Quercetin and Its Metabolites Inhibit Recombinant Human Angiotensin-Converting Enzyme 2 (ACE2) Activity

Xiaocao Liu, Ruma Raghuvanshi, Fatma Duygu Ceylan, and Bradley W. Bolling*

ABSTRACT: Angiotensin-converting enzyme 2 (ACE2) is a host receptor for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Inhibiting the interaction between the envelope spike glycoproteins (S-proteins) of SARS-CoV-2 and ACE2 is a potential antiviral therapeutic approach, but little is known about how dietary compounds interact with ACE2. The objective of this study was to determine if flavonoids and other polyphenols with B-ring 3’,4’-hydroxylation inhibit recombinant human (rh)ACE2 activity. rhACE2 activity was assessed with the fluorogenic substrate Mca-APK(Dnp). Polyphenols reduced rhACE2 activity by 15–66% at 10 μM. Rutin, quercetin-3-O-glucoside, tamarixetin, and 3,4-dihydroxyphenylacetic acid inhibited rhACE2 activity by 42–48%. Quercetin was the most potent rhACE2 inhibitor among the polyphenols tested, with an IC50 of 4.48 μM. Thus, quercetin, its metabolites, and polyphenols with 3’,4’-hydroxylation inhibited rhACE2 activity at physiologically relevant concentrations in vitro.

KEYWORDS: quercetin, angiotensin-converting enzyme 2, enzyme kinetics, polyphenols

INTRODUCTION

Human angiotensin-converting enzyme 2 (ACE2) is an 805 amino-acid transmembrane protein that contains an extracellular domain with a typical HEMGH metalloproteinase zinc-binding site.12 ACE2 is localized at the lung alveolar epithelial cells, arterial and venous endothelial cells, renal tubular epithelium, and the epithelia of the small intestine.3−5 This protein acts as monocarboxypeptidase that exclusively cleaves a single C-terminal residue from angiotensin II (Ang II), generating angiotensin-(1–7).6 It counterbalances the accumulation of Ang II formed by the action of the angiotensin-converting enzyme (ACE). The anti-inflammatory properties of ACE2 are mediated by the activation of the Mas receptor in the renin–angiotensin–aldosterone system (RAAS).7−9 More recently, ACE2 was identified as a receptor for S-proteins of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to infiltrate host cells.10−12 Inhibiting the interaction between S protein and the host ACE2 is predicted to inhibit SARS-CoV-2 infection.13−15

A preliminary computational molecular docking study identified quercetin, luteolin, and eriodictyol as potential inhibitors of the interaction between S protein and ACE2.16 It is predicted that polyphenols inhibit the entry of SARS-CoV-2 into cells by restraining the viral S protein—human ACE2 interface; however, detailed studies that describe the interaction of flavonoids with ACE2 are scarce. It is evident from earlier studies that the kaempferol and polyphenol-containing vegetable extracts inhibit rhACE2 activity.17 Quercetin, luteolin, and eriodictyol are structurally similar flavonoids having 3’,4’-hydroxylated B-rings. Therefore, we hypothesized that flavonoids and phenolic acids with B-ring 3’,4’-hydroxylation would inhibit ACE2 activity (Figure 1).

Since polyphenols are extensively metabolized upon consumption, we further evaluated the potential for quercetin metabolites to also inhibit rhACE2. In this study, we describe the validation of an rhACE2 assay and the extent these polyphenols act as rhACE2 inhibitors.
assay buffer used in the rhACE2 activity assay was made in-house, having 0.05 M 2-morpholinoethane-sulfonic acid (MES), 0.3 M NaCl, and 10 μM ZnCl₂, pH 6.8. It was stored at 4 °C when not in use. Phenol standards (quercetin, quercetin-3-O-glucoside, quercetin-3-O-galactoside, rutin, tamarixetin, isorhamnetin, (±)-eriodictyol, (−)-epicatechin, luteolin, and 3,4-dihydroxyphenylacetic acid) and nicotianamine were first dissolved in methanol with a concentration of 2 mM as stock solutions. A methanol vehicle was used as a background control. The rhACE2 enzyme, the McAPK(Dnp) substrate, and DX600 stock solutions were further diluted to conduct experiments in the absence of methanol, aliquots of these stock solutions were evaporated at room temperature and then reconstituted in the assay buffer. 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was used to determine the amount of the final product in pmol of Mca. In this study, 1 RFU = 40.09 pmol of Mca at 37 °C in the presence of 11.25 μM substrate. For routine testing, the rhACE2 enzyme activity of thawed protein aliquots was normalized. Briefly, the rhACE2 stock solution aliquot at 10 μg/mL was diluted in the assay buffer, then incubated with the substrate (final concentration: rhACE2 50 ng/mL, substrate 11.25 μM) for 10 min at 37 °C. Based on the catalytic product as a reference of 1.358 RFU for 50 ng/mL rhACE2 enzyme, the rhACE2 stock solution was then diluted to be equivalent to the reference activity.

