.5 mDa) or $C_{11}H_{13}NO_2$ (deviation, −2.6 mDa). The second subfraction, F3-B, was pale yellow with a λ_{max} at 285 nm. A positive molecular ion $[M + 1]^+$ at m/z 127 and a fragment at m/z 109 indicate loss of one H_2O molecule. F3-B had an accurate mass of $[M + Na]^+$ 149.0218 Da and an empirical formula of $C_6H_6O_3$ (deviation, 0.3 mDa). This compound was identified as HMF after comparison of the color, retention time, λ_{max}, molecular weight, and molecular formula with those of a pure HMF standard. F3-C had white color and a λ_{max} at 260 nm. The mass spectrum of F3-C showed a positive molecular ion $[M + 1]^+$ at m/z 143, a negative molecular ion $[M - 1]^-$ at m/z 141, and a fragment at m/z 125, also indicating the loss of one H_2O molecule. The accurate mass of $[M - 1]^-$ was 141.0193 Da, indicating that the likely empirical formula was $C_6H_6O_4$ (deviation, 0.5 mDa). This compound was identified as HMFA after comparison of the color, retention time, λ_{max}, molecular weight, and molecular formula with those of a pure HMFA standard.

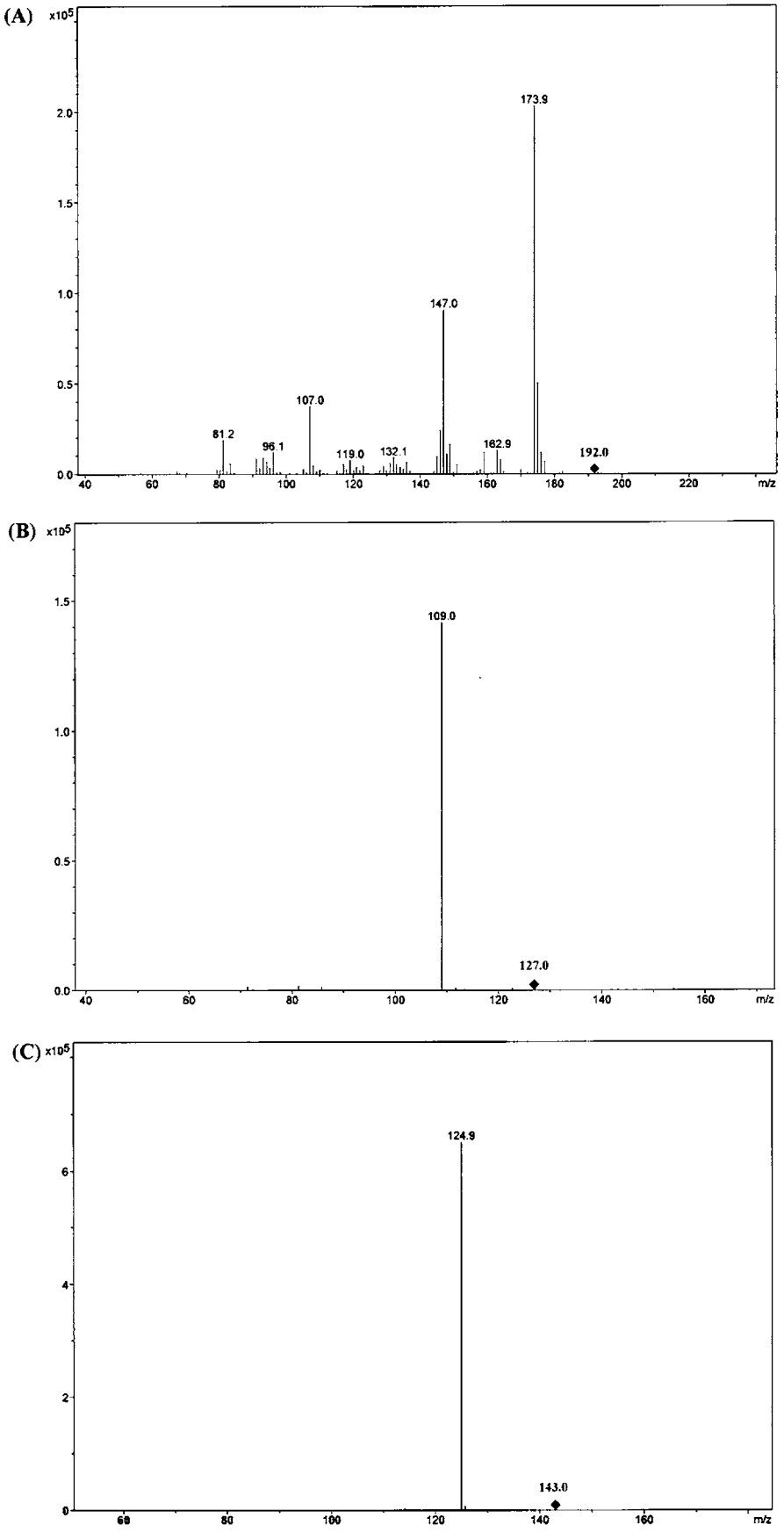

Figure 4. LC-ESI-MS/MS results of F3 components: (A) F3-A, Rt 6.59 min, λ_{max} 370 nm, MW 191 Da, molecular formula $C_6H_{13}N_3O_4$ or $C_{11}H_{13}NO_2$; (B) F3-B (HMF), Rt 7.45 min, λ_{max} 285 nm, MW 126 Da, molecular formula $C_6H_6O_3$; (C) F3-C (HMFA), Rt 7.99 min, λ_{max} 260 nm, MW 142 Da, molecular formula $C_6H_6O_4$.

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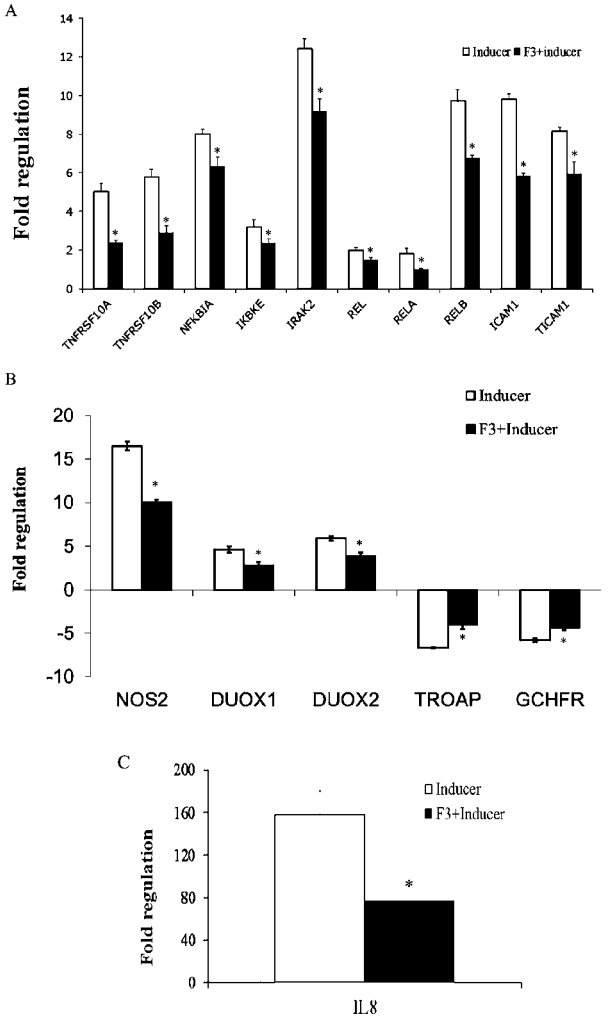

Figure 5. Effect of cytokine inducers and F3 on NF-κB and NO signaling pathways: (A) cytokine receptors (TNFRSF10A and TNFRSF10B), receptor-associated proteins (IRAK2 and TICAM1), inhibitor kB kinase (IKBKE), NF-κB inhibitor (NFKBIA), NF-κB subunits (REL, RELA, and RELB), and NF-κB responsive gene (ICAM1); (B) NOS2 (iNOS gene), two peroxidases genes dual oxidase 1 and 2 (DUOX1 and DUOX2), GCFHR regulator of iNOS biosynthesis cofactor BH4, and TROAP, a gene suppressed by NO; (C) NF-κB responsive gene IL-8. ∗ denotes significant difference from cytokine inducer.

