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Manipulation of citrulline availability in humans

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Rougé C, Des Robert C, Robins A, Le Bacquer O, Volteau C, De La Cochetière M-F, Darmaun D. Manipulation of citrulline availability in humans. *Am J Physiol Gastrointest Liver Physiol* 293: G1061–G1067, 2007. First published September 27, 2007; doi:10.1152/ajpgi.00289.2007.—To determine whether circulating citrulline can be manipulated in vivo in humans, and, if so, whether citrulline availability affects the levels of related amino acids, nitric oxide, urinary citrulline, and urea nitrogen, 10 healthy volunteers were studied on 3 separate days: *1*) under baseline conditions; *2*) after a 24-h treatment with phenylbutyrate (0.36 $g \cdot kg^{-1} \cdot day^{-1}$), a glutamine "trapping" agent; and *3*) during oral L-citrulline supplementation (0.18 $g \cdot kg^{-1} \cdot day^{-1}$), in randomized order. Plasma, erythrocyte (RBC), and urinary citrulline concentrations were determined by gas chromatography-mass spectrometry at 3-h intervals between 1100 and 2000 on each study day. Regardless of treatment, RBC citrulline was lower than plasma citrulline, with an RBC-to-plasma ratio of 0.60 ± 0.04, and urinary citrulline excretion accounted for <1% of the citrulline load filtered by kidney. Phenylbutyrate induced an ~7% drop in plasma glutamine ($P = 0.013$), and 18 ± 14% ($P < 0.0001$) and 19 ± 17% ($P < 0.01$) declines in plasma and urine citrulline, respectively, with no alteration in RBC citrulline. Oral L-citrulline administration was associated with *1*) a rise in plasma, urine, and RBC citrulline (39 ± 4 vs. 225 ± 44 μmol/l, 0.9 ± 0.3 vs. 6.2 ± 3.8 μmol/mmol creatinine, and 23 ± 1 vs. 52 ± 9 μmol/l, respectively); and *2*) a doubling in plasma arginine level, without altering blood urea or urinary urea nitrogen excretion, and thus enhanced nitrogen balance. We conclude that *1*) depletion of glutamine, the main precursor of citrulline, depletes plasma citrulline; *2*) oral citrulline can be used to enhance systemic citrulline and arginine availability, because citrulline is bioavailable and very little citrulline is lost in urine; and *3*) further studies are warranted to determine the mechanisms by which citrulline may enhance nitrogen balance in vivo in humans.

nutrition; gut; glutamine; protein metabolism

CITRULLINE IS A NONPROTEIN amino acid found as a free amino acid in most biological fluids such as plasma, urine, and cerebrospinal fluid (20, 24, 31, 38, 39, 41). Two main pathways account for citrulline production (9, 32, 38, 39; Rougé C, Des Robert C, Robins A, Le Bacquer O, De La Cochetière MF, Darmaun D, unpublished observations): *1*) citrulline is synthesized from glutamine in enterocytes, by condensation of ornithine and carbamyl phosphate (a reaction catalyzed by ornithine carbamyl-transferase); and *2*) the conversion of arginine to nitric oxide produces citrulline as well, in a reaction catalyzed by nitric oxide (NO) synthetase (NOS) in most NO-producing tissues. Using stable isotope-labeled arginine and citrulline, other workers have found the contribution of arginine to citrulline synthesis to account for ~10% of circulating citrulline flux, and nearly 90% arise from glutamine (6, 38, 39, 43). Citrulline produced by gut is released as such into the bloodstream. Approximately 83% of the circulating citrulline is taken up by kidney (38, 39, 43), where citrulline is converted to arginine by arginosuccinate synthase (ASS) and arginosuccinate lyase (ASL) in cells of the proximal tubules (22). The production of arginine from citrulline accounts for 60% of the overall rate of de novo arginine synthesis in the body, but only 5–15% of circulating arginine (6, 26). Released as such into the bloodstream, arginine is used by most tissues for protein synthesis and other purposes (11, 14).

Interest about citrulline has risen substantially in the last decade, because plasma citrulline has been proposed as a noninvasive index of intestinal function. Plasma citrulline level indeed correlated with residual bowel length after extensive small intestinal resection (7, 33) or with small intestinal mass in celiac disease (8), intestinal transplantation (30, 16), or radiation-induced enteritis (23). In addition, recent animal studies suggest that citrulline may have a protein anabolic effect under specific settings: a citrulline-enriched diet indeed enhanced nitrogen balance after extensive small intestinal resection (28) and muscle protein synthesis in old, undernourished rats (29).

