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Inhibition of advanced glycation end product formation on collagen by rutin and its metabolites

Daniel Cervantes-Laurean[a,e,*], Derek D. Schramm[c], Elaine L. Jacobson[a], Ihab Halaweish[e], Geza G. Bruckner[b,c], Gilbert A. Boissonneault[b,c,d]

[a]*Department of Pharmacology and Toxicology, College of Pharmacy, Arizona Cancer Center, University of Arizona, Tucson, AZ 85724, USA*
[b]*Department of Clinical Sciences/Clinical Nutrition, University of Kentucky, Lexington, KY 40536, USA*
[c]*Graduate Center for Nutritional Sciences, University of Kentucky, Lexington, KY 40536, USA*
[d]*Lucille P. Markey Cancer Center, University of Kentucky, Lexington, KY 40536, USA*
[e]*Chemistry and Biochemistry Department, South Dakota State University, Brookings, SD 57006, USA*

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Abstract

Several lines of evidence suggest that rutin, flavonoid in fruits and vegetables, or one of its metabolites may effectively modulate advanced glycation end product (AGE) formation. Following ingestion, rutin forms metabolites that include 3,4-dihydroxyphenylacetic acid (3,4-DHPAA), 3,4-dihydroxytoluene (3,4-DHT), m-hydroxyphenylacetic acid (m-HPAA), 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA) and 3,5,7,3′,5′-pentahydroxyflavonol (quercetin). We studied the effects of rutin and its metabolites on the formation of AGE biomarkers such as pentosidine, collagen-linked fluorescence, N^{ϵ}-carboxymethyllysine (CML) adducts, glucose autoxidation and collagen glycation, using an in vitro model where collagen I was incubated with glucose. Rutin metabolites containing vicinyl dihydroxyl groups, i.e., 3,4-DHT, 3,4-DHPAA and quercetin, inhibited the formation of pentosidine and fluorescent adducts, glucose autoxidation and glycation of collagen I in a dose-dependent manner, whereas non-vicinyl dihydroxyl group-containing metabolites, i.e., HVA and m-HPAA, were much less effective. All five metabolites of rutin effectively inhibited CML formation. In contrast, during the initial stages of glycation and fluorescent AGE product accumulation, only vicinyl hydroxyl group-containing rutin metabolites were effective. These studies demonstrate that rutin and circulating metabolites of rutin can inhibit early glycation product formation, including both fluorescent and nonfluorescent AGEs induced by glucose glycation of collagen I in vitro. These effects likely contribute to the beneficial health effects associated with rutin consumption.

1. Introduction

Glycation is a reversible, nonenzymatic reaction of the aldehyde groups of reducing sugars with amino groups of proteins, forming Schiff bases that are subsequently rearranged to more stable ketoamines (Amadori rearrangement) [1]. Such ketoamines lead, through oxidation, to the formation of a variety of fluorescent and nonfluorescent advanced glycation end products (AGEs) associated with numerous pathologies. Pentosidine and N^{ϵ}-carboxymethylysine (CML) are AGEs that are increased in skin collagen I during both intrinsic aging and diabetes [2]. Pentosidine is an example of a fluorescent AGE, whereas CML is nonfluorescent. Advanced glycation end products formed by nonoxidative mechanisms, methylglyoxal and 3-deoxyglucosone, also occur, forming structures such as N^{ϵ}-carboxyethyllysine, pyrraline and imidazolone [3]. A variety of sugars including glucose, glucose autoxidation products (arabinose and glyoxal) and pentoses contribute to AGE formation [4–6]. In addition to sugars, oxidation of lipids and amino acids can result in CML formation, e.g., during inflammation [7].

Advanced glycation end products accumulate on long-lived proteins such as collagen [8,9], altering structural, biochemical and physical properties. The concentration of AGE-modified collagen increases in tissues with

Abbreviations: AGEs, advanced glycation end products; CML, N^{ϵ}-carboxymethylysine; 3,4-DHPAA, 3,4-dihydroxyphenylacetic acid; 3,4-DHT, 3,4-dihydroxytoluene; m-HPAA, m-hydroxyphenylacetic acid; HVA (homovanillic acid), 3-methoxy-4-hydroxyphenylacetic acid; OPA, *ortho*-phthaldehyde; PBS, phosphate buffer saline.

* Corresponding author. Chemistry and Biochemistry Department, South Dakota State University, Box 2202, Brookings, SD 57007, USA. Tel.: +1 605 6886732; fax: +1 605 6886364.

E-mail address: daniel.cervantes@sdstate.edu (D. Cervantes-Laurean).

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increasing age and may contribute to the reduction in elasticity of artery, heart and lung tissues that occurs as organisms age [1,10–12]. In turn, an AGE-related reduction in elasticity may lead to the age-dependent decline in physiological functions such as cardiac index, vital capacity, renal blood flow and maximum oxygen uptake. Additional clinical conditions possibly accelerated by AGEs include neuropathy, nephropathy, retinopathy, joint stiffness, senile cataracts, Alzheimer's disease and cardiovascular disease [13].

Protein fluorescence at 370–440 nm is an estimate of AGE-related protein damage [1,9,11,14]. A specific AGE with these fluorescence properties has been reported as vesperlysine A [15]. In 1990, Odetti et al. [16] identified aminoguanidine and rutin, a flavonol, as inhibitors of hyperglycemia-induced collagen-linked fluorescent adduct formation in streptozotocin-induced diabetic rats. Mechanisms by which aminoguanidine could inhibit hyperglycemia-induced collagen-linked fluorescent adduct formation were discussed [16]. Recently, the inhibition of AGEs in tissue proteins of streptozotocin-induced diabetic rats by rutin and a rutin derivative has been described [17,18]. Rutin is a common dietary flavonoid that is consumed in fruits, vegetables and plant-derived beverages such as tea and wine. Little or no dietary rutin is absorbed intact since gut microflora metabolize rutin to a variety of compounds that may be absorbed [19]. Rutin's aglycone, quercetin and the monophenols 3,4-DHT, 3,4-DHPAA, m-HPAA and HVA have been identified in the blood of animals following oral administration of rutin (Fig. 1) [19,20].

