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Effect of Iron−Quercetin Complex on Reduction of Nitrite in in Vitro and in Vivo Systems

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This study investigated whether reducing agents such as quercetin and iron(II) facilitate formation of nitric oxide (NO) gas from orally ingested nitrite in an vivo study. When 3 mg/kg $Na^{15}NO_2$ was orally administered to rats with or without iron(II) or quercetin, $Hb^{15}NO$, which is indicative of systemic ^{15}NO, was detected in the blood, with the maximum blood concentration of $Hb^{15}NO$ at 15 min after nitrite or nitrite plus quercetin treatment, whereas after administration of nitrite plus iron(II) or nitrite plus iron(II) and quercetin, the time was shortened to 10 min. Interestingly, iron(II), quercetin, or iron(II) plus quercetin did not affect the total amount of $Hb^{15}NO$ generated from orally administered $Na^{15}NO_2$. However, the systemic nitrite concentration was significantly decreased in the presence of iron(II) or iron(II) plus quercetin. These results may indicate that iron(II) is critical to the generation of NO gas from nitrite, whereas quercetin contributed little under the in vivo experimental conditions.

KEYWORDS: Nitric oxide; quercetin; nitrite; EPR; rat; iron

INTRODUCTION

Nitric oxide (NO) is a free radical molecule that has numerous roles in various physiological functions, such as regulation of the cardiovascular, immune, and nervous systems. It has long been believed that NO is synthesized from L-arginine, NADPH, tetrahydrobiopterin (BH_4), and molecular oxygen and that the synthesis is catalyzed by NO synthases (NOSs). However, an alternative pathway for NO production in biological systems has been described in the past decade. In addition to NOSs, xanthine oxidoreductase can serve as an alternative enzymatic pathway for NO production through reduction of the therapeutic organic nitrate nitroglycerin, as well as inorganic nitrate and nitrite under hypoxic conditions in the presence of NADH (*1*). Besides enzymatic production of NO, nonenzymatic nitrite-derived mechanisms for the generation of NO were reported, including hemoglobin-derived reduction (*2*) and the following acidic reactions (*3*):

$$NO_2^- + H^+ \leftrightarrow HNO_2 \quad (1)$$

$$HNO_2 + H^+ \leftrightarrow H_2NO_2^+ \leftrightarrow NO^+ + H_2O \quad (2)$$

$$H_2NO_2^+ + NO_2^- \leftrightarrow N_2O_3 + H_2O \quad (3)$$

$$N_2O_3 \leftrightarrow NO + NO_2 \quad (4)$$

It was reported that blood pressure was lowered in a dose-dependent manner by nitrite treatment of spontaneously hypertensive rats (*4*). Furthermore, Cosby et al. demonstrated that nitrite acts as a vasodilator at physiological levels (*5*). In a previous paper, we demonstrated that orally administered nitrite is detectable in the circulation as HbNO and that nitrite treatment attenuates L-NAME-induced hypertension in a dose-dependent manner (*6*).

The low pK_a value (3.3) (*7*) of reaction 1 makes it unlikely that nonenzymatic NO is formed from nitrite (NO_2^-) at physiological pH (6.8−7.4). In fact, researchers reported that

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NO_2^--derived NO formation is dominant in acidic environments such as the stomach (*8*) and ischemic rat heart (*9*). In addition, several researchers have demonstrated that NO production from nitrite was facilitated in the presence of ascorbate (*10*) or quercetin (*7*) at acidic pH due to the reduction of nitrosonium cation by these reducing agents.

$$NO^+ + e^- \rightarrow NO \quad (5)$$

Quercetin is one of the most abundant flavonoids in the diet; 2−250 mg of quercetin/kg of wet weight is found in fruits, 0−100 mg/kg in vegetables, 4−16 mg/L in red wine, 10−25 mg/L in tea, and 3−13 mg/L in fruit juices (*11*), and the daily consumption of quercetin has been estimated to be 16−23 mg (*12*).

In 2000, Cheng and Breen demonstrated the formation of a quercetin−iron complex at neutral pH, and it was reported that the redox potential of quercetin was altered (*13*). This led us to predict that the quercetin−iron complex may alter the generation of NO from nitrite in vivo. Therefore, in this study, we investigated whether quercetin or the quercetin−iron complex affected NO production from nitrite in both in vitro and in vivo systems.

MATERIALS AND METHODS

Materials. *N*-Methyl-D-glucamine dithiocarbamate (MGD) was synthesized according to a previously published method (*14*) from *N*-methyl-D-glucamine (Sigma, St. Louis, MO) and carbon disulfide (Sigma). The purity and molecular weight of synthesized MGD were verified by comparison with commercially available MGD by high-performance liquid chromatography and mass spectrometry, respectively. Sodium nitrite and other chemicals were obtained from Wako Pure Chemical Industries (Tokyo, Japan). The stable sodium nitrite ($Na^{15}NO_2$) isotope was obtained from Cambridge Isotope Laboratories (Andover, MA). Argon gas was purchased from Shikoku Acetylene Kogyo (Kagawa, Japan). NO gas was obtained commercially from Sumitomo Seika Chemicals (Osaka, Japan), and higher oxides such as NO_2 and N_2O_3 (NO_x) were removed by passing the gas through a trap containing 1 M KOH. A NO-saturated aqueous solution was prepared by bubbling NO gas for 15 min through water that had been previously deoxygenated by bubbling with purified argon gas for 15 min (*15*).