Even though several workers have reported plasma and urinary citrulline levels in various age groups in humans (5, 18, 24, 27), the factors that may regulate citrulline availability have not been specifically investigated. Since glutamine is the main precursor of citrulline, several groups have shown that enteral glutamine supplementation resulted in a rise in plasma citrulline concentration: for instance, plasma citrulline rose ~33, 30, and 27% in healthy volunteers who received enteral glutamine at rates of 0.04 (12), 0.05 (25), and 0.09 $g \cdot kg^{-1} \cdot h^{-1}$ (35), respectively. Yet, the effects of glutamine depletion, such as observed in stress, on plasma citrulline have not been addressed. Moreover, little is known on the effect of exogenous citrulline administration on the circulating levels and excretion rates of citrulline, other related nitrogen substrates, or urinary urea in vivo in humans.

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The aims of the present study therefore were *1*) to determine whether circulating citrulline levels can be decreased in vivo in humans, by depleting glutamine, its main endogenous precursor, and raised by oral citrulline supplementation, and *2*) if so, whether citrulline availability affects the levels of related amino acids, nitric oxide, urinary citrulline, and urea nitrogen.

MATERIAL AND METHODS

Chemicals

All chemicals (propan-1-ol, acetylchloride, hydrogen chloride, ammonium hydroxide, potassium hydroxide, 5-sulfosalicylic acid, ethylacetate, and heptafluorobutyric anhydride), citrulline (L-2-amino-5-ureidovaleric acid), and ion exchange resins (Dowex 50WX8–200 and Dowex 1X8) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). L-[2,3,3,4,4,5,5-$^2H_7$] citrulline (d7-citrulline) was obtained from CDN Isotopes (Cil Cluzeau Info Labo; SteFoy la Grande, France) and homocitrulline (ε-carbamoyl lysine) from Advanced Asymetrics (Millstadt). Phenylbutyrate (Ammoniaps) was purchased from Orphan Europe (Paris-La Défense, France), and L-citrulline (Stimol) from Biocodex (Gentilly, France).

Subjects

Ten young, healthy adult male volunteers were enrolled in studies performed in the Clinical Investigation Center at the Hôtel-Dieu Hospital, Nantes, France after signing an informed consent form, according to protocols approved by the local Institutional Review Board (Comité de Protection des Personnes n°2 des Pays de la Loire). Only subjects who were free of any gastrointestinal, metabolic, renal, or hematological disease and were not taking any medication were recruited. Subjects were 24.2 ± 3.3 yr old (range 20–32) and had a mean body mass index (weight/height^2) of 22.5 ± 2.3 kg/m^2.

Experimental Design

Each subject underwent three separate studies with a 1-wk washout period in between study days. On each study day, subjects reported after an overnight fast to the Clinical Investigation Center at Hotel-Dieu Hospital, Nantes, France, where they stayed all day. They remained at rest and received a standard diet (35 kcal·kg^{-1}·day^{-1}, 16% protein, 30% fat, and 54% carbohydrate) supplied as three daily meals at 0830, 1230, and 1900. Each subject was studied on three occasions: *1*) under baseline conditions; *2*) during oral L-citrulline administration (0.18 g·kg^{-1}·day^{-1}) divided into five equal doses given at 3-h intervals between 0700 and 1900 to increase circulating citrulline; and *3*) after 24 h of treatment with phenylbutyrate per os (0.36 g·kg^{-1}·day^{-1}) split in six equal doses given at 4-h intervals to decrease circulating citrulline.

The baseline study was always performed first, the order of the other two studies was randomized. The dose of phenylbutyrate chosen was shown to result in an ~25% decline in plasma glutamine concentration (10). The dose of citrulline was chosen so as to represent approximately four times plasma citrulline flux, based on studies by other workers (6, 38, 39).

On each study day, 5 ml of blood were obtained at 1100, 1400, 1700, and 2000 into sampling tubes containing EDTA, and urine was collected two times, between 0800 and 1400 and between 1400 and 2000. Blood cells and plasma were immediately separated by centrifugation at 3,000 *g* for 10 min at 4°C, and the resulting samples were stored at −80°C until analysis. Urine samples were aliquoted and stored immediately at −80°C until analysis.