To elucidate the possible mechanisms of rutin's action, this study was conducted to establish the relative efficacy of various flavonoids and rutin metabolites as inhibitors of glucose autoxidation, glycation and specific AGE formation, estimated as CML and pentosidine formation and glucose-induced collagen-linked fluorescence, under conditions of hyperglycemia using glucose and type I collagen as in vitro models. The data presented here show that, in addition to rutin itself, its common biological metabolites effectively inhibited the formation of AGE biomarkers, glucose autoxidation and glycation.

2. Materials and methods

2.1. Materials

Type I collagen, phosphate buffers, Folin & Ciocalteu's reagent, bovine serum albumin, 2,4,6,-trinitrobenzenesulfonic acid, sodium borate, sodium azide, HCl, NaOH, copper sulfate, Na/K tartrate, sodium dodecyl sulfate, glucose, 3,4-dihydroxytoluene (3,4-DHT), 3,4-dihydroxyphenylacetic acid (3,4-DHPAA), 3-methoxy-4-hydroxyphenylacetic acid (HVA), m-hydroxyphenylacetic acid (m-HPAA), sp-Sephadex C25, Dowex 2X8-100 and *ortho*-phthaldehyde (OPA) were purchased from Sigma (St. Louis, MO). Quercetin was purchased from ICN Biochemicals (Costa

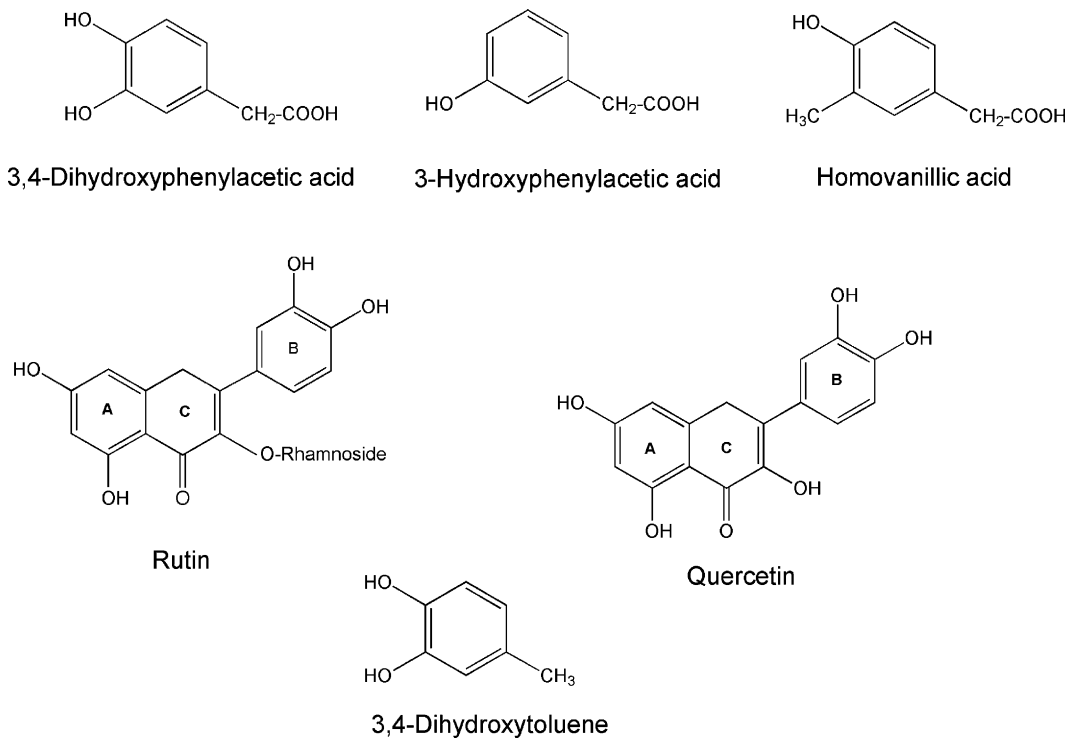

Fig. 1. Structures of rutin and rutin metabolites.

Mesa, CA). A μBondapak C18 reverse-phase column and an Xterra C18 column were purchased from Waters (Milford, MA).

2.2. Synthesis of standard CML and ^{14}C-CML

The preparation of a CML standard was as follows: iodoacetamide (27.7 mg/ml; 150 mM) was added to 1 ml of 100 mM Na_2CO_3 pH 11.3 containing 5 mg of *N*-acetyl lysine (~20 mM). The mixture was incubated for 24 h at 37°C. The reaction mixture was then hydrolyzed with 6 M HCl at 110°C for 12 h under nitrogen in 12×32-mm screw-top test tubes. N^{ε}-Carboxymethylysine was purified from the mixture using sp-Sephadex C25 and Dowex 2X8.

To prepare ^{14}C-CML, 846 nmol (50 μCi) of ^{14}C-iodoacetamide and 2 mg of *N*-acetyl lysine (~10 μmol) were mixed in 100 μl of 1 M Na_2CO_3 (pH 11.5) for 24 h at 37°C. The reaction mixture was hydrolyzed with 6 M HCl at 110°C for 12 h. ^{14}CML was purified using the combination of sp-Sephadex C25 (40–125 μm) and Dowex 2X8-100 (cross-linkage 8%, 50–100 dry mesh). The preparation of a quantitative standard CML solution was as follows: iodoacetamide (54.4 mg/ml; 300 mM) was added to 1 ml of 100 mM Na_2CO_3, pH 11.3 containing 20 mg of hippuryllysine (66 mM). The mixture was incubated for 24 h at 37°C and monitored by high-performance liquid chromatography (HPLC; C18 μBondapak column, 3.9×300 mm, particle size 10 μm, pore size 125 Å) using 50 mM ammonium formate, pH 6.0 and 10% methanol (v/v). The flow rate was 1 ml/min, and the detection was performed using an ISCO UV detector at 254 nm. The product of the reaction was isolated and lyophilized. The absorbance at 240 nm was used to calculate the concentration of CML hippuryllysine adduct. The sample was then hydrolyzed with 6 M HCL at 110°C for 12 h and reconstituted with water.