Statistical Analysis. All incubations were conducted in triplicate with three independent experiments. Quantitative data are presented as the mean ± standard deviation (SD) or the standard error of the mean (SEM), as specified in the figure legends. Statistical tests were performed using GraphPad Prism 7.0. Two-sided Student’s t-tests were used for comparisons between two groups, whereas one-way ANOVA or two-way ANOVA with Tukey’s post hoc was used for comparisons among multiple independent groups. Significant differences were considered at P < 0.05.

RESULTS

Optimization of the rhACE2 Activity Assay. An rhACE2 protein dose response was established from 3.13 to 250 ng/mL with 11.25 μM substrate at 37 °C (Figure 2). After 10 min of incubation with the substrate, enzymatic activity declined (Figure 2A). At 10 min of incubation, the amount of the product formed was linear from 3.13 to 250 ng/mL rhACE2 (Figure 2B). Thus, a 10 min incubation period was used for subsequent experiments.

The intra- and inter-assay reproducibility were determined to test the robustness of the assay (Table 1). The intra-assay coefficient of variance (CV) for RFU with 25, 50, and 100 ng/mL of rhACE2 and the 11.25 μM substrate at 37 °C was 6.04–7.88%, whereas the inter-assay CV was 2.52–9.96%.

Quercetin Inhibits rhACE2 Activity. The reaction rate of 50 ng/mL rhACE2 in the presence of 5–100 μM quercetin was time- and concentration-dependent at 37 °C (Figure 3A). In contrast, the reaction rate of rhACE2 was consistent at 0.100 RFU/min (P > 0.05). After 2.5 min, 100 μM quercetin reduced rhACE2 activity to 0.004 ± 0.029 RFU/min (P < 0.0001 vs control), while 10 μM quercetin reduced activity to 0.048 ± 0.022 RFU/min (P = 0.0007 vs control). Incubation with 5 μM quercetin reduced rhACE2 activity from 2.5 to 8.5 min, but at 10.5 min, the difference became nonsignificant relative to the control (P > 0.05). The impact of time on rhACE2 inhibition was greatest for 5 μM quercetin (Figure 3B). To further describe the time dependency of inhibition, the quercetin IC50 value was determined at 2.5 and 10.5 min (Figure 3C,D). The rhACE2 IC50 of quercetin increased from approximately 4.48 μM after 2.5 min to 29.5 μM at 10.5 min.

Inhibition of rhACE2 was dependent on temperature (Figure 4). rhACE2 activity increased with temperature (Figure 4A). The amount of Mca formed at 10 min was 2.2-fold when temperature increased from 25 to 37 °C. At each temperature, 10 μM quercetin inhibited rhACE2 activity relative to the control (Figure 4B).

Kinetics of Enzyme Inhibition. A kinetic study was performed at increasing substrate concentrations to determine how rhACE2 activity is linked to catalytic efficiency. At the optimal reaction conditions (Figure 2A), activity over a 10 min incubation period was linear over varying protein concentrations, indicating that the reaction rate was stable over this period. The Michaelis–Menten constant Km and maximum velocity Vmax were determined by coinubcation of 0.7–90 μM of the substrate and 50 ng/mL rhACE2 in the presence of quercetin (10 μM), rutin (10 μM), or DX600 (1 μM), as a positive control (Figure 5, Table 2). Overall, these compounds decreased the affinity of rhACE2 to the substrate (e.g., higher Km values compared to the rhACE2 control) and lowered the velocity (with the exception of rutin), resulting in a decreased catalytic efficiency (Kcat/Km). DX600 reduced the maximum initial velocity (3.49 ± 0.66 pmol/min) by 74% compared with the ACE2 control (13.3 ± 0.5 pmol/min) and weakened the affinity of rhACE2 for the substrate with a 5.8-fold increased Km value and a reduced Kcat/Km value. A similar trend was also
observed with quercetin, as it had 28% lower $V_{\text{max}}$ (9.56 ± 0.36 pmol/min) and 1.7-fold increased $K_{\text{m}}$ compared to the control. For rutin, although the $K_{\text{m}}$ value was lower than rhACE2 alone, the maximum velocity was higher than the rhACE2 control. Thus, the $K_{\text{cat}}/K_{\text{m}}$ value for rutin was not similar to quercetin (Table 2).