Analytical Methods

GC-MS. Blood samples (5 ml) were drawn into sampling tubes containing EDTA. Blood cells and plasma were immediately separated by centrifugation at 3,000 *g* for 10 min at 4°C, and the resulting samples were stored at −80°C until analysis. The concentration of citrulline in plasma, erythrocytes (RBCs), and urine was measured by GC-MS as described (Rougé et al., unpublished observations).

Briefly, before analysis of plasma and RBC, a 10-nmol aliquot of 0.1 mol/l homocitrulline, an analog of citrulline, was added to 200 μl of sample, as an internal standard. Citrulline was extracted from deproteinized plasma and blood cells on Dowex 50 cation exchange resin. Urine samples were aliquoted soon after collection and stored immediately at −80°C until analysis. Before analysis of urine, a 10-nmol aliquot of d_7-citrulline was added to 400 μl of urine, as an internal standard. Urine samples were first passed on a Dowex 1X8 anion exchange resin and then on Dowex 50WX8 cation exchange resin.

Citrulline from plasma, RBC, or urine extracts was derivatized to its propyl heptafluorobutyryl derivative (Rougé et al., unpublished observations), and analysis was performed using an HP 5890 series II gas chromatograph coupled with a 5971 mass selective detector (Hewlett-Packard, Palo Alto, CA) using a DB-1 (30 m × 0.25 mm ID, 0.25 μm film thickness; J & W Scientific, Courtaboeuf, France) capillary column operated in the splitless mode. The mass spectrometer was operated in the electron impact ionization mode. The acquisition time was 14 min. Ions at m/z = 114, 118, and 128, representing natural citrulline, d_7-citrulline, and homocitrulline, respectively, were selectively monitored.

Amino acid analyzer. Glutamine, ornithine, and arginine analyses were performed by using a Biotronik LC5001 automatic amino acid analyzer.

Nitrate and nitrite assay. Urinary nitrates and nitrites were quantified by a commercial kit (Roche-Diagnostic, Meylan, France), following the Griess method.

Total urinary nitrogen excretion was quantified by the Kjeldhal method.

Calculations

The renal clearance of creatinine, a measure of glomerular filtration rate (GFR; ml/min), was calculated as GFR = $[creat]_u \times V/[creat]_p$, where $[creat]_u$ and $[creat]_p$ are the concentrations of creatinine in urine and plasma (nmol/ml), respectively, and V is urinary output (ml/min). Citrulline clearance (ml/min) was calculated as: $V \times [cit]_u/[cit]_p$, where $[cit]_u$ is urinary citrulline concentration (nmol/ml), and $[cit]_u$ is the concentration of citrulline in urine (nmol/ml). The fraction (%) of citrulline filtered by glomerulus that was excreted into urine was calculated as $(100 \times V \times [cit]_u)/([cit]_p \times GFR)$. Nitrogen balance (N balance, mmol N/12 h) was estimated as N balance = $N_{in} - N_{out}$, where N_{in} is overall nitrogen intake (mmol nitrogen/12 h). Nitrogen intake was calculated as the sum of dietary protein nitrogen (based on food composition tables), plus nitrogen provided as oral citrulline, and N_{out} is total urinary nitrogen output, as quantified by the Kjeldhal method, between 0800 and 2000.

Statistical Analysis

Results are reported as means ± SD for amino acid quantification. Mixed linear models were used to test order of treatment and the effects of time, phenylbutyrate treatment, and oral citrulline on amino acids concentrations and to assess the relationships between plasma and RBC citrulline levels and between plasma and urine citrulline levels. The use of a mixed linear model provided correlations that did not suffer from pseudoreplication, since it is especially designed for repeated data points obtained at various time points (13), contrary to conventional linear models.

RESULTS

Order of Regimens

Because the order of regimens per se affected neither plasma nor RBC or urine citrulline (data not shown), the 10 subjects

were considered as a single group studied under three separate regimens: control (baseline), phenylbutyrate treatment, and oral citrulline supplementation.

Diurnal Variation of Plasma and RBC Citrulline

On the control day, plasma citrulline tended to decline at 1400 and 2000, compared with the earlier samples (Fig. 1; Table 1). In contrast, RBC citrulline level did not vary significantly throughout the day.