2.3. Glucose autoxidation

Glucose (50 mM) was incubated in PBS, 0.01% sodium azide (w/v) at 37°C for 10 days. Aliquots were assayed for glyoxal in a manner similar to that used by Wells-Knecht et al. [6]. Briefly, 100 μl of sample, 800 μl of 0.5N sodium formate (pH 2.9) and 100 μl of 0.5N Girard-T (pH 2.9) were incubated at 37°C for 30 min. Absorbance at 294 nm was read on a Milton Roy Genesys 5 spectrophotometer, and sample concentrations were calculated using a standard curve for glyoxal.

2.4. Collagen glycation

Type I collagen (1 mg/ml) was incubated in PBS with 50 mM glucose, 0.01 sodium azide, 1.5 μCi D(U-^{14}C) glucose. Samples were incubated in Sarsedt 75×12-mm polyethylene tubes for 5 days in a Forma Scientific incubator at 37°C. After 5 days, samples were removed and loaded to a Sepharose PD 10 columns equilibrated with 0.1 M sodium acetate (pH 5.0) from which glycosylated protein containing only Amadori products was eluted. Incorporation of radioactivity in rutin metabolite treatment groups was compared to control groups. After conversion of radioactivity to milligram of glucose incorporated, data were expressed as glucose (mg)/collagen (g).

2.5. CML isolation and quantification

Two consecutive 1 ml anion (sulfonic acid) and cation (ammonium) exchange resins were used. The sample was dissolved in 10 ml of 200 mM formic acid and applied to a previously equilibrated 1-ml sp-Sephadex resin. The sample was washed two times each with 200, 100, 50, 10 mM formic acid solutions and eluted with 10 ml of 25 mM ammonium formate, pH 10.5. This solution was applied to a 1-ml Dowex X-100 cation resin previously equilibrated with 2 M potassium hydroxide. The column was washed twice with 10 ml of 25 mM ammonium formate, pH 10.5, and twice with 10 ml of 12.5 mM ammonium formate, pH 9.5. Then, 10 ml of water was added to the column, and the sample was eluted with 5 ml of 100 mM HCl and 5 ml of 25 mM HCl. Ten milliliters more of 25 mM HCl was added, and the aliquots pooled in a 50-ml tube. The collected sample was lyophilized and dissolved in water. An aliquot of ^{14}CML was used to monitor recovery.

A Varian ProStar 230 HPLC was used to analyze CML and lysine content. The HPLC conditions for CML analysis were as follows: a C18 reverse-phase column Xterra (3.0×250 mm, particle size 5 μm) was used. A 50 mM NaCl solution containing 1% sodium azide and 10% methanol was used as a running buffer at a flow rate of 1ml/min. The column was heated to 37°C, and the sample was monitored with an Agilent fluorescent detector at 340/450 nm. Standard amino acid analysis was used to determine lysine content by OPA derivatization using a C18 μBondapak column (3.9×300 mm, particle size 10 μm, pore size 125 Å) [21]. The flow rate was 2 ml/min, and the sample was monitored in the same manner as described for CML.

2.6. Glucose-induced collagen-linked fluorescent adduct and pentosidine formation

Collagen suspensions were generated by stirring 100 mg type I collagen into 50 ml of sterile PBS for 12 to 16 h at 45°C. Suspensions were centrifuged at 3000×*g* for 10 min, and the supernatants were collected. Multiple supernatants were combined where necessary and assayed for total protein content.

Glucose was added to control samples at a final concentration of 50 mM. Treatment groups received 50 mM glucose plus a rutin metabolite (quercetin, m-HPAA, 3,4-DHPAA, 3,4-DHT or HVA). Rutin metabolites were dissolved in phosphate buffer and tested at concentrations up to 150 μM. Negative control groups were employed: a negative control lacking glucose to test for fluorescence native to collagen incubated in PBS, and control groups containing rutin metabolites but no glucose to account for fluorescence native to collagen when incubated with test compounds. The collagen concentration used was 1 g/L. Reactions were incubated at 37°C for 16 days. Glucose-

induced collagen-linked fluorescent adduct formation was measured as fluorescence (335/385 and 370/440 nm) on a Hitachi F2000 fluorometer and expressed as arbitrary units of fluorescence/mg protein.

2.7. Pentosidine quantification

Pentosidine formation was quantified using a modification of the method of Dyer et al. [22]. Samples were dialyzed against phosphate buffer at a ratio of 1000:1 for 24 h, changing the dialysate every 8 h. Samples were concentrated (Jouan Speed Vac, RCT 60/RC 1010), then hydrolyzed in 6 M HCl for 12 h at 110°C, and the sample was purged with nitrogen and sealed. After hydrolysis, hydrochloric acid was removed by evaporation (Jouan Speed Vac, RCT 60/RC 1010), water was added and the sample was neutralized by the addition of NaOH. An aliquot containing approximately 100 μg collagen normalized by hydroxyproline content was analyzed for pentosidine by reversed-phase HPLC. The 100-μg samples averaged 16% hydroxyproline and 3% lysine (amino acid content of collagen samples was determined by P.C. Andrews, Ph.D., director of the University of Michigan's Protein and Carbohydrate Structure Facility).

