Supplementation

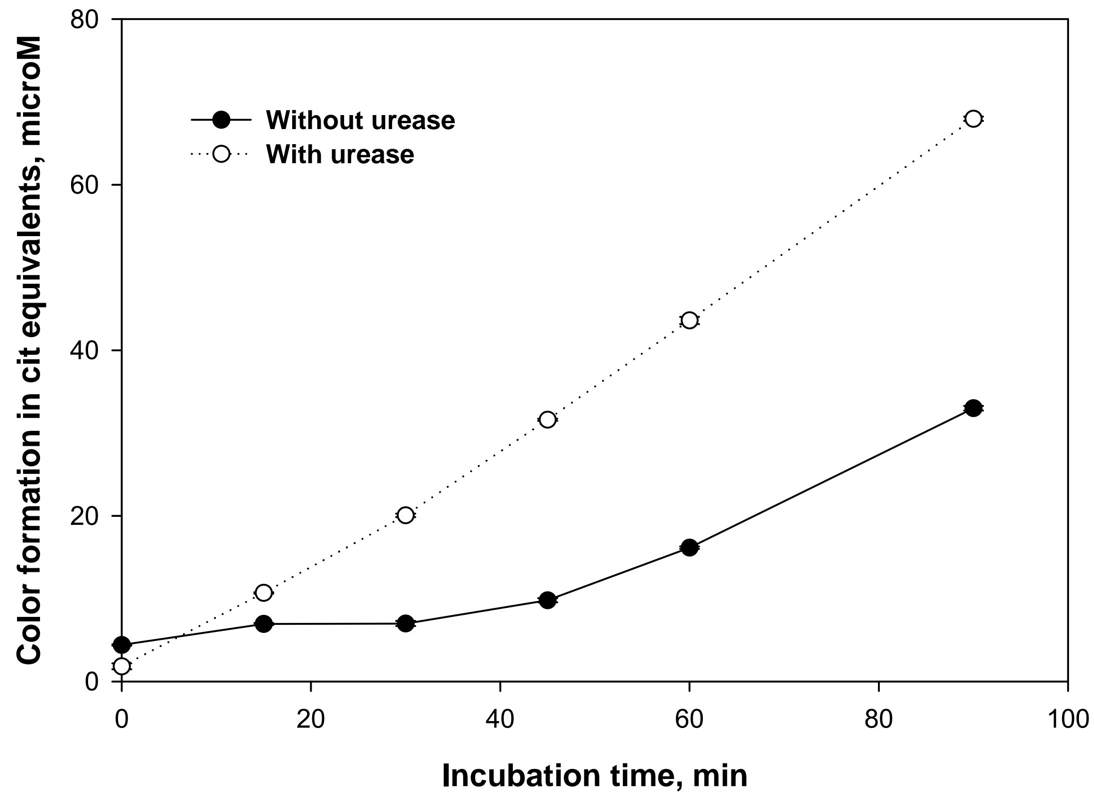
Table S1 Effect of deproteinization reagents on absorbance of blank.

	Absorbance
2mg/ml kidney homogenate without deproteinzation	1.114±0.275
Sulfosalicylic acid	
4%	0.227±0.006
10%	0.254±0.023
Trichloroacetic acid	
10%	0.790±0.016[a]
20%	1.332±0.085[a]
Sulfuric acid 4%	0.355±0.022
Hydrochloric acid 1N	0.357±0.011

All measurements were analyzed in triplicate.