Effect of Oral Phenylbutyrate

As expected, treatment with phenylbutyrate (0.36 $g \cdot kg^{-1} \cdot day^{-1}$) induced a significant, 7.3 ± 9.2% ($P = 0.013$) decline in plasma glutamine, from 501 ± 103 μmol/l to 464 ± 106 μmol/l ($n = 10$ subjects, 1 sampling time per subject at 1100). As a consequence, and as expected, phenylbutyrate treatment was associated with a significant, 18 ± 14% ($P < 0.0001$) decline in plasma citrulline (39 ± 4 μmol/l vs. 32 ± 3 μmol/l; $n = 10$ subjects, 4 sampling times per subject; baseline vs. phenylbutyrate treatment), and a 19 ± 17% ($P < 0.01$) decline in urinary citrulline (0.9 ± 0.3 μmol/mmol of creatinine vs. 0.7 ± 0.2 μmol/mmol of creatinine; baseline vs. phenylbutyrate treatment). RBC citrulline was not altered ($P = 0.19$), nor was plasma ornithine ($P = 0.26$), plasma arginine ($P = 0.26$), or urinary arginine ($P = 0.14$).

Phenylbutyrate treatment was associated with a significant reduction in urinary nitrate (15 ± 39%; $P = 0.06$), and urinary urea excretion (17 ± 15% $P < 0.0001$), without any alteration in plasma urea ($P = 0.40$).

Effect of Oral Citrulline Supplementation

As expected, oral L-citrulline administration induced a dramatic, 488 ± 92% rise in plasma citrulline (from 39 ± 4 to 225 ± 44 μmol/l; $P < 0.0001$). Urinary citrulline excretion rose accordingly (+571 ± 266%; from 0.9 ± 0.3 to 6.2 ± 3.8 μmol/mmol of creatinine; $P < 0.001$), whereas RBC citrulline only rose 139 ± 52% (from 23 ± 1 to 52 ± 9 μmol/l; $P < 0.0001$). Plasma glutamine, ornithine, and arginine rose 24 ± 21% (from 501 ± 103 to 614 ± 110 μmol/l; $P = 0.0007$), 73 ± 42% (from 90 ± 29 to 150 ± 46 μmol/l; $P = 0.0003$), and 92 ± 57% (from 134 ± 33 to 247 ± 62 μmol/l; $P = 0.00005$), respectively. Oral citrulline supplementation did not alter urinary arginine ($P = 0.15$), nor plasma urea ($P = 0.23$), urinary urea ($P = 0.56$), or urinary nitrate excretion ($P = 0.55$).

Because GFR was assessed by using creatinine clearance, the load of citrulline filtered by glomerulus could be calculated (see *Calculations*), as well as the fraction of this load that is eventually excreted into urine. During exogenous citrulline supplementation, 0.2 ± 0.1% of the citrulline load filtered by glomerulus, and 0.4 ± 0.2% of the arginine filtered, were excreted in urine. The ratio of urinary urea nitrogen to total urinary nitrogen was 77 ± 11% on the control day, and 85 ± 8% on the citrulline supplementation day ($P = 0.15$; not significant). Oral citrulline supplementation was associated with an appreciable rise in nitrogen balance (+57%, from 683 ± 246 to 970 ± 187 mmol nitrogen/12 h; $P = 0.0053$) (Fig. 2).

RBC vs. Plasma Citrulline Concentrations

On each of the 3 study days, RBC citrulline was significantly lower than plasma citrulline (Table 1). Even though the average ratio of RBC-to-plasma citrulline concentration (RBC/P) was 0.60 ± 0.04, this ratio varied widely between subjects, ranging between 0.26 and 1.09. Five subjects indeed had RBC/P ratios between 0.74 and 1.09 (not different from 1.0), i.e., they had no significant citrulline concentration gradient across red cell membrane, whereas five other subjects had RBC/P ratios that were below 1.0 (0.26–0.40). The RBC/P ratio was unaltered (0.71 ± 0.07; $P = 0.126$) after phenylbutyrate treatment but declined to 0.23 ± 0.03 ($P = 0.001$) during oral citrulline supplementation.