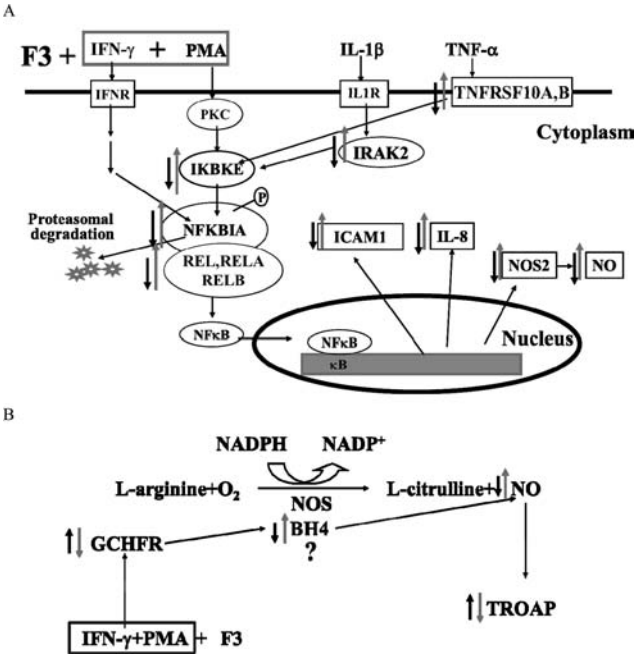

Figure 6. Proposed schemes for anti-inflammatory mechanisms of MRPs: (A) NF-κB pathway; (B) alternative pathway. Direction of cell signaling induced by cytokines alone is shown by the gray arrows. Mitigation of cell signaling induced by cytokines due to pre-exposure to F3 is shown by black arrows.

Fraction F3-A produced a concentration-dependent inhibition of nitric oxide with an IC_{50} of 0.076 mmol/L (Figure 4). The relative activities of F3-B and F3-C to inhibit nitric oxide were not dependent on concentration and had relatively less activity. Although we were unable to identify the F3A fraction, it is known that F3-B or HMF is present in such foods as coffee, breakfast cereals, honey, jam, wine, beverages, and dried fruit[48] and in many traditional Chinese medicines and herbs.[49,50] HMF is a major product of hexose degradation under acidic conditions, where aldo−keto isomerization of aldohexose is followed by the dehydration of furanose tautomers[51] and by the decomposition from 3-deoxyhexosone produced by MR or caramelization[52] or by methylglyoxal recombination with glyceraldehyde.[53] HMF has no health risks at high concentrations in specific foods[54,55] and possesses free radical scavenging[49] and affinity to inhibit H_2O_2-induced NO in LO2 hepatocytes.[50] HMFA is a metabolite of HMF via aldehyde oxidation as well as through the aldol reaction, intramolecular ketalization, and dehydration of its precursor glyceraldehyde and pyruvate.[56] Although relatively less is known about the bioactivity of HMFA, one study has reported antitumor activity.[57]

Mechanism of Anti-inflammatory Activity of LMW MRPs. Pretreatment of Caco-2 cells with IFN-γ + PMA produces maximum activation of NF-κB after 30 min.[4] We report here that a total of 11 genes implicated in the Caco-2 NF-κB signaling pathway (TNFRSF10A and B, NFKBIA, IKBKE, IRAK2, REL, RELA and B, ICAM1, and TICAM1) were up-regulated (range of 1.8−157-fold) after 60 min of exposure to the cytokine mixture (Figure 5A). Our observation that the inflammation induced in Caco-2 cells using the combination of IFN-γ and PMA produced up-regulation of NFKBIA has also been reported by Sun et al.[58] in Jurkat T cells. In addition, three genes involved in the NO signaling pathway, namely, NOS2, DUOX1, and DUOX2, were up-regulated (range of 6−16-fold), whereas two genes (TROAP and GCHFR) were down-regulated (−6.7- and −5.8-fold, respectively) when treated with IFN-γ + PMA (Figure 5B). Preincubating Caco-2 cells with 50 μg/mL F3 for 24 h produced significant ($P < 0.05$) reductions in cytokine-induced NFKBIA gene expression in both the up-regulated and down-regulated genes. This result confirmed that the NF-κB subunit was inactivated or that binding to the promoter region of NFKBIA gene was inhibited by components of the F3 fraction. Fraction F3 also down-regulated the induced gene expression of both receptor genes (TNFRSF10A) and associated proteins (e.g., IRAK2, TICAM1), known to be required for NF-κB activation.

A proposed scheme for the bioactivity of F3 to mitigate an inflammatory response is presented in Figure 6. Cytokine inducers that react with the receptors on the cytoplasm

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membrane, or directly diffuse into the cell, activate the inhibitor kB kinase (IKK) complex. The activated IKK will further phosphorylate the NF-κB inhibitor IkB, which releases a NF-κB dimer, and once translocated into the nucleus, it will bind to the target gene that initiates iNOS and other pro-inflammatory cytokine gene expressions, such as TNF-α and IL-1b, and IL-8. The up-regulated iNOS triggers NO production, which in turn will up-regulate cytokines that lead to further activated NF-κB. In our study, when Caco-2 cells were treated with IFN-γ and PMA, these different signaling levels were up-regulated; however, when cells were preincubated with our F3 fraction prior to cytokine treatment, the expression of these up-regulated genes was significantly reduced as shown by the down-positioned arrows.