Preparation of Iron−MGD Complex for NO Collection. Stock solutions of $FeSO_4 \cdot 7H_2O$ (0.1 M) and MGD (0.5 M) were prepared immediately prior to measurement and were used within a few hours. All solutions were prepared in argon-purged distilled water. The iron−MGD complex was prepared by adding 2 mL of iron (5 mM) and 2 mL of MGD (25 mM) from stock solutions to 0.5 M HEPES buffer (pH 7.4) that had been deoxygenated with purified argon for 30 min as reported previously (*16*). We conducted NO trapping by using the iron(II)−MGD complex under anaerobic conditions because it is rapidly oxidized in air to iron(III)−MGD ($k = 5 \times 10^5\ M^{-1}s^{-1}$) (*16*), which is inferior to the iron(II)−MGD complex for NO trapping.

The apparatus for NO collection is shown in **Figure 1**. To trap gaseous NO from sample solutions, 20 mL/min of Ar gas flow was introduced into the Fe−MGD solution for 15 min, and then the solution was aspirated into a 1 mL plastic syringe and stored in liquid nitrogen until use.

A calibration curve was prepared by mixing various amounts of NO-saturated solution with Fe−MGD complex (5 mM) and then storing as described above.

Animals. Male Sprague−Dawley rats (5 weeks old, weighing 100−110 g) were obtained from Japan SLC (Shizuoka, Japan) and kept in cages at a controlled temperature (25 °C) under a controlled lighting condition (12/12 h light/dark cycle). The animals were fed a commercial diet and had access to tap water ad libitum until the day of the experiments. Animals were fasted for 12 h before experiments. All animal care and treatments were conducted in accordance with the guidelines of the animal use and care committee of the University of Tokushima. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication 85-23, revised 1985).

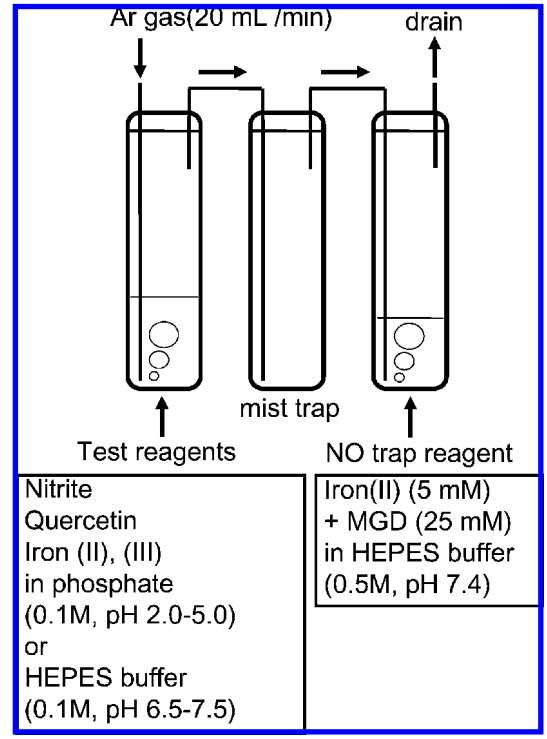

Figure 1. Experimental apparatus used for the experiments to trap gaseous NO produced by iron−MGD complex.

Application of Nitrite with or without Quercetin−Iron Complex to Rats. Rats were divided into five groups of five rats each: group 1, control; group 2, ^{15}N-nitrite; group 3, ^{15}N-nitrite + 13 mg/kg quercetin; group 4, ^{15}N-nitrite + 13 mg/kg $FeSO_4 \cdot 7H_2O$; and group 5, ^{15}N-nitrite + 13 mg/kg quercetin + 13 mg/kg $FeSO_4 \cdot 7H_2O$. All animals except the controls received $Na^{15}NO_2$ (3 mg/kg) with or without the other compounds by oral gavage, and the total volume of infusate (1 mL/kg) was adjusted by 1.5% carboxy methyl cellulose sodium (CMC-Na). We used sodium nitrite instead of potassium nitrite to prevent possible effects of potassium on the cardiovascular system and blood pressure (*17*). Rats were anesthetized with pentobarbital sodium (40 mg/kg, ip), and venous blood was obtained from the vena cava by direct puncture with a 1 mL plastic syringe equipped with an 18 gauge needle at 5, 10, 15, 20, 40, and 60 min after $Na^{15}NO_2$ administration and stored in liquid nitrogen until use. Plasma nitrite and nitrate concentrations were measured according to the Griess method as reported previously (*18*) on the day of preparation to avoid artifactual changes during storage. Briefly, the plasma was deproteinized by centrifugation through a 10 kDa molecular weight filter (Microcon 10, Millipore) at 10000 rpm for 1 h at 4 °C for a 200 μL sample. We avoided acidic deproteinization due to the acid decomposition of nitrite (reactions 1−4).