Relationship Between Changes in Red Cell and Plasma Citrulline

There was a significant linear relationship between RBC citrulline and plasma citrulline levels under baseline conditions ($P = 0.024$), and during oral citrulline supplementation ($P < 0.0001$) (Fig. 3). The slope of the regression line describing the correlation was, however, weak, and no significant linear relation was observed between plasma and RBC citrulline during phenylbutyrate treatment ($P = 0.49$).

Relationship Between Changes in Urine and Plasma Citrulline

Even though urinary citrulline excretion and plasma citrulline concentration changed in the same direction, and even though the rise with oral citrulline supplementation and the decline during phenylbutyrate treatment were of the same

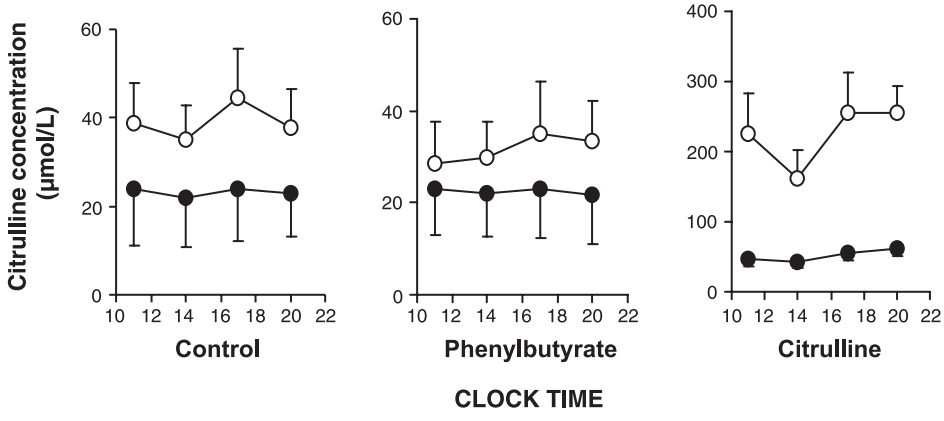

Fig. 1. Composite time course of plasma citrulline (○) and erythrocyte (RBC) citrulline (●) concentration under baseline conditions (*left*), after phenylbutyrate treatment (*middle*), and during oral citrulline supplementation (*right*) in 10 healthy adult subjects.

Table 1. *Plasma citrulline concentration, and urinary excretion of citrulline and related nitrogen compounds under control conditions, and during treatment with phenylbutyrate or oral citrulline supplementation*

		Regimen								
		Baseline			Phenylbutyrate			Citrulline Supplementation		
	Time	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median
Plasma citrulline, μmol/l	1100–2000	39	4	39	32	3	30	225	44	32
RBC citrulline, μmol/l	1100–2000	23	1	21	22	1	20	52	9	50
RBC/plasma citrulline	1100–2000	0.60	0.04	0.57	0.71	0.07	0.62	0.23	0.03	0.23
Urinary citrulline, μmol/mmol of creatinine	0800–2000	0.90	0.14	0.8	0.65	0.07	0.6	6.60	0.99	5.2
Plasma glutamine, μmol/l		501	103	528	464	106	483	614	110	635
Plasma ornithine, μmol/l	1100	90	29	88	83	25	79	150	46	127
Plasma arginine, μmol/l	1100	134	33	137	122	37	122	247	62	245
Urine arginine, μmol/mmol of creatinine	1100	1.2	0.8	1.3	3.4	2.0	3.0	8.6	14.7	3.5
Urine nitrates, mmol N/12 h	0800–2000	1.0	0.6	0.8	0.8	0.5	0.6	0.9	0.7	0.6
Urinary urea excretion, mmol N/12 h	0800–2000	415.2	133.3	227.5	374.0	84.6	124.5	437.7	98.9	163
Plasma urea, mmol/l	0800–2000	5.2	1.3	5.1	4.2	0.9	4.0	5.0	1.1	4.5
Total urinary nitrogen, mmol N/12 h*	0800–2000	683	246	776	538	135	494	970	187	975
GFR, ml/min†	0800–2000	134.4	22.6	132.2	129.2	22.6	139.4	151.5	28.1	154.6
Clearance of citrulline, ml/min	0800–2000	0.27	0.12	0.21	0.27	0.12	0.23	0.36	0.24	0.27
Clearance of arginine, ml/min	0800–1200	0.16	0.23	0.09	0.40	0.39	0.32	0.58	0.33	0.59
% Citrulline excreted into urine	0800–2000	0.20	0.08	0.18	0.21	0.07	0.20	0.22	0.12	0.17
% Arginine excreted into urine	0800–1200	0.11	0.14	0.06	0.32	0.29	0.22	0.37	0.20	0.34