HPLC was performed on a Waters Nova-Pak C-18 reverse-phase column using a Varian 9010 solvent delivery system, a Varian Fluorichrom fluorometer and a Varian Aerograph D2 lamp. Flow rate was 0.8 ml/min. Solvent A was 0.1% trifluoroacetic acid, and solvent B 100% acetonitrile. A step gradient was formed by adjusting the volumes of solutions A and B at the times indicated: from 0 to 87% A for 85 min; from 87% to 100% A for 20 min; and 100% for 15 min. A pentosidine standard was purchased from Dr. Vincent Monnier, Case Western Reserve University, Cleveland, OH. The pentosidine standard was detected by fluorescence at ex 325/em 385 nm and eluted at approximately 67 min.

2.8. Statistical analyses

Significance ($P \leq .05$) for all assays was determined by ANOVA and Student's *t*-test on Microsoft Excel, correlation by Pearson correlation on SYSTAT and IC_{50} values by regression on SPSS.

Table 1
Inhibition of glucose-induced collagen-linked fluorescent adduct formation by rutin metabolites[a]

	IC_{50} values (μM)[b]	
	370/440 nm	335/385 nm
Rutin	60	16
3,4-DHPAA	68	22
3,4-DHT	77	22
Quercentin	65	18
HVA	>150	>150
m-HPAA	>150	>150

[a] Type I collagen (1 mg/ml) was incubated in PBS (pH 7.4) containing 50 mM glucose and 0.01 sodium azide for 30 days at 37°C. Metabolites were tested up to 150 μM.

[b] IC_{50} values were calculated by regression analysis of dose–response curves.

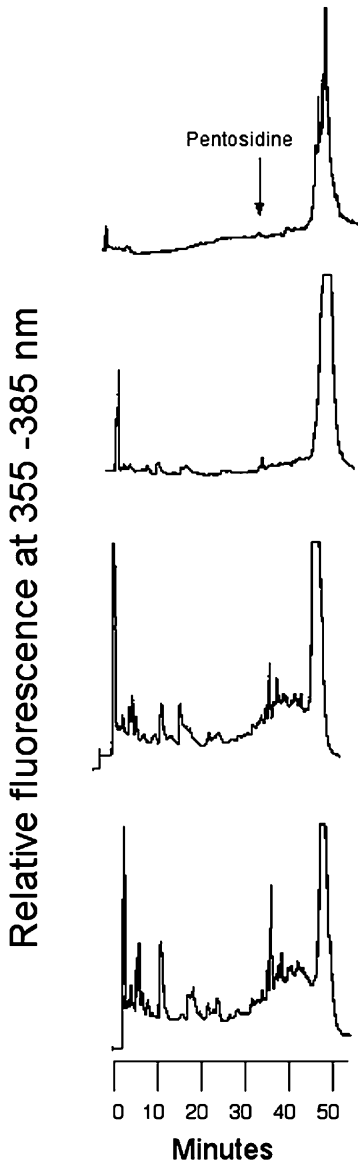

Fig. 2. Pentosidine formation monitored by HPLC during collagen glycation by glucose. Type I collagen (1 mg/ml) was incubated in PBS containing 50 mM glucose, glucose and 0.01% sodium azide for 10, 20 and 30 days at 37°C. An aliquot was loaded onto reverse-phase HPLC using a fluorescent detector at 335/385 nm. A standard of pentosidine was used to identify the corresponding peak in the protein digest. Representative chromatograms illustrate the accumulation of pentosidine over this period ($n = 3$.).

3. Results

3.1. Fluorescence inhibition by rutin metabolites on collagen incubated with glucose

Odetti et al. [16] demonstrated that streptozotocin-induced diabetic rats had increased skin collagen fluorescence indicative of glycation and that aminoguanidine or

rutin provided protection against formation of these fluorescent adducts. These investigators suggested that this effect was related to the possible inhibition of aldose reductase activity. In the studies described here, a possible alternative explanation for the inhibitory effect of rutin or its metabolites was tested. Since rutin is readily converted in the small intestine to a number of metabolites, the ability of rutin and selected rutin metabolites to inhibit glucose-induced collagen fluorescence using concentrations of glucose relevant to hyperglycemia was quantified [23]. Fluorescence at 370/440 nm was used to measure general monosaccharide-induced protein damage of the AGE type. Fluorescence at 335/385 nm was used to measure monosaccharide-induced protein damage of the pentosidine type [4]. The results in Table 1 show that rutin and its metabolites potently inhibited AGE fluorescence formation of both the general and pentosidine-like types. Rutin and its metabolites containing vicinyl dihydroxyl groups, i.e., quercetin, 3,4-DHPAA and 3,4-DHT, inhibited the formation of collagen-linked fluorescence by glucose-induced glycation in a dose-dependent manner with mean values ranging from 65 to 77 μM at 370/440 nm and 18 to 22 μM at 335/385 nm. Appropriate controls were used to establish that the metabolites tested were not fluorescence quenchers. The monophenols HVA and m-HPAA were not effective inhibitors of glucose-induced collagen fluorescence at either wavelength (>155 μM). Furthermore, we found that flavonoids with vicinyl dihydroxyl groups in the B ring such as rhamnetin, myricetin, fisetin, quercitrin and miricitrin inhibited fluorescence formation (335/385 nm) with IC_{50} values of 25, 20, 22, 19, 19 μM, respectively, thereby proving to be more potent inhibitors of glucose-induced collagen fluorescence formation than flavonoids such as galangin, kaempferol and kaempferol-7-neohespridoside, with IC_{50} values of 110, 99 and 131 μM, respectively.