EPR Measurement. All EPR measurements were carried out according to our previous paper (*19*). Briefly, the frozen blood or Fe−MGD samples were directly transferred to a liquid nitrogen-filled quartz EPR finger dewar, which was placed in the cavity of a JES TE 300 ESR spectrometer (JEOL, Tokyo, Japan) with an ES-UCX2 cavity (JEOL) to collect EPR spectra at the X band (9.5 GHz). Typical instrument conditions were 20 mW microwave power, 6.3 Gauss modulation amplitude, 0.3 s time constant, 8 min scan time, 3200 ± 250 Gauss scan range for HbNO, and 9.045 GHz microwave frequency. Spectra were stored on an IBM personal computer for analysis.

The relative concentration of HbNO was obtained from the peak-to-peak amplitude of the first signal of the doublet signal of the *z* factor of $Hb^{15}NO$ at $g = 2.01$ (*6*), and the NO−Fe−MGD complex was quantified by double integration of its EPR signal.

UV−Visible Spectrophotometry. Reduction of iron(III) by quercetin was measured using a U-3000 spectrophotometer (Hitachi, Tokyo,

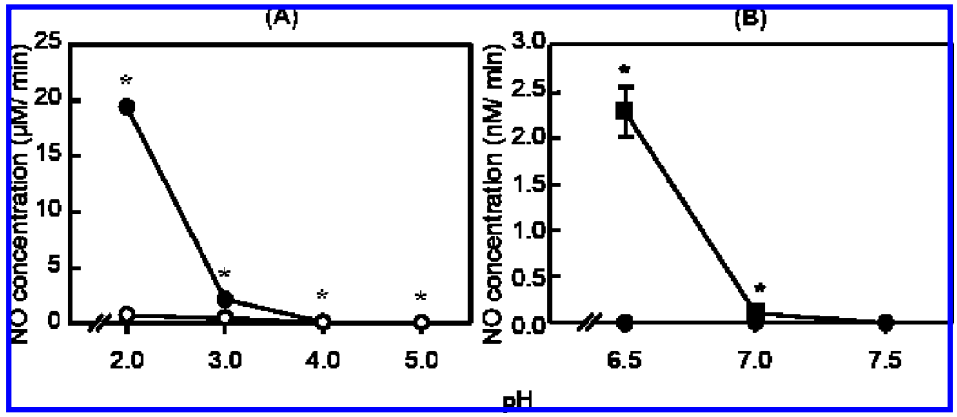

Figure 2. Formation of gaseous NO associated with reduction of nitrite. Nitrite (1 mM) (○), nitrite (1 mM) with quercetin (1 mM) (●), or nitrite (1 mM) with iron(III)−quercetin (1 mM) (■) was mixed in phosphate buffer (0.1 M, pH 2.0−5.0, **A**) or in HEPES buffer (0.1 M, pH 6.5−7.5, **B**) at room temperature under anaerobic condition, and then argon gas (20 mL/min) was introduced to carry gaseous NO into the iron−MGD solution for NO gas measurement. Each sample was stored in liquid nitrogen, and the EPR signal of the NO−iron−MGD complex was measured using EPR spectroscopy. The data are presented as NO concentration per minute. Values are expressed as means ± SE of three independent experiments. ∗, $p < 0.01$ vs nitrite (**A**) and vs nitrite with quercetin (**B**).

Japan) at room temperature. The reaction mixture contained 50 μM quercetin, 50 μM $FeCl_3$, and 500 μM 1,10-phenanthroline in 50 mM HEPES buffer (pH 6.5). The reaction was started by adding iron. The reduction of iron(III) was measured by monitoring the appearance of iron(II)−phenanthroline complex at 510 nm under anaerobic conditions because iron(II) is susceptible to oxidation.

Statistical Analysis. Data are expressed as mean ± SE in figures and mean ± SD in tables. Data were analyzed by two-way ANOVA, followed by the Tukey test for comparisons between groups. $p < 0.05$ was accepted as statistically significant.

RESULTS

Quercetin-Facilitated NO Generation from Nitrite. Figure 2 shows NO generation from the reaction mixture containing 1 mM nitrite with or without 1 mM quercetin in 100 mM phosphate buffer under acidic (**Figure 2A**, pH 2.0−5.0) and neutral (**Figure 2B**, pH 6.5−7.5) conditions at room temperature. Nitrite spontaneously produced NO gas under the acidic condition, and the coexistence of quercetin facilitated its generation (**Figure 2A**). However, the NO production decreased with increasing pH and reached an undetectable level at pH 5.0 under our experimental conditions, even in the presence of quercetin. When 1 mM nitrite was mixed with 1 mM quercetin and 1 mM iron(III), apparent NO gas production of 2.35 ± 0.15 nM/min was observed at pH 6.5 and 0.12 ± 0.04 nM/min at pH 7.0 (**Figure 2B**). No NO production was observed from nitrite even in the presence of quercetin at pH 6.5 or above. Conversely, when 1 mM nitrite was mixed with iron(II), NO production was observed at pH 7.0 (0.10 ± 0.03 μM/min), and it was facilitated in the presence of 1 mM quercetin to the rate of 0.95 ± 0.12 μM/min (**Figure 3**).