Amino acid concentrations, clearance, and % of amino acids excreted into urine were averaged for each subject on each study day. RBC, erythrocytes; N, nitrogen. *Total urinary nitrogen was quantified in 8 of the 10 volunteers. †Glomerular filtration rate (GFR) was calculated as urinary creatinine excretion (μmol/min)/plasma creatinine concentration (μmol/ml).

magnitude in urine and plasma, no significant linear relationship was observed between urine citrulline and plasma citrulline levels under any of the regimens.

DISCUSSION

The findings of the present study suggest plasma citrulline levels can be lowered by glutamine depletion and raised by oral citrulline administration. The data further suggest incomplete equilibration of citrulline between plasma and RBCs and extensive reabsorption of citrulline by renal tubule, because <1% of an oral dose of citrulline is excreted in urine. Finally, to the best of our knowledge, the present study is first to demonstrate that oral citrulline can be used to enhance the availability of citrulline and arginine in systemic bloodstream, whereas it does not enhance urea excretion and may thus improve nitrogen balance in vivo in humans.

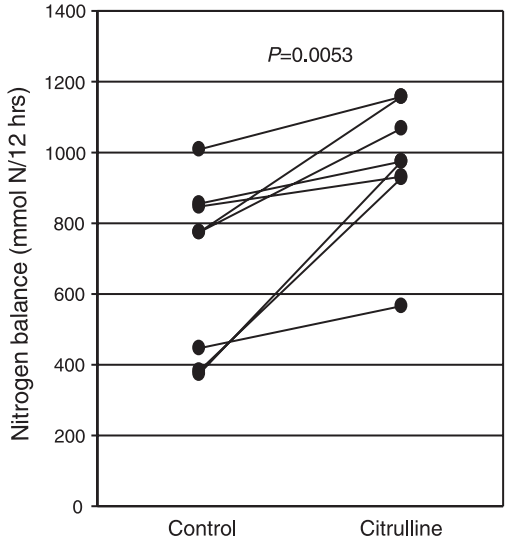

Fig. 2. Nitrogen balance under baseline conditions and during oral citrulline supplementation in 8 healthy volunteers.

Even though the plasma and urine citrulline levels found in our subjects are consistent with earlier reports (7, 15, 17, 21, 24, 27), we observed fluctuations in plasma citrulline through the day under baseline conditions. The exact mechanism for the fluctuations in plasma citrulline on the control day is unclear: although protein does not contain any citrulline, meal protein supplies amino acids that are potential precursors for citrulline synthesis, such as glutamate and glutamine, which could potentially increase plasma citrulline. However, we observed a decline in plasma citrulline: this suggests that either the amino acids derived from meal protein are predominantly used for purposes other than citrulline synthesis (e.g., protein synthesis) or citrulline utilization is increased after meals. Accordingly, Rabier and Kamoun (32) showed that plasma citrulline declined after a high-protein meal and returned to normal value 4 h later. This finding implies that if plasma citrulline is used as a noninvasive marker to monitor intestinal function attention should be paid to the time of sampling.

The present data suggest plasma citrulline can be depleted by treatment with phenylbutyrate, a glutamine-trapping agent. The ~18% decline in plasma citrulline observed in the present study during phenylbutyrate treatment is likely secondary to the 7% drop in plasma glutamine, the main precursor of citrulline. The lesser degree of glutamine depletion observed in this study, compared with our previous study (~7% vs. ~25%; $P = 0.01$) despite the same dose of phenylbutyrate (0.36 $g \cdot kg^{-1} \cdot day^{-1}$) (10), likely reflects differences in study design, since subjects were fed in the present study, rather than fasted as in earlier reports (10). The ~17% decline in plasma and urinary urea during treatment with phenylbutyrate is consistent with the fact that glutamine is the main precursor of urea. The fact that plasma citrulline depends on prevailing glutamine