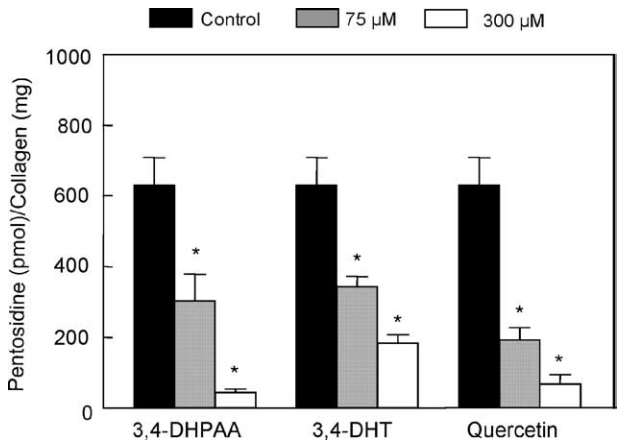

Fig. 3. Pentosidine inhibition by vicinyl dihydroxyl group containing rutin metabolites during collagen glycation by glucose. Type I collagen (1 mg/ml) was incubated in PBS containing 50 mM glucose and 0.01% sodium azide at 37°C in the absence (black bars) or presence of 75 μM (white bars) and 300 μM (stripped bars) of 3,4-DHPAA, 3,4-DHT, quercetin. Pentosidine was quantified after an incubation of 30 days by reverse-phase HPLC. *Indicates statistically significant difference ($P<.01$) ($n=5$.).

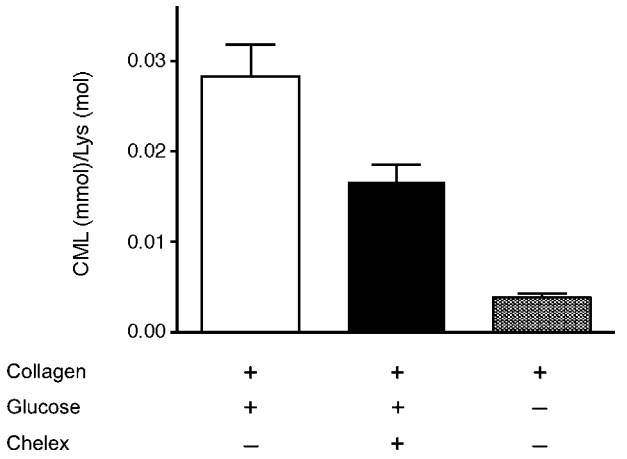

Fig. 4. CML formation during glycation of collagen I by glucose under metal chelation conditions. Type I collagen (1 mg/ml) was incubated in PBS with and without chelex in the absence or presence of 50 mM glucose and 0.01% sodium azide at 37°C for 30 days. Samples were processed as described under Materials and Methods.

The latter compounds contain one dihydroxyl group on the B ring. These results indicate the importance of vicinyl dihydroxyl groups on the ability of rutin metabolites to inhibit glucose-induced collagen-linked fluorescence formation, likely related to the well-documented increased radical scavenging activity provided by vicinyl dihydroxyl groups in phenolic and polyphenolic flavonoids [24]. Rutin metabolites containing vicinyl dihydroxyl groups also inhibited glycation-induced protein fluorescence formation by sugars other than glucose, e.g., ADP-ribose, a potent glycation pentose, on histones (data not shown) [5,25–27]. Diethylenetriaminepentaacetic acid, a potent transition metal chelator, did not inhibit the formation of fluorescence at 370–440 and 335–385 nm induced by ADP-ribose glycation of histones (data not shown). However, metabolites containing vicinyl dihydroxyl groups were effective inhibitors (data not shown). Although inhibition by rutin metabolites of fluorescence may involve different mechanisms according to the sugar source, at least some mechanisms of inhibition of AGE fluorescence do not involve metal chelation. Inhibition of fluorescence may involve trapping of reactive dicarbonyl and reactive oxygen species [28].

3.2. Rutin metabolite inhibition of pentosidine formation

Since AGE fluorescence represents a broad spectrum of protein modifications, the formation of a specific AGE, pentosidine, in collagen incubated with 50 mM glucose was studied. Pentosidine formation in collagen increased from 0.11 pmol/mg of collagen at Day 0 to 881 pmol of pentosidine/mg of collagen at Day 30 (Fig. 2).

Based on fluorescence data shown in Table 1, we hypothesized that rutin metabolites containing vicinyl dihydroxyl groups would inhibit the formation of pentosidine, an important fluorescent AGE found to increase with age and diabetic complications [24,28]. Two concentrations

Table 2
CML inhibition by rutin metabolites during collagen glycation by glucose[a]

	% CML inhibition			
	75 μM		300 μM	
Chelex	–	+[b]	–	+[b]
Control	0	57	0	57
3,4-DHPAA	86.9±2.1	85.3±1.5	96.7±1.5	92.3±3.0
3,4-DHT	93.9±0.6	84.1±4.2	93.4±2.2	93.0±2.0
Quercetin	87.9±2.1	76.9±3.1	90.1±3.3	89.1±0.7
m-HPAA	89.0±2.7	68.8±3.9	94.0±2.0	85.7±4.1
HVA	95±1.3	74.3±4.2	96.7±1.1	88.5±5.0

[a] Type I collagen (1 mg/ml) was incubated in PBS containing 50 mM glucose and 0.01% sodium azide at 37°C for 30 days with and without chelex in the absence or presence of 75 or 300 μM of 3,4-DHT, quercetin, m-HPAAm or HVA.

[b] Percentage of CML inhibition was calculated using as a control the incubation of glucose and collagen.

of rutin metabolites, 75 and 300 μM, were chosen based on the IC_{50} values for inhibition of pentosidine-like fluorescence. Each of the three rutin metabolites containing vicinyl dihydroxyl groups, 3,4-DHPAA and 3,4-DHT, and quercetin was an effective inhibitor of pentosidine formation, as was the flavonoid quercetin (Fig. 3). In contrast, rutin metabolites containing non-vicinyl dihydroxyl groups, i.e., HVA and m-HPAA, were not inhibitors (data not shown).

3.3. Rutin metabolite inhibition of CML formation

N^{ε}-Carboxymethylysine is another important specific AGE biomarker that increases in skin collagen in diabetes and aging [2,29] and that correlates with the severity of diabetic complications in tissue proteins [30]. N^{ε}-Carboxymethylysine, a nonfluorescent AGE, is formed during glucose glycation of proteins via three different pathways. These include (1) oxidative cleavage of the Amadori product [30] (Hodge pathway), (2) autoxidation of glucose [31–33] (Wolff pathway) and (3) Schiff base retro aldol cleavage (Namiki pathway) [34]. We evaluated whether rutin metabolites could inhibit CML formation during collagen I glycation by glucose.