Polyphenol Inhibition of rhACE2 Activity. Polyphenols with 3′,4′-hydroxylation and known quercetin metabolites (isorhamnetin, tamarixetin, quercetin-3-O-glucuronide, and 3,4-dihydroxyphenylacetic acid) were evaluated for rhACE2 inhibition. Nicotianamine, a natural product from soybeans, and an ACE inhibitor19 as well as DX600 were used as controls (Table 3). All of these polyphenols inhibited rhACE2 activity. The extent of rhACE2 inhibition was dependent on time. After 10 min of incubation, the inhibition was significantly decreased relative to 2 min for all polyphenols except (+)-eriodictyol. DX600, nicotianamine, and quercetin were more potent inhibitors than the other tested polyphenols at 2 min. However, at 10 min, quercetin inhibition of rhACE2 was less than that of DX600 and nicotianamine.

Figure 3. Quercetin inhibits rhACE2 activity. (A) rhACE2 velocity (ΔRFU/min) and (B) rhACE2 inhibition (% of control) in the absence or presence of 5–100 μM quercetin incubation with 50 ng/mL rhACE2, 11.25 μM substrate, for up to 10.5 min at 37 °C. For (A), rate differences were analyzed by two-way ANOVA, followed by Tukey’s multiple-comparison test. Incubation time accounted for 5.92% of total variation ($P = 0.0002$), whereas the quercetin concentration accounts for 78.2% with $P < 0.0001$, however their interaction was not significant ($P > 0.05$). Within each time interval, bars bearing different letters indicate significant differences ($P < 0.05$). For (B), the % inhibition was determined relative to the untreated control at each time point. Differences were assessed by one-way ANOVA, followed by Tukey’s multiple-comparison test among different quercetin concentrations at each time interval, *$P < 0.005$ compared to the control. For (A) and (B), the data were obtained by three independent experiments with triplicates and shown as means ± SDs. (C, D) Analysis of rhACE2 inhibition by quercetin concentration at 2.5 min (C) and 10.5 min (D). Data were fitted using a variable slope [inhibitor]-normalized response model in GraphPad Prism 7. Data are represented by three independent experiments with triplicates, and IC$_{50}$ values are means ± SEMs.

Figure 4. Temperature affects rhACE2 activity. (A) Temperature dependence on the time course of product formation with or without 10 μM quercetin. (B) Effect of temperature on product formation after 10 min of incubation with or without 10 μM quercetin. Assays were performed at 50 ng/mL rhACE2 enzyme concentration with 11.25 μM substrate. Statistical analysis was performed by one-way ANOVA, followed with Tukey’s multiple-comparison testing, ***$P < 0.0005$ and ****$P < 0.0001$. Differences between the treated and untreated samples are denoted by upper and lowercase letters, respectively. Data are means ± SDs and are of three experiments performed with triplicate samples.
DISCUSSION

Angiotensin-converting enzymes, ACE and ACE2, share considerable homology with 41.8% sequence identity at the catalytic domain, and both belong to the M2 family of metallopeptidases with HEMGH zinc-binding motifs as an amino-terminal catalytic domain.20 22 Structural and functional studies have revealed that flavonoids inhibit ACE because of the double bond between C2 and C3 on the C-ring; the ketone group of C4 at the C-ring; and the 3′,4′-catechol group in the B-ring.23 25 Although the ACE inhibition mechanism by flavonoids has not yet been fully understood, the catechol group in the B-ring may exert a charge-charge interaction with the Zn²⁺ ion in the ACE active site.21

Specifically, luteolin exhibits the highest capacity to inhibit ACE activity among 17 flavonoids with an IC₅₀ value of 23 μM, followed by quercetin with an IC₅₀ value of 43 μM.25