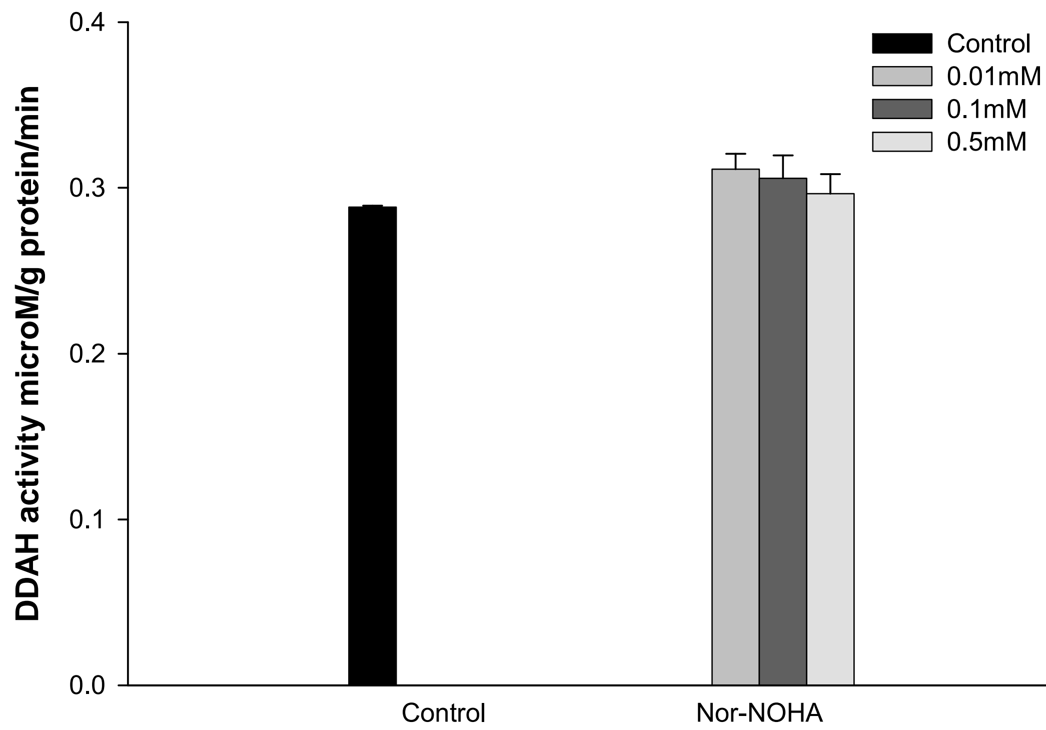
[a] The supernatant was opalescent.

Figure S1



Time course of color formation in citrulline equivalents in the presence of the DDAH substrate (ADMA) in the absence (solid circle) and presence of urease (open circle). Each time point was determined in triplicate.

Figure S2



The effect of arginase on the L -citrulline assay to detect renal DDAH activity. Nor-NOHA was used as arginase inhibitor. All measurements were analyzed in triplicate.

Supplementary methods

We tested 4 different homogenization buffers: HB1, pH=6.8 which contained 20 mM Tris, 1% Triton X-100, 5 mM EDTA, 10 mM EGTA, 2mM DTT, 1 mM sodium orthovanadate, 0.1 mg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and aprotinin; HB2 contained 0.1M sodium phosphate, pH=6.5 containing 2mM 2-mercaptoethanl; HB3 contained 0.1M sodium phosphate, pH=6.5; and HB4 was RIPA buffer (Santa Cruz), which contained 20 mM Tris, pH=7.6, 137mM sodium chloride, 0.2% Nonidet P-40, 0.1% sodium deoxycholate, 0.02% SDS, 0.0008% sodium azide, and protease inhibitor cocktail.

L-citrulline, sulfosalicylic acid, trichloroacetic acid, sulfuric acid, antipyrine, sodium nitrite and urease were purchased from Sigma. ADMA and diethylamine NONOate (DEA NONOate) were purchased from Cayman, 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ) was from AG Scientific Inc., N^{W}-Hydroxy-nor-L-arginine (nor-NOHA) was from Calbiochem. Diacetyl monoxime was from Fisher. The 96-well polystyrene plate and thermo resistant sealing tape were from Costar Corning Inc.