Effect of Quercetin/Iron(III) Ratio on Formation of NO from Nitrite. Because quercetin was reported to bind iron(III) (*13*) and the quercetin−iron complex facilitated NO formation from nitrite (**Figure 2**), we investigated the optimal ratio between quercetin and iron(III) for NO production from nitrite. As shown in **Figure 4**, NO production from nitrite increased with the increase in iron(III) concentration and reached a plateau when the ratio of quercetin/iron(III) = 1:1.

Absorption Spectrum of Iron−Quercetin Complex. Figure 5 shows the change in the absorption spectra of 50 μM quercetin in the presence of iron(III) in 50 mM HEPES buffer (pH 6.5) under aerobic conditions at room temperature. The absorption maxima at 250 and 370 nm were characteristic of quercetin (*7*).

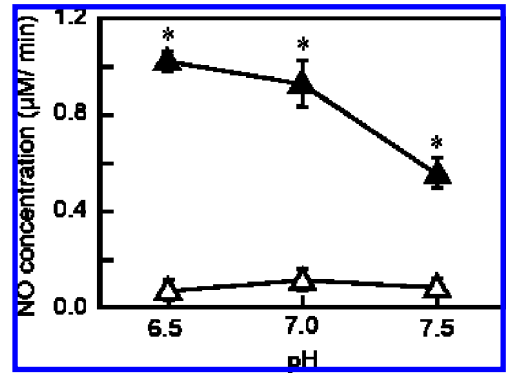

Figure 3. Formation of gaseous NO associated with reduction of nitrite. Nitrite (1 mM) with 1 mM iron(II) (△) or 1 mM nitrite with 1 mM iron(II) and 1 mM quercetin (▲) was mixed in 0.1 M HEPES buffer (pH 6.5−7.5) at room temperature. Experimental conditions were the same as in **Figure 2**. Values are expressed as means ± SE of three independent experiments. ∗, $p < 0.01$.

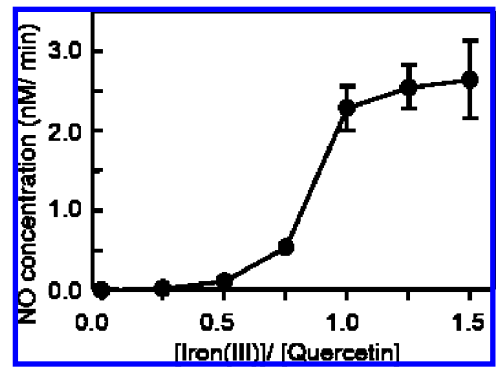

Figure 4. Effect of molar ratios of iron(III)/quercetin on amount of gaseous NO generated. A reaction mixture containing 1 mM nitrite, 1 mM quercetin, and various concentrations of iron(III) was prepared in HEPES buffer (0.1 M, pH 6.5) and then bubbled with Ar gas to measure gaseous NO. Experimental conditions were as shown in **Figure 2**. Values are expressed as mean ± SE of three independent experiments.

When 50 μM iron(III) was introduced into 50 μM quercetin, it gave two peaks at 290 and 420 nm, suggesting the formation of the one-electron oxidation of quercetin (290 nm) (*20*) and iron(III)−quercetin complex (420 nm), respectively (*21*). Next, we investigated whether iron(III) was reduced to iron(II) by

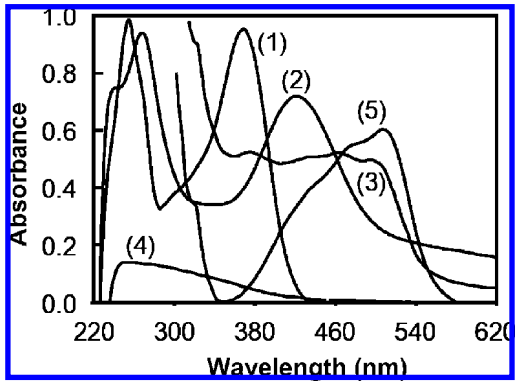

Figure 5. Absorption spectra of quercetin (spectrum 1), quercetin with iron(III) (spectrum 2), quercetin with iron(III) in the presence of 1,10-phenanthroline (spectrum 3), iron(III) (spectrum 4), and iron(II) in the presence of 1,10-phenanthroline (spectrum 5). Quercetin (50 μM) gave inherent absorption bands at 254 and 370 nm, which were in turn shifted to 420 nm by the addition of 50 μM iron(III), demonstrating the formation of a 1:1 ratio of quercetin−iron(III) complex. Adding 500 μM 1,10-phenanthroline to the reaction mixture containing iron(III) plus quercetin and then incubating if for 1 min gave an absorption band at 510 nm indicating the formation of iron(II) from iron(III) under anaerobic conditions. Spectrum 5 represents the absorption of 50 μM iron(II) with 500 μM 1,10-phenanthroline. Spectra were measured in 50 mM HEPES buffer solutions (pH 6.5) at room temperature.