Under optimized assay conditions, quercetin inhibited rhACE2 activity by reducing its affinity for the Mca-AKP(Dnp) substrate and led to a lower catalytic efficiency (Kcat/Km). In addition, quercetin had mixed rhACE2 inhibition as it reduced Vmax and increased Km. Structurally related polyphenols also inhibited rhACE2. Quercetin metabolites (isorhamnetin, tamarixetin, 3,4-dihydroxyphenylacetic acid, and quercetin-3-glucuronide), rutinosides and glycosides of quercetin, and other flavonoids (luteolin, (±)-eriodictyol, and (−)-epicatechin) also inhibited rhACE2. Notably, polyphenols were less potent inhibitors than DX600. However, flavonoids still decreased Km and Kcat/Km values, indicating interaction with the ACE2 active site. Further analysis by computational simulation, X-ray crystallography, or NMR needs to be employed to define the specific interactions of flavonoids with 3′,4′-dihydroxylation and ACE2.

Quercetin is mainly distributed in foods as glycosides and rutinosides. It is metabolized by dehydroxylation, glucuronidation, sulfation, and methylation, or further catabolism by gut microbiota to phenolics such as 3,4-dihydroxyphenylacetic acid have been reported as high as 13989

Table 2. Steady-State Kinetic Parameters of 10 μM Rutin, 10 μM Quercetin, and 1 μM DX600 with rhACE²

| class | polyphenol | inhibition (%)² |
|-------|-----------|-----------------|
| flavonoids | quercetin | 66.2 ± 2.2²² |
| flavonoids | quercetin-3-O-glucoside | 47.7 ± 3.7²² |
| flavonoids | quercetin-3-O-galactoside | 34.2 ± 3.7²² |
| flavonoids | rutin | 48.3 ± 4.7²² |
| flavonoids | quercetin-3-O-glucuronide | 33.1 ± 4.9²² |
| flavonoids | tamarixetin | 41.5 ± 5.0²² |
| flavonoids | isorhamnetin | 14.7 ± 1.4²² |
| flavonoids | flavanone (±)-eriodictyol | 24.4 ± 1.4²² |
| flavonoids | flavanone (−)-epicatechin | 27.4 ± 5.7²² |
| flavonoids | flavones luteolin | 37.1 ± 0.6²² |
| phenolic acids | 3,4-dihydroxyphenylacetic acid | 42.1 ± 3.4²² |
| known ACE2 inhibitors | nicotianamine | 1.0 ± 0.3²² |
| known ACE2 inhibitors | DX600 | 67.7 ± 1.3²³ |

²²“Screening experiments were performed by co-inoculation of 50 ng/mL rhACE2 enzyme and 11.25 μM substrate at 37 °C for 2 or 10 min in the presence of 10 μM of compounds, except for 1 μM of DX600.

²³Statistical analysis was by one-way ANOVA with Dunnet post hoc test, with P < 0.05 considered significant, denoted by uppercase letters. Two-tailed t-tests were conducted for each inhibitor to evaluate differences by time, denoted by * at 10 min, where P < 0.05 was considered significant. Data are means ± SDs from three individual experiments with triplicates.

Table 3. Polyphenols Inhibit rhACE2 Enzyme Activity²²

| class | polyphenol | 2 min | 10 min |
|-------|-----------|-------|--------|
| flavonoids | quercetin | 66.2 ± 2.2²² | 38.1 ± 1.9²² |
| flavonoids | quercetin-3-O-glucoside | 47.7 ± 3.7²² | 20.9 ± 2.4²² |
| flavonoids | quercetin-3-O-galactoside | 34.2 ± 3.7²² | 12.9 ± 2.8²² |
| flavonoids | rutin | 48.3 ± 4.7²² | 14.5 ± 2.7²² |
| flavonoids | quercetin-3-O-glucuronide | 33.1 ± 4.9²² | 10.2 ± 3.3²² |
| flavonoids | tamarixetin | 41.5 ± 5.0²² | 19.6 ± 3.2²² |
| flavonoids | isorhamnetin | 14.7 ± 1.4²² | 12.2 ± 0.5²² |
| flavonoids | flavanone (±)-eriodictyol | 24.4 ± 1.4²² | 25.6 ± 0.8²¹ |
| flavonoids | flavanone (−)-epicatechin | 27.4 ± 5.7²² | 4.39 ± 3.06²⁵ |
| flavonoids | flavones luteolin | 37.1 ± 0.6²² | 26.1 ± 1.7²¹ |
| phenolic acids | 3,4-dihydroxyphenylacetic acid | 42.1 ± 3.4²² | 6.48 ± 2.61²³ |
| known ACE2 inhibitors | DX600 | 67.7 ± 1.3²³ | 80.3 ± 1.3²³ |