1mM ADMA in sodium phosphate buffer 0.1M, pH=6.5 were prepared for use as substrate. 4-20% sulfosalicylic acid, 4% sulfuric acid, and 1N hydrochloric acid were prepared as deproteinization solutions. As shown in Table S1, in the absence of deproteinization the absorbance was very high (due to detection of protein-bound L-citrulline and increased turbidity) and the optimum deproteinization solution (giving the minimal absorbance was 4% sulfosalicylic acid). L-citrulline standard solution was made by adding 17.5mg of L-citrulline to 1000ml sodium phosphate buffer to make 100μM standard, used as stock solution. Oxime reagent (0.8%) was made by adding 0.8g of

diacetyl monoxime in 100ml of 5% (v/v) acetic acid. This solution was stored in the dark at 4°C. Antipyrine/H_2SO_4 reagent (0.5%) was made by adding 0.5g antipyrine in 100ml of 50% (v/v) sulfuric acid. 1mM and 0.1mM DMNQ was prepared as superoxide donor; 1mM and 0.1mM sodium nitrite and DEA NONOate were used as NO donors; 0.01mM, 0.1mM, and 0.5mM nor-NOHA was prepared for inhibition of arginase.

Pilot studies for optimization of assay

In pilot studies, in the presence of 25μM L-citrulline, we tested the effect on background color of 4 different homogenization buffers (HB1-4) and the following common additives: 0.1M sodium phosphate buffer (pH=6.5), 1% Triton X-100, 1M HEPES, 0.3M sucrose, 100nM urea, 0.9% NaCl, 0.1M DTT, 1% 2-mercaptoethanol, 0.5% Tween, 1% SDS, 0.5M EDTA, and 0.2M EGTA. 0.1M sodium phosphate buffer, pH=6.5 containing protease inhibitors (0.1 mg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and aprotinin) was examined for the effect of protease inhibitors on color formation. We also determined whether protease inhibitors were required for stability of DDAH in this assay. We found that protease inhibitors were not required and that the simple HB3 gave optimal color. Other reagents, 1mM ADMA and 4% sulfosalicylic acid, used in this assay were also examined for their impact on color formation. The effect of protein content was tested by using BSA and kidney homogenate at the concentrations of 1 and 2 mg/ml. Serial diluted L-citrulline standards (0-100 μM) were prepared in distilled water. All samples were analyzed in triplicate. The contribution of buffer/additive to color formation was represented as percent of mean absorbance of each sample compared to

25μM L-citrulline (=100%). The findings from these pilot studies were used to optimize the assay for kidney tissue.

Another time course study was conducted to decide the ideal incubation time. This was similar to the urea time course study except that 400 μl of 5mM ADMA was used to replace sodium phosphate buffer. Moreover, the effect of different blanks was also analyzed: The first blank (B1) simply omitted the substrate ADMA; the second blank (B2) is boiled homogenate to stop the reaction at t=0; and the third blank (B3) is treated with acid to stop the reaction at t=0. It is theoretically possible that citrulline could be simultaneously consumed by the ASS/ASL enzymes that are abundant in kidney. We incubated kidney homogenate with excess citrulline (200μM) in the absence of ADMA (n=4). At incubation time was 0 and 90min, 0.5ml of 4% sulfosalicylic acid was added for deproteinization and assayed. We found citrulline formation at t=0 and 90 min were similar, suggesting no citrulline consumption. The urea effect was examined by incubation of 100μl of kidney cortex and medulla homogenate (20mg/ml) and different concentrations of urea solutions (1, 5, 10, 50, and 100nM) in the presence or absence of urease (100U/ml homogenate) at 37ºC for 15min.

When the assay had been optimized for kidney we also investigated the impact of arginase inhibition (with nor-NOHA) and possible inhibitory actions by NO or superoxide (using DEA NONOate, nitrate, and DMNQ) on DDAH activity. Kidney homogenate was pre-incubated with urease at 37C for 15min, then 400ul of mixture of ADMA and drugs were added to the homogenate and incubated at 37ºC for 45min.

To determine the inter- and intra-assay variability we ran supernatant of kidney cortex (which after deproteinization could be stored at -80°C and remained stable after 1 freeze/thaw cycle) in 12 different assays and 9 times in one assay.