quercetin. We adopted 1,10-phenanthroline as a indicator for iron(II) because it binds iron(II) and then gives an absorbance at 510 nm for the iron(II)−phenanthroline complex (*22*). When 500 μM 1,10-phenanthroline was introduced into a reaction mixture containing 50 μM quercetin and 50 μM iron(III) in HEPES buffer (pH 6.5) under anaerobic conditions, absorbance of the iron(II)−phenanthroline complex at 510 nm was observed, suggesting that iron(III) was reduced to iron(II) by quercetin.

Effect of Quercetin−Iron Complex on Systemic HbNO Production from Nitrite. In a previous paper, we demonstrated that orally administered nitrite was detectable in the circulation as HbNO using a stable isotope of nitrogen and EPR spectroscopy (*6*). It has been reported that the antioxidant agent ascorbate facilitates NO production from nitrite (*10*). In addition, Takahama et al. reported that quercetin also enhanced the formation of NO from nitrite in in vitro systems (*7, 20*). However, it was unclear whether quercetin or the quercetin−iron complex facilitated the circulating NO in vivo. To address this question, we administered a stable isotope of sodium nitrite ($Na^{15}NO_2$) instead of naturally abundant (>99%) sodium nitrite ($Na^{14}NO_2$) in the presence or absence of quercetin or quercetin−iron complex. The shape of the EPR signal of $Hb^{15}NO$ is different from that of $Hb^{14}NO$, which enabled us to distinguish exogenous nitrite-derived NO from endogenous NO. As shown in **Figure 6**, when 3 mg/kg $Na^{15}NO_2$ was orally administered to rats, marked $Hb^{15}NO$-derived doublet EPR signals (A_z = 23.4 Gauss (*23*)) were observed in the blood, which indicated that orally administered nitrite can be a source of circulating NO in the form of HbNO.

Next, we investigated the time course of changes in blood HbNO concentration after ingestion of nitrite and quercetin or quercetin−iron complex. As shown in **Figure 7**, the peak blood HbNO concentration occurred at the measurement after intake for both nitrite alone and nitrite plus quercetin treatment (15 min), and the changes of blood HbNO concentration were almost the same for both.

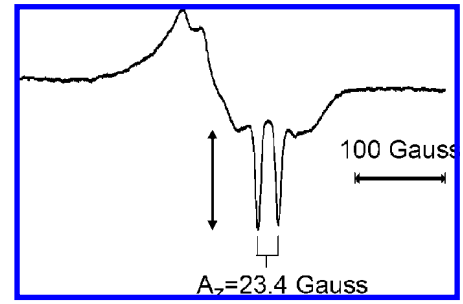

Figure 6. Representative EPR spectra of $Hb^{15}NO$ in whole blood measured at 77 K from a rat given 3 mg/kg $Na^{15}NO_2$. The rat was orally administered $Na^{15}NO_2$ at a rate of 1 mL/kg of body weight, and then blood was taken from the vena cava under anesthesia using pentobarbital sodium (40 mg/kg of body wt, ip). The $Hb^{15}NO$ concentration was obtained by measuring the peak height of the A_z region of the $Hb^{15}NO$ (indicated as two-headed arrow). Spectrometer conditions are described under Materials and Methods.

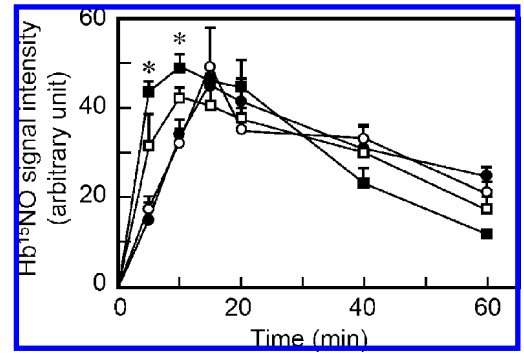

Figure 7. Time course of blood $Hb^{15}NO$ concentration changes after oral $Na^{15}NO_2$ treatment. Rats were orally administered 3 mg/kg of body weight $Na^{15}NO_2$ (○), 3 mg/kg $Na^{15}NO_2$ with 13 mg/kg quercetin (●), 3 mg of $Na^{15}NO_2$ with 13 mg/kg $FeSO_4 \cdot 7H_2O$ (□), and 3 mg of $Na^{15}NO_2$ with 13 mg/kg $FeSO_4 \cdot 7H_2O$ and 13 mg/kg quercetin (■), and then blood was drawn from the vena cava under anesthesia. The HbNO concentration was obtained by measuring the peak height of the region of the A_z region of the EPR signal. The HbNO concentration is expressed as mean ± SE. Each point represents the average of five experiments. * indicates a significant difference from 3 mg of $Na^{15}NO_2$ treatment ($p < 0.05$).