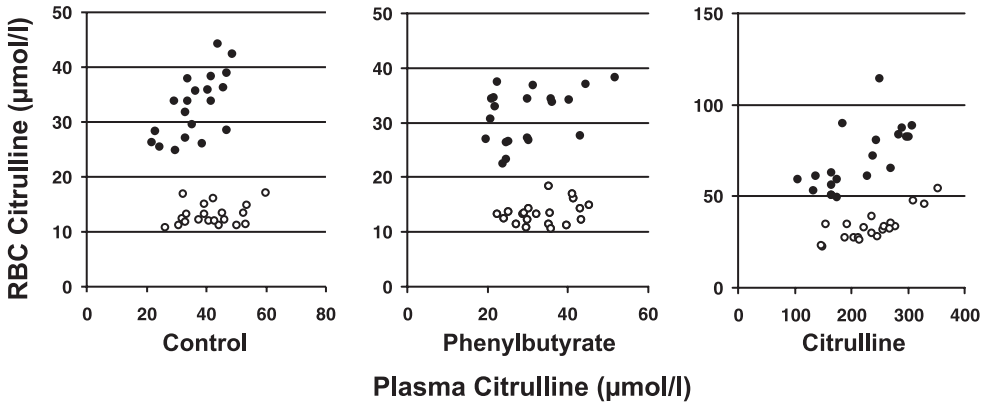

Fig. 3. Relationship between citrulline concentrations in plasma (P) and RBCs under baseline conditions (*left*), after phenylbutyrate treatment (*middle*), and during oral citrulline supplementation (*right*) in 10 healthy adult subjects. ●, Subjects in whom baseline RBC/P citrulline concentration ratio did not significantly differ from 1 (0.74–1.09); ○, subjects in whom baseline RBC/P ratio was below 1 (0.26–0.40).

levels suggests that caution should be exercised when using plasma citrulline as an index of intestinal function in clinical situations known to produce glutamine depletion, such as acute illness and stress (40).

We are not aware of earlier studies on RBC citrulline in humans. The concomitant assessment of RBC and plasma citrulline concentration revealed a wide range of variation in the RBC/P citrulline concentration ratio. Under baseline conditions, citrulline concentration was consistently lower in RBC than plasma; yet, whereas RBC/P ratio was close to 1.0 in five subjects, it was below 1.0 (0.26–0.40) in the remaining five. When the entire data set is considered as a whole, the correlation between plasma and RBC citrulline levels is weak (Fig. 3). This may be due to the heterogeneity of the population studied. When the population was divided in two subgroups, based on the RBC/P citrulline concentrations under baseline conditions, a different picture emerged. In subjects who had a low baseline RBC/P ratio (<1.0), changes in RBC citrulline did not correlate with changes in plasma citrulline. In contrast, in the five other subjects in whom baseline RBC/P ratio was close to 1.0, the changes in RBC citrulline induced by phenylbutyrate and oral citrulline tended to correlate with changes in plasma.

The observation that two groups were identified with regard to plasma-to-RBC citrulline ratio is intriguing. This heterogeneity may reflect differences either in citrulline metabolism or in citrulline transport across the membrane of RBC. Citrulline uptake involves an active, Na^+-dependent transport in rat small intestine (37) and in Caco-2 cells, a model of human enterocytes (3). Although citrulline transport has been characterized in arterial smooth muscle (42) and neurons as well (36), we are not aware of studies of citrulline transport across the RBC membrane. Studies using incubation of fresh RBCs in the presence of labeled citrulline would be required to address this issue. On the other hand, differences in the expression of enzymes involved in RBC citrulline metabolism among subjects could explain the results. In a population of healthy volunteers, Apostol and Tayek (2) observed two different patterns in the response of plasma citrulline to arginine supplementation: some increased plasma citrulline, whereas others did not. They speculate that NO synthase activity differed widely between these groups (2). The differences we observed in RBC citrulline could result from differences in NOS activities; Kang et al. (19), however, reported that NOS is not active in RBCs, so this mechanism cannot account for our findings. In contrast, arginase is abundant in RBC. Differences in arginase activities in RBC have been reported in humans, since patients with sickle cell disease had a 60% elevation in RBC arginase activity, compared with controls (26). As arginine is both a product and a precursor of citrulline via ornithine, differences in arginase activity could play a role as well. This is, however, unlikely, because differences in arginase activity were reported in African Americans who suffer from sickle cell disease, and our subjects were Caucasian and had normal blood counts. Further studies would be warranted to determine the precise mechanisms responsible for the apparent compartmentation of citrulline between plasma and RBCs observed in a substantial fraction of the healthy volunteers.