Fig. 4 shows that there was a significant increase in the CML content of collagen incubated with glucose, although some CML was observed in native collagen I, suggesting its in vivo formation. Two concentrations of rutin metabolites were used (75 and 300 μM). These concentrations were chosen based on the IC_{50} values for vicinyl dihydroxyl group-containing metabolites shown to inhibit fluorescent adduct formation (Table 1). At 75 μM, all five rutin metabolites tested inhibited CML formation by at least 87% (Table 2). Inhibition of CML formation by rutin metabolites was more effective than inhibition of pentosidine formation (Fig. 3 and Table 2).

It is known that transition metals and oxygen are required for CML formation following protein glycation by glucose. In order to test only the effect of metal chelation on the formation of CML during the glycation of collagen I, chelex, a metal chelating agent, was used (Fig. 4). Unexpectedly, we detected CML formation in the presence of chelex, although the amount was significantly reduced compared with glucose-induced collagen I glycation in the absence of chelex.

We next tested whether rutin metabolites have any effect on the formation of CML during glucose-induced glycation of collagen under conditions of metal chelation. A dose-related inhibition of CML was observed by rutin metabolites at 75 and 300 μM (Table 2). These data show that the inhibition by rutin metabolites containing vicinyl dihydroxyl groups in the presence of chelex at 75 μM was as effective as without chelex. However, metabolites containing non-vicinyl dihydroxyl group were less effective in inhibiting CML formation with chelex when compared to conditions without chelex. The percentage of inhibition of CML by all rutin metabolites at 300 μM was similarly effective in the presence or absence of chelex. Since chelex alone inhibited CML formation by 57% and rutin metabolites increased the inhibition to approximately 90%, the inhibition of CML observed by rutin metabolites must involve mechanism(s) other than metal chelation.

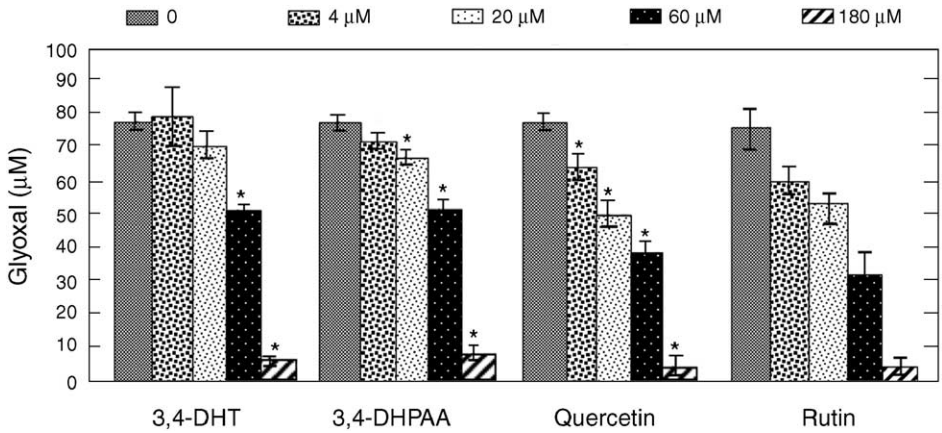

Fig. 5. Inhibition of glucose autoxidation by rutin metabolites. PBS containing 50 mM glucose and 0.01% sodium azide were incubated for 10 days at 37°C. Glyoxal concentrations were measured as described under Materials and Methods. Vicinyl dihydroxyl group containing rutin metabolites 3,4-DHT, 3,4-DHPAA and quercetin were added at 0, 4, 20, 60 and 180 μM.

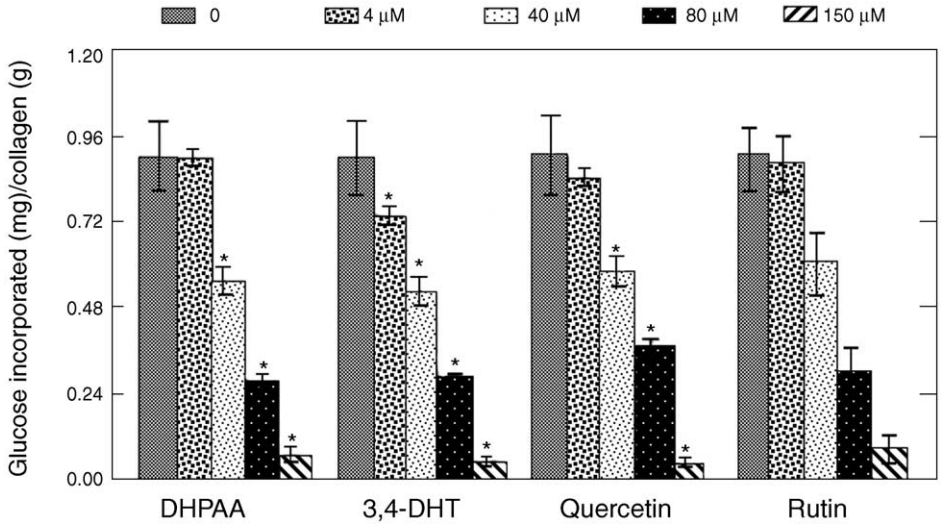

Fig. 6. Inhibition of glycation by rutin metabolites. Type I collagen (1 mg/ml) was incubated in PBS containing 50 mM glucose and 0.01% sodium azide at 37°C for 5 days. Vicinyl dihydroxyl group containing rutin metabolites 3,4-DHT, 3,4-DHPAA and quercetin were added at 0, 4, 40, 80 and 150 μM. Neither HVA nor m-HPAA inhibited glycation of collagen type I (data not shown). *Significance at $P \leq .05$, compared with 0 (control).