Co-administration of nitrite and quercetin−iron(II) complex or nitrite plus iron(II) facilitated the production of HbNO and shortened the time-to-maximum blood concentration to 10 min.

Effect of Quercetin−Iron Complex on Systemic Nitrite/Nitrate Concentrations. We measured the plasma nitrite and nitrate concentrations at the same time points as shown in **Figure 7**, and it was found that plasma nitrite was significantly reduced by the co-administration of iron(II) or quercetin−iron(II) complex (5 min) compared to the control group, whereas plasma nitrate concentrations were almost the same between groups (**Figure 8**).

DISCUSSION

We undertook the present study to assess the effect of quercetin on circulating HbNO formation from an orally administered stable isotope of nitrite ($^{15}NO_2^-$) using the EPR technique.

It has been believed that NO is enzymatically synthesized by NOSs with L-arginine as a substrate. Recently, in addition to NOSs, xanthine oxidoreductase and hemoglobin are reported to be capable of producing NO from nitrite (*1, 2*). Besides

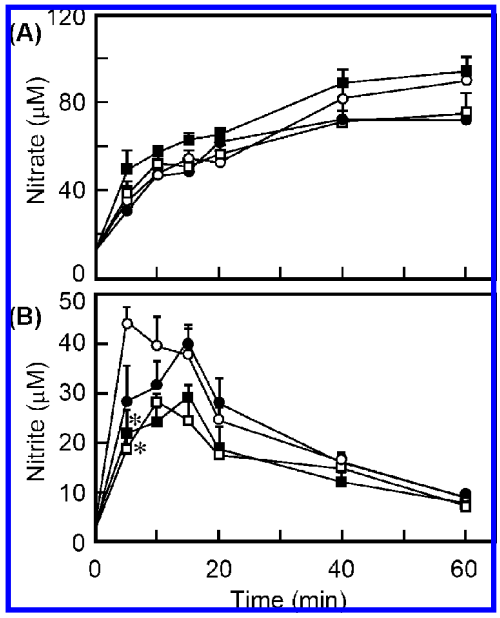

Figure 8. Time course of changes in blood nitrate (**A**) and nitrite (**B**) concentrations after oral $Na^{15}NO_2$ treatment. Rats were orally administered 3 mg/kg body weight $Na^{15}NO_2$ (○), 3 mg/kg $Na^{15}NO_2$ with 13 mg/kg quercetin (●), 3 mg of $Na^{15}NO_2$ with 13 mg/kg $FeSO_4 \cdot 7H_2O$ (□), and 3 mg of $Na^{15}NO_2$ with 13 mg/kg $FeSO_4 \cdot 7H_2O$ and 13 mg/kg quercetin (■), and then blood was drawn from the vena cava under anesthesia. The blood nitrate and nitrite concentrations were measured by Griess method as described under Materials and Methods. Each value is expressed as the mean ± SE of five experiments. * indicates significant difference from 3 mg of $Na^{15}NO_2$ treatment ($p < 0.05$).

enzymatic production of NO, NO generation by acid decomposition of nitrite was reported (eqs 1−4) (*3*). However, these reactions are dominant below pH 4 due to the low pK_a value (3.3) of the reaction (eq 1) (*7*). In fact, researchers reported that NO_2^--derived NO formation is restricted to acidic environments such as the stomach (*8*) and ischemic rat heart (*9*). In a previous study, we detected orally administered nitrite in the circulation as HbNO using a stable isotope of nitrogen (^{15}N) and EPR spectroscopy and demonstrated that nitrite treatment attenuated L-NAME-induced hypertension in a dose-dependent manner. The NO from nitrite reached maximum concentration in the blood within 20 min (*6*), suggesting that orally ingested nitrite can be a source of NO. Previously, several pathways have been proposed for NO generation from orally ingested nitrite. One is that when nitrite mixes with gastric acid, it interacts with hydrogen ion to form nitrous acid in accordance with reaction 1 (*8*). Then, nitrous acid forms gaseous NO by spontaneous decomposition through reactions 2−4; alternatively, several reducing species such as ascorbic acid are expected to be good candidates for NO production from nitrous acid in the stomach (*24*) (reaction 5).

In 2002, Takahama et al. reported that quercetin facilitated NO formation from nitrite at acidic pH (≈2) in an in vitro system because the phenolic moiety of quercetin can reduce nitrous acid to produce NO (*7*). Certainly, as shown in **Figure 2**, the facilitation of gaseous NO production from nitrite occurred in the presence of quercetin at acidic pH (<4) because of the pK_a of nitrite (*20*).