Little is known about the handling and potential effects of an exogenous load of citrulline in humans. In the present study, plasma citrulline rose nearly fivefold (~490%) in volunteers supplemented with oral citrulline, implying that exogenous citrulline is efficiently absorbed in the gut and may escape uptake in splanchnic territory without any significant conversion to urea, as plasma urea remained unaltered (Table 1). Even though low levels of citrulline have been reported in urine from subjects of various age groups, the fraction of citrulline reabsorbed by kidney is not known. Because urine was collected throughout the study, and because creatinine clearance reflects glomerular filtration rate in healthy subjects, we were able to calculate that $<1\%$ of the amount of citrulline filtered by glomerulus is excreted as such; the rest, $>99\%$, is therefore either reabsorbed or converted to arginine. Accordingly, the fivefold rise in plasma citrulline resulted in a near doubling in plasma arginine. Regardless of the specific mechanisms of citrulline reabsorption and/or metabolic conversion in kidney, the present data therefore suggest that orally administered citrulline is highly bioavailable, since systemic citrulline rose dramatically, whereas urinary citrulline loss was minimal. This finding implies that the oral route could be used effectively to increase citrulline and arginine availability in specific clinical situations.

During exogenous citrulline supplementation, the bulk of the citrulline administered was thus presumably converted to arginine. Arginine can be utilized by three main pathways: urea synthesis, NO production, and incorporation into protein synthesis (11, 14). In the present study, neither plasma urea nor urea excretion rose during oral citrulline administration, suggesting that little arginine underwent conversion to urea. Similarly, the excretion of nitrate and nitrite remained unaltered.

We therefore speculate that the incorporation of arginine into protein synthesis may have risen. Even though rates of protein synthesis were not assessed in the present study, calculated nitrogen balance rose appreciably (+57%, from 683 to 970 mmol of nitrogen/12 h; $P = 0.0053$) during citrulline administration. This improved nitrogen balance is consistent with a putative rise in the rate of whole body protein synthesis and agrees with the enhanced rate of nitrogen accretion observed upon citrulline supplementation in animals by Osowska et al. (28, 29).

Taken together, the findings of the present study lead us to speculate oral citrulline could be used, either as a means to deliver arginine to the systemic circulation or as a putative protein anabolic agent, in specific clinical situations. Arginine indeed may become a conditionally essential amino acid in conditions associated with intestinal damage, such as radiation-induced enteritis (23), celiac disease (8), short bowel syndrome (7, 33), or premature birth (4, 44). For instance, after intestinal resection, the main site of citrulline production is essentially removed, and consequently plasma arginine level is reduced. Accordingly, citrulline supplementation increased arginine pools and restored nitrogen balance in rodents who had undergone extensive intestinal resection (28). The authors argue that citrulline may be a better candidate than arginine for supplementation, because extensive uptake and metabolism of arginine by the liver may cause excessive ureagenesis (9). In preterm infants, the limited expression of pyrroline-5-carboxylase, ASS, and ASL results in low rates of citrulline and arginine synthesis (4, 44). Because arginine is a precursor of NO, arginine deficiency results in decreased NO production, which could lead to intestinal dysfunction or necrotizing enterocolitis. Accordingly, in a clinical trial, arginine supplementation prevented necrotizing enterocolitis in a group of very-low-birth-weight infants (1). ASS and ASL activities can be enhanced by corticosteroid treatment (44). To our knowledge, the effects of combined corticosteroids and citrulline to raise arginine availability and potentially prevent necrotizing enterocolitis have yet to be examined in the latter population of patients.

In summary, the present study demonstrates that plasma citrulline can be depleted by depletion of glutamine and raised by oral citrulline supplementation, implying that citrulline is efficiently absorbed in the gut and reabsorbed by the kidney. In addition, to the best of our knowledge, this study is the first to suggest that oral citrulline can be used to raise arginine availability without affecting urea excretion and may enhance nitrogen balance in healthy humans. Further studies are warranted to determine whether citrulline has a true protein anabolic effect in vivo in humans and, if so, determine what mechanism(s) could mediate this putative effect.

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