3.4. Inhibition of early adduct formation by rutin and its metabolites

We tested whether rutin and its metabolites could inhibit early adduct formation during glucose autoxidation and glycation that could contribute to the formation of AGEs. Autoxidation of glucose has been reported to contribute to the formation of reactive intermediates which are able to react with protein lysine residues to form AGEs, e.g., CML. Glyoxal and arabinose are two key intermediates formed during glucose autoxidation. Therefore, glyoxal formation was measured to monitor the extent of glucose autoxidation and the effect of rutin metabolites on this process as shown in Fig. 5. While rutin and its metabolites containing vicinyl dihydroxyl group, quercetin, 3,4-DHT and 3,4-DHPAA inhibited the formation of glyoxal with IC_{50} values of 70, 92, 97 and 100 μM, respectively, metabolites containing non-vicinyl dihydroxyl groups had no effect (data not shown). Flavonoids containing vicinyl dihydroxyl groups were also effective inhibitors of autoxidation, e.g., rhamnetin, myricetin, fisetin, quercitrin and miricitrin, exhibiting IC_{50} values of 115, 33, 38, 45, and 68 μM, respectively, while flavonoids such as galangin, kaempferol and kaempferol-7-neohesperidoside containing non-vicinyl dihydroxyl groups did not inhibit autoxidation. It is known that metals are required for glucose autoxidation to form glyoxal and arabinose [28]. Therefore, it is possible that the inhibition of autoxidation of glucose by rutin metabolites may have been mediated via metal chelation and/or radical oxygen species quenching since both are involved in the oxidative degradation of glucose.

Recent reports demonstrated inhibition of glycation by rutin and a rutin derivative in tissue proteins from streptozotocin-induced diabetic rats [18,34]. Whether rutin metabolites affect the inhibition of glycation by glucose on collagen was tested by using ^{14}C-radiolabeled glucose labeled in all carbons and prepurified to remove reactive decomposition products as described by Edelstein and Brownlee [28]. After acid precipitation and gel filtration, Amadori products were measured by quantifying radioactive protein. It was calculated that 0.08% of the original glucose (50 mM) was incorporated into collagen type I in the glycation assays. Since it is likely that intermediates formed from autoxidation of glucose contribute to glycation, the amount of glyoxal and arabinose [6] was quantified. It was found that these two intermediates contributed only 2.2% of the radiolabel in glycated collagen. However, due to the minimal contribution of glyoxal and arabinose, glycation by molecules other than glucose is not likely to contribute significantly to glycation as measured. Vicinyl dihydroxyl group-containing rutin and rutin metabolites, DHPAA, 3,4-DHT, and quercetin, inhibited glycation with IC_{50} values of 58, 49, 47 and 55 μM, respectively (Fig. 6), while HVA and m-HPAA did not significantly inhibit at any dose or time tested (data not shown). We also observed that flavonoids containing two or three vicinyl dihydroxyl groups on the B ring such as fisetin, quercitrin, myricetin, myricitrin and rhamnetin inhibited type I collagen glycation with IC_{50} values of 62, 54, 52, 62 and 67 μM, respectively. Flavonoids containing non-vicinyl dihydroxyl groups on the B ring such as galangin, morin, kaempferol and kaempferol-7-neohesperidoside did not inhibit glycation at concentrations up to 150 μM (data not shown). These results taken together indicate that inhibition by rutin and its metabolites inhibit the formation of early products such as ketoamines (glycation) and glyoxal (autoxidation) as well as the formation of late fluorescent and nonfluorescent protein adducts such as pentosidine and CML (AGEs), respectively, under conditions of hyperglycemia.

4. Discussion

Flavonoids are dietary constituents consumed in fruits, vegetables and other plant-derived foods such as wine and tea. Their beneficial effects on cardiovascular disease and cancer risk are well documented. The observation by Odetti et al. [16] that rutin diminishes the accumulation of diabetes-induced skin collagen fluorescence in rats suggested that rutin or its metabolites might be responsible for this effect. Although the authors suggested that rutin might inhibit this fluorescence formation on skin collagen through the inhibition of aldose reductase, the mechanism of inhibition was not elucidated. In addition, a recent study by Nagasawa et al. [18,34] reported inhibition by rutin and a rutin derivative of N^{ϵ}-fructoselysine, an early glycation product, and AGEs using immunohistochemistry in tissue proteins from streptozotocin-induced diabetic rats. The current study reports the influence of rutin metabolites, specifically its aglycone quercetin and its monophenol derivatives, 3,4-DHPAA, 3,4-DHT, m-HPAA and HVA, on glucose-induced protein damage and on early to late stages of AGE formation.

Rutin and its metabolites, quercetin, 3,4-DHPAA and 3,4-DHT, containing vicinyl dihydroxyl groups were found to inhibit autoxidation of glucose more effectively than the non-vicinyl hydroxyl group-containing metabolites m-HPAA and HVA. The presence of vicinyl dihydroxyl groups was shown by others to affect the ability of phenols to inhibit iron and copper-catalyzed production of initiating radical species [35,36]. Thus, it is likely that metal chelation and/or free radical scavenging properties contribute to the inhibition of glucose autoxidation by rutin metabolites containing vicinyl dihydroxyl groups. Furthermore, our data show that flavonoids containing vicinyl dihydroxyl groups in the B-ring were also effective inhibitors of glucose autoxidation.

Rutin and its metabolites also inhibited glycation. Since early glycation, in particular ketoamine formation, does not involve reactive oxygen species or metal-catalyzed oxidation, the inhibition observed by rutin and its metabolites may involve mechanisms such as the trapping of reactive amino groups from type I collagen by the keto-quinone intermediates of rutin metabolites, making them unable to react with glucose. Inhibition of glycation was also observed with rutin and other flavonoids containing vicinyl dihydroxyl groups, further suggesting their importance. Inhibition of glycation in tissue proteins in streptozotocin-induced diabetic rats by rutin and a rutin derivative was also reported by Nagasawa et al. [18,34]. Nagasawa et al. [18,34] did not show whether the inhibition was due to rutin directly and/or to its metabolites, whereas the current study shows that rutin and its metabolites are effective inhibitors of glycation.