However, contrary to the in vitro experiments, when nitrite was administered to fasting rats with or without quercetin, the $Hb^{15}NO$ formation from ^{15}N-nitrite was unrelated to the presence of quercetin (**Figure 7**). In addition, the value of the area under the curve of plasma nitrite concentration

Table 1. AUC of Systemic Nitrite and Nitrate

exptl conditions	AUC (μM·min) (mean ± SD) nitrite	nitrate
$Na^{15}NO_2$	1377 ± 113	3776 ± 423
$Na^{15}NO_2$ + quercetin	1320 ± 299	3115 ± 945
$Na^{15}NO_2 + FeSO_4 \cdot 7H_2O$	943 ± 189*	3627 ± 801
$Na^{15}NO_2 + FeSO_4 \cdot 7H_2O$ + quercetin	933 ± 344*	4427 ± 551

*, $p < 0.05$ vs $Na^{15}NO_2$.

(**Figure 8B**) in rats treated with nitrite alone (1377 ± 113 μM·min, mean ± SD) was equal to that of nitrite plus quercetin (1320 ± 299 μM·min) (**Table 1**), suggesting that quercetin did not affect either nitrite absorption from the upper gastrointestinal tract or NO production from nitrite in fasting rats. It has been reported that the gastric pH of fasting rats is below 2 (*25*), and the pH values of the $Na^{15}NO_2$ solution and quercetin plus $Na^{15}NO_2$ were 6.5 and 6.4, respectively. Therefore, one possible reason quercetin did not facilitate the generation of NO gas from nitrite was the elevation of the gastric pH by administration of quercetin.

It has been reported that quercetin readily forms complexes with iron and that complex formation changes its redox potential (*13*). Therefore, we investigated whether the quercetin−iron complex generates more NO gas from nitrite than the quercetin−nitrite system. As shown in **Figure 2B**, the iron(III)−quercetin complex facilitated much more NO gas formation from nitrite, and it was observed even at pH 7.0. To investigate the optimum ratio for NO gas production from nitrite by the quercetin−iron complex, we used a mole ratio method. As shown in **Figure 4**, the NO gas generation was maximum when the ratio of iron(III)/quercetin was 1:1. At this time, iron(III) was reduced to iron(II) in the presence of quercetin, as evidenced by the appearance of the absorbance at 510 nm, which is the λ_{max} for the iron (II)−phenanthroline complex. These results are in good agreement with a previous report that quercetin forms 1:1 and 2:1 complexes with iron(III) at the 3-hydroxychromone site and subsequent catechole site depending on the amount of iron(III) and that an electron is transferred from quercetin to iron(III) to form iron(II) (*26*).

It has been reported that metallic iron acts as a reductant of nitrite, thus producing nitrogen gas and ammonium (*27*). We reported the production of a NO−iron(II)−MGD complex when nitrite was mixed with the iron(II)−MGD complex at neutral pH, and the rate constant was calculated to be 1.46 ± 0.08 M^{-2} s^{-1} at neutral pH (*16*). However, it is still unclear whether the iron(II) ion is able to reduce nitrite to form NO gas in water without any catalyst (*28*). Therefore, we investigated the effect of iron(II) on NO gas production from nitrite. Interestingly, the NO production from nitrite with iron(II) was 0.10 ± 0.03 μM·min at pH 7.0 (**Figure 3**), which is by 3 orders of magnitude greater than that of quercetin plus iron(III) (**Figure 2B**). In addition, this NO gas formation was stimulated in the presence of quercetin (**Figure 3**). These phenomena may suggest that nitrite was directly reduced by iron(II), and then NO gas and iron(III) were generated, followed by reduction of iron(III) to iron(II), by quercetin (**Figure 5**). To examine whether the NO gas generation from nitrite is modulated in the presence of iron(II) or quercetin−iron(II) complex in an in vivo system and whether the iron(II) or quercetin affects the total NO production from nitrite, we administered nitrite, nitrite plus iron(II), and nitrite plus iron(II) and quercetin to rats and then measured the systemic HbNO as an index of the NO produced (*19*). When $Na^{15}NO_2$ was orally administered to rats, apparent EPR signals

Table 2. AUC of Systemic $Hb^{15}NO$

exptl conditions	AUC (arbitrary units of $Hb^{15}NO \cdot min$) (mean ± SD)
$Na^{15}NO_2$	1801 ± 357
$Na^{15}NO_2$ + quercetin	1809 ± 191
$Na^{15}NO_2$ + $FeSO_4 \cdot 7H_2O$	1817 ± 479
$Na^{15}NO_2$ + $FeSO_4 \cdot 7H_2O$ + quercetin	1915 ± 343

corresponding to the $Hb^{15}NO$ were observed in the blood, meaning that the ^{15}NO originated from the administered $Na^{15}NO_2$, rather than from systemic nitrite, because the natural abundance of ^{15}N is <0.1%. As shown in **Figure 7**, quercetin did not alter the systemic HbNO dynamics in comparison to nitrite alone. However, nitrite plus iron(II) facilitated the HbNO production in the early period of the experiment (<10 min), and nitrite plus iron(II) and quercetin significantly increased the HbNO production at 5 and 10 min after oral administration. Meanwhile, the areas under the curve of the $Hb^{15}NO$ concentration of each group shown in **Figure 7**, which represents the total amount of $Hb^{15}NO$ from orally administered nitrite, had the same values (**Table 2**), indicating that the existence of reducing compounds, such as quercetin, iron(II), and the colocalization of iron(II) with quercetin investigated in this experiment, were unrelated to the total amount of NO production from nitrite in vivo.