²²Statistical analysis was by one-way ANOVA with Dunnet post hoc test, with P < 0.05 considered significant, denoted by uppercase letters. Two-tailed t-tests were conducted for each inhibitor to evaluate differences by time, denoted by * at 10 min, where P < 0.05 was considered significant. Data are means ± SDs from three individual experiments with triplicates.
~100 μM.\(^\text{29}\) ACE2 is primarily a membrane-bound enzyme located in the heart, lungs, vasculature, kidneys, and oral and digestive tracts.\(^\text{30}\) In rats, quercetin and its methyl metabolites accumulate to nmol/g (dw) concentrations in the lung, kidney, heart, and muscle after consumption of 1% quercetin for 11 weeks.\(^\text{31}\) After a single dose of [2-\(^{14}\)C]quercetin-4′-glucoside to rats, 71% of the dose was recovered in the GI tract at 6 h, which later declined to 3.4% by 24 h.\(^\text{32}\) Thus, ACE2 inhibition is plausible in the oral cavity and digestive tracts.

Increased flavonoid consumption is associated with reduced mortality from cardiovascular diseases in older women.\(^\text{33}\) Meta-analyses of randomized controlled trials have associated the intake of >500 mg quercetin per day with reduced blood pressure.\(^\text{34,35}\) Yet the precise antihypertensive mechanism of quercetin is still not clear,\(^\text{36–38}\) partly due to the variation of administration dosage, experimental duration, type of experiment design, and participant population.\(^\text{39}\) In \textit{in vitro} studies have demonstrated that flavonoid-rich foods are capable of inhibiting ACE activity.\(^\text{40–42}\) Also, an acute hypertensive rat model supports the antihypertensive potential of quercetin through reduced plasma ACE activity.\(^\text{43}\) In contrast, ACE inhibition by flavonoids has not been observed in other human and animal studies.\(^\text{44–47}\) On the bias of homology between ACE and ACE2, it would be expected that inhibition of ACE2 by quercetin would be independent of its ability to modulate blood pressure. Further mechanistic studies are needed to define the impact of polyphenol ACE2 inhibition on the RAAS.

Inhibition of ACE2 may be undesirable because functional ACE2 inhibits inflammation by reducing activation of the angiotensin II type 1 receptor pathway.\(^\text{15}\) SARS-CoV-2 uses ACE2 as a receptor to enter cells, and the resulting proteolysis of ACE2 contributes to lung damage.\(^\text{15,48}\) While disrupting S protein and ACE2 interactions might prevent SARS-CoV-2 entry to cells, inhibiting ACE2 activity could be detrimental to infection recovery. Therefore, it is important to clarify if polyphenol–ACE2 interactions inhibit S-protein binding and ACE2 activity in tissues. If both occur, it will be important to understand if the antioxidant and anti-inflammatory activity of polyphenols through other pathways (e.g., inhibition of nuclear factor κB and activation of nuclear factor erythroid 2-related factor 2) would negate the proinflammatory aspects of ACE2 inhibition during SARS-CoV-2 infection.

In summary, polyphenols with 3′,4′-dihydroxylated inhibit rhACE2 activity \textit{in vitro}. Among the polyphenols tested, quercetin was the most effective rhACE2 inhibitor, and several of its known metabolites also function as inhibitors. In the context of flavonoid metabolism, it is plausible that dietary polyphenol intake could inhibit ACE2, particularly in the digestive tract. Given these findings, it is urgent to further investigate the functional effects of polyphenols on ACE2 \textit{in vivo}.

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\section*{ABBREVIATIONS}

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; S protein, spike glycoprotein; RAAS, renin–angiotensin–aldosterone system; rhACE2, recombinant human angiotensin-converting enzyme 2; ACE, angiotensin-converting enzyme; Ang II, angiotensin II; RFU, relative fluorescence unit; Mca, 7-methoxycoumarin-4-acetic acid

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