Since rutin and its metabolites containing vicinyl dihydroxyl groups were effective inhibitors of glucose-induced collagen-linked fluorescent adduct formation, we hypothesized that these metabolites would likewise inhibit the accumulation of pentosidine in glucose-exposed collagen. Pentosidine is an imidazo[4,5*b*]pyridinium ring formed by cross-linking sugars between lysine and arginine residues [22]. The data presented demonstrate a close relationship between the inhibition of pentosidine-like fluorescence (335/385 nm) and the inhibition of the formation of material comigrating with the pentosidine standard on HPLC analyses. This observation suggests a close relationship between the role of these rutin metabolites in inhibiting accumulation of pentosidine and pentosidine-like fluorescence.

Information that may help establish a possible mechanism for the inhibition of glucose-induced collagen fluorescence by vicinyl dihydroxyl group containing rutin metabolites and flavonoids comes from a recent study which showed that rutin, a rutin derivative and quercetin, all of which contain vicinyl dihydroxyl groups, inhibited the formation of AGEs in tissue proteins and that this inhibition was similar to that induced by aminoguanidine, a known inhibitor of glycation that traps reactive carbonyl species such as glyoxal and 3-deoxyglucosone [37]. Each of these three flavonoid compounds reduced protein carbonyl accumulation. Additionally, in the present study, the formation of fluorescent adducts and pentosidine, using ADP-ribose as the glycating sugar to modify histone H1, did not depend on the presence of metals and was inhibited by aminoguanidine (data not shown). Likewise, their formation was strongly inhibited by vicinyl dihydroxyl group-containing metabolites (data not shown). The combined data suggest that rutin metabolites, which contain vicinyl dihydroxyl groups, may inhibit fluorescent adduct and pentosidine formation by trapping reactive oxygen and carbonyl intermediates.

The formation of CML was inhibited with equal effectiveness by both vicinyl and non-vicinyl dihydroxyl group containing rutin metabolites, unlike that observed for the formation of pentosidine and fluorescent adducts. These data support the argument that the formation of fluorescent (i.e., pentosidine) and nonfluorescent (i.e., CML) adducts involves different metabolic intermediates and that these are differentially sensitive to rutin metabolites. The inhibition of CML formation is very complex since rutin metabolites could interfere at different stages, from the autoxidation reaction to the oxidative degradation of Amadori products and the retro aldol condensation of the Schiff base adducts. Furthermore, rutin metabolites could scavenge reactive oxygen species (superoxide and H_2O_2), and/or the reactive carbonyl species, glyoxal, making it difficult to pinpoint at what level(s) rutin metabolites are inhibiting the formation of CML. Our data also show that CML formation is inhibited to the same extent by 300 μM rutin metabolites in the presence or absence of the metal chelator chelex, suggesting a mechanism independent of metal chelation.

Glucose is hypothesized to induce much of the extracellular monosaccharide-induced in vivo protein damage noted with diabetes [38,39]. The blood glucose concentration in normoglycemic humans is approximately 5 mM. If the ratio of rutin metabolite to monosaccharide (0.001) examined in this study is a good measure of an effective inhibitory

concentration, then only micromolar concentrations of rutin metabolites may be necessary for efficacy in vivo. To achieve a ratio of rutin metabolite to monosaccharide in vivo that is similar to that required for 50% inhibition of fluorescent adduct formation in our in vitro glucose-induced collagen-linked fluorescent adduct formation assay, the total blood concentration of active metabolites would need to be approximately 5 μM; however, rutin metabolite association with albumin would be expected to increase required concentrations. Recent studies looking at the pharmacokinetics of rutin and its aglycone, quercetin, have reported blood concentrations in human volunteers of up to 3.5 μM of quercetin when doses of 50 mg of either rutin or quercetin were used in their diets [40]. Since our data suggest that micromolar concentrations of rutin metabolites would reduce monosaccharide-induced protein damage, and flavonoid intake has been estimated to be as high as 1 g/day [41], and that plasma levels of such flavonoids are markedly increased following consumption [42], dietary consumption of flavonoids may offer some protection against glucose-induced protein damage. It should be mentioned that recent studies with flavonoids such as rutin and quercetin have demonstrated intracellular uptake, although the mechanism(s) involved remain unknown [18,43].

The results of our investigation suggest that rutin and its vicinyl dihydroxyl group-containing metabolites which are present in body fluids after oral administration of rutin may inhibit autoxidation, glycation and hyperglycemia-induced collagen-linked fluorescent adduct formation as observed by Odetti et al. [16,44] and in Nagasawa's recent studies [18,34]. In addition, polyphenol containing green tea extracts have been shown to delay the appearance of diabetic complications in rat models [45]. Rich polyphenol containing extracts from the plant *Ilex paraguariensis* have been shown to inhibit the formation of AGEs using an in vitro experimental model [46]. Although only a few thousand flavonoids have been identified, there are more than 20 million possible flavonoid family members [47]. Furthermore, flavonoid conjugates and monophenols such as flavonoid glucuronides, phenylacetic acids, phenylpropionic acids and phenyl-γ-valerolactones can arise during the metabolism of each flavonoid [48,49]. Since up to 1 g of mixed metabolites may be consumed daily in the human diet, these compounds are likely to be important in the health-promoting effects of consuming a diet high in plants and plant products [50] and may contribute to the protective effect observed by consumption of wine in the so-called French paradox diet. The characterization of flavonoid metabolites, metabolite biochemical effects and metabolite structure–function relationships will lead to a better understanding of the role these compounds have in disease prevention and progression.

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