It has been reported that removal of orally administered nitrite from the stomach is very rapid and due predominantly to absorption into the blood stream (*29*), with total nitrite concentrations declining to less than half the initial levels in 10 min (*30*). The absorbed nitrite then interacts with deoxyhemoglobin to form HbNO (*31*). If nitrite were completely absorbed from the stomach and systemic HbNO were derived from systemic nitrite, the time course and the concentration of systemic HbNO would depend on the systemic nitrite concentration. Our present experiments indicated that the systemic nitrite concentration was significantly lower in the nitrite plus iron(II) group and the nitrite plus iron(II) and quercetin group (**Figure 8B** and **Table 1**). Nevertheless, there was no difference of total HbNO formation between groups. Taking into account the relevance of the HbNO concentration to the blood nitrite level, it is conceivable that rapid decomposition of nitrite in the stomach is responsible for the NO gas formation (*24*), which in turn enters the blood and then interacts with deoxyhemoglobin to form HbNO (rapid NO formation). On the other hand, the remaining nitrite would be absorbed from the stomach and then interact with deoxyhemoglobin to form HbNO as reported previously (*31*).

Quercetin, iron, and nitrite are common components of food. Quercetin is the most abundant flavonoid in food (*11*). In addition, vegetables and fruits contain large amounts of nitrate and nitrite (*32*) and supply 86% of the daily nitrate intake in the general U.S. population (an average of 75 mg of NO_3^- by nonvegetarians and 268 mg by vegetarians) and 16% of the daily nitrite intake (an average of 0.77 mg of NO_2^- by nonvegetarians and 1.7 mg in diets high in cured meat) (*33*). When nitrate is ingested, it is rapidly absorbed in the upper small intestine, and up to 75% is excreted in the urine within 24 h (*24*). The remaining ingested nitrate (≈25%) undergoes enterosalivary recirculation, and it is concentrated in the salivary glands and then secreted in the saliva (*34*). The rate of microbial reduction of nitrate to nitrite in the oral cavity is reported to be around 10−20% of total ingested nitrate (*35*), and the nitrite is moved into the stomach by swallowing. Vegetarians take in 14.2 mg of nitrite per day maximum. Daily intake of iron by Japanese subjects is estimated to be 9.82 mg/day (*36*). According to the in vitro result it was expected that nitrite is rapidly reduced to NO gas in the presence of iron(II) with quercetin. However, in in vivo experiments, it was found that iron(II) and quercetin did not affect the total HbNO amount but did modulate the initial rate of HbNO production from nitrite (**Figure 7**). Iron occurs in food largely as either iron(III) or heme-iron. Some iron(III) is reduced by dietary constituents (such as quercetin) and intestinal secretions to iron(II), which is soluble at neutral pH (*37*). In addition, dietary iron(III) is reduced to iron(II) by intestinal iron reductase on the apical side of the duodenal epithelia of the small intestine, and then divalent metal transporter 1 (DMT1), which locates in the duodenum, transports the iron(II) to the basolateral side (*38*). Meanwhile, some ingested nitrite is absorbed from the stomach (*31*), and some exits the stomach via passage into the duodenum (*39*). Interestingly, when we administered the same molar concentrations of iron(III) as iron(II) to ^{15}N-nitrite (3 mg/kg)-treated rats with or without quercetin, the changes in the $Hb^{15}NO$ signal intensity of rats treated with nitrite plus iron(III) plus quercetin were the same as those of rats treated with nitrite plus iron(II) plus quercetin. Likewise, the changes of $Hb^{15}NO$ signal intensity of rats treated with nitrite plus iron(III) were the same as those of rats treated with nitrite plus iron(II) (data not shown). Taking these data into account, the combination of iron and nitrite may affect the formation of NO gas as described in this paper. Recently, Gladwin et al. proposed that the nitrite is reduced by iron(II) porphyrins (*40*−*42*).

In conclusion, we demonstrated that the quercetin−iron complex facilitated the formation of NO gas from nitrite both in vitro and in vivo, although the complexes did not affect the nitrite availability of the NO source in vivo. The metabolic pathways from nitrite to NO in rats (*43*) seem similar to those of humans (*44*), but we need further study to apply these conclusions directly to humans.

ABBREVIATIONS USED

NO, nitric oxide; EPR, electron paramagnetic resonance; NOSs, nitric oxide synthases; NO_2^-, nitrite; MGD, *N*-methyl-D-glucamine dithiocarbamate; L-NAME, N^G-nitro-L-arginine methyl ester; HbNO, iron(II) nitrosyl hemoglobin.

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