NO is synthesized by the conversion of L-arginine to L-citrulline by iNOS, and the cofactor tetrahydrobiopterin (BH4) is also needed for NO synthesis.[59] When Caco-2 cells were induced with IFN-r + PMA, the expression of GTP cyclohydrolase feedback receptor (GCHFR) was down-regulated, which we hypothesize would increase BH4 synthesis and lead to an increase in NO production. The increased NO will in turn down-regulate trophinin-associated protein (TROAP) expression. In our study, the pretreatment of Caco-2 cells with F3, prior to cytokine treatment, attenuated the extent of GCHFR down-regulation, which interfered with NO synthesis. The reduced NO production lessened the down-regulation signal of TROAP, which is otherwise suppressed by NO.[60]

Conclusion. This study examined the antioxidant and anti-inflammatory activities of MRPs generated from MR sugar−amino acid model systems. It is important to note that conclusions concerning the bioactive properties of MRPs, when evaluated solely on chemical, cell-free antioxidant assay data, underestimate the relative importance of LMW MRP components, which have been shown in this study to have both intracellular antioxidant and anti-inflammatory activities. A bioactive fraction (F3) recovered from <1 kDa MRPs reduced the inflammation in Caco-2 cells induced with IFN-γ + PMA, mediated through the transcriptional down-regulation of genes involved in the NF-κB pathway as well as the translational inhibition of iNOS and IL-8 expression. The identification of HMF and HMFA, together with the unidentified F3-A, as the bioactive components that comprise the LMW MRP collectively inhibited iNOS, IL-8, and other genes that are involved in the NF-κB and NO signaling pathways. In conclusion, LMW MRP components derived from a heated Glu-Lys mixture have bioactive properties with antioxidant and anti-inflammatory activities that can be derived from our diet and which may potentially protect against the etiology of intestinal inflammation, IBD, or other inflammatory diseases. Further studies are required to determine the chemical structure of F3-A and the additive or synergistic anti-inflammatory mechanisms of action for each of the F3 subfractions recovered from Glu-Lys or other MRPs.

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ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane) hydrochloride; AGEs, advanced glycation end-products; BH4, tetrahydrobiopterin; COX-2, cyclooxygenase 2; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DUOX1, dual oxidase 1; DUOX2, dual oxidase 2; FI, fluorescence intensity; GCHFR, GTP cyclohydrolase I feedback regulator; GFRP, GTP cyclohydrolase feedback regulator protein; Glu, glucose; HMF, 5-hydroxymethyl-2-furfural; HMFA, 5-hydroxymethyl-2-furoic acid; IBD, inflammatory bowel disease; ICAM1, intercellular adhesion molecule 1; IECs, intestinal epithelial cells; IKBKE, inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon; IKK, inhibitor of kappa B kinase; iNOS, inducible nitric oxide synthase; IRAK2, interleukin-1 receptor-associated kinase 2; Lys, lysine; MEKK-1, mitogen-activated protein kinase 1; MEM, minimum essential medium; MR, Maillard reaction; MRPs, Maillard reaction products; MW, molecular weight; NFKBIA, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; NIK, NF-κB inducing kinase; NO, nitric oxide; $ORAC_{FL}$, oxygen radical absorbance capacity−fluorescein; PMA, phorbol 12-myristate 13-acetate; REL, V-rel reticuloendotheliosis viral oncogene homologue (avian); RELA, V-rel reticuloendotheliosis viral oncogene homologue A (avian); RELB, V-rel reticuloendotheliosis viral oncogene homologue B; ROS, reactive oxygen species; RNS, reactive nitrogen species; TICAM1, toll-like receptor adaptor molecule 1; TNF-α, tumor necrosis factor alpha; TNFRSF10A, tumor necrosis factor receptor superfamily, member 10a; TNFRSF10B, tumor necrosis factor receptor superfamily, member 10b; TRAILR1, TNF-related apoptosis-inducing ligand receptor 1; TRAILR2, TNF-related apoptosis-inducing ligand receptor 2; TROAP, trophinin associated protein.

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