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RESEARCH ARTICLE | *Renal Hemodynamics*

Calcium channel blockade blunts the renal effects of acute nitric oxide synthase inhibition in healthy humans

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Montanari A, Lazzeroni D, Pelà G, Crocamo A, Lytvyn Y, Musiari L, Cabassi A, Cherney DZ. Calcium channel blockade blunts the renal effects of acute nitric oxide synthase inhibition in healthy humans. *Am J Physiol Renal Physiol* 312: F870–F878, 2017. First published February 8, 2017; doi:10.1152/ajprenal.00568.2016.—Our aim was to investigate whether blockade of calcium channels (CCs) or angiotensin II type 1 receptors (AT1R) modulates renal responses to nitric oxide synthesis inhibition (NOSI) in humans. Fourteen sodium-replete, healthy volunteers underwent 90-min infusions of 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ N^G-nitro-L-arginine methyl ester (L-NAME) on 3 occasions, preceded by 3 days of either placebo (PL), 10 mg of manidipine (MANI), or 50 mg of losartan (LOS). At each phase, mean arterial pressure (MAP), glomerular filtration rate (GFR; inulin), renal blood flow (RBF; p-aminohippurate), urinary sodium (UNaV), and 8-isoprostane (U8-iso-PGF2αV; an oxidative stress marker) were measured. With PL + L-NAME, the following changes were observed: +6% MAP ($P < 0.005$ vs. baseline), −10% GFR, −20% RBF, −49% UNaV ($P < 0.001$), and +120% U8-iso-PGF2αV ($P < 0.01$). In contrast, MAP did not increase during LOS + L-NAME or MANI + L-NAME ($P > 0.05$ vs. baseline), whereas renal changes were the same during LOS + L-NAME vs. PL + L-NAME (ANOVA, $P > 0.05$). However, during MANI + L-NAME, changes vs. baseline in GFR (−6%), RBF (−12%), and UNaV (−34%) were blunted vs. PL + L-NAME and LOS + L-NAME ($P < 0.005$), and the rise in U8-iso-PGF2αV was almost abolished (+37%, $P > 0.05$ vs. baseline; $P < 0.01$ vs. PL + L-NAME or LOS + L-NAME). We conclude that, since MANI blunted L-NAME-induced renal hemodynamic changes, CCs participate in the renal responses to NOSI in healthy, sodium-replete humans independent of changes in MAP and without the apparent contribution of the AT1R. Because the rise in U8-iso-PGF2αV was essentially prevented during MANI + L-NAME, CC blockade may oppose the renal effects of NOSI in part by counteracting oxidative stress responses to acutely impaired renal NO bioavailability.

renal hemodynamic function; nitric oxide; N^G-nitro-L-arginine methyl ester; manidipine; losartan

NITRIC OXIDE (NO) is an endogenous vasodilator that plays a major role in the endothelial control of vascular function. NO is produced by NO synthase (NOS) in response to receptor-dependent agonists (i.e., acetylcholine and bradykinin) and physiochemical stimuli (i.e., shear stress) (36). In the kidney, NO-mediated control of the vascular tone contributes to the maintenance of renal blood flow (RBF), glomerular filtration rate (GFR) (5–8, 12–15, 17, 18, 23, 27, 31–33, 36–38, 40, 41, 43–45), blood pressure (BP)-induced RBF autoregulation (28, 29), sodium (Na^+) excretion (21), renin release (30), and tubule glomerular feedback (49). In various pathological conditions NOS activity may be reduced, and this is associated with enhanced production of reactive oxygen species (ROS), such as superoxide (O_2^-), that rapidly reacts with NO to form a highly reactive peroxynitrite intermediate ($ONOO^-$). The resulting decrease in NO bioavailability leads to abnormal endothelium-dependent vasomotor responses, which are defined as "endothelial dysfunction" (ED) (10, 11, 19, 22, 36, 48). ED is implicated in the pathogenesis and progression of clinical conditions with vascular risk such as hypertension, diabetes, dyslipidemia, heart failure, and chronic kidney disease (4, 6, 10, 11, 19, 22, 48).

Because of the significant role of ED in human cardiovascular and renal diseases, it is important to know whether pharmacological interventions blunt the renal hemodynamic response to an acute NOS inhibition. This issue has been addressed previously in a few studies in humans undergoing systemic infusion of NOS inhibitors, using angiotensin II (AII) type 1 receptor (AT1R)-antagonists (ARBs) (7, 33, 37, 38, 40) and calcium channel blockers (CCBs) (17, 18).

Because NOS inhibitors are known to markedly potentiate renal actions of exogenous AII, whereas AII accentuates renal vasoconstriction to NOS inhibition (1, 2, 36), an attenuation by AT1R blockade of NOS inhibition-induced renal changes could be expected. However, in most studies, ARBs did not influence the renal hemodynamic changes due to NOS inhibition in unstressed experimental animals (2) or humans (7, 33, 37, 38, 40).

CCBs act on L- and/or T-type voltage-dependent calcium channels (CCs) (9, 16, 20, 24, 25, 29), the activity of which is also inhibited by NO in different tissues, including the kidney (9, 16, 20, 29). CCBs also scavenge O_2^- and decrease oxidative stress, thereby increasing NO bioavailability (9, 16). As a consequence, CCBs attenuate renal vasoconstriction responses caused by NOS inhibition in animals (2) and humans (17). However, NOS inhibitors such as N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine methyl-ester (L-NAME),

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when infused systemically, also result in substantial blood pressure (BP) increases, which are blunted or reversed by CCBs (2, 17). Therefore, the relative contribution of reduced intrarenal NO bioavailability to CC-mediated renal vasoconstriction vs. the nonspecific influences of simultaneous BP variations remains uncertain.

The aim of the present study was to investigate whether and to what extent CCs participate in the renal responses to NOS inhibition independent of BP changes. For this purpose, healthy humans on Na^+ repletion underwent NOS inhibition experiments with low-dose, systemic L-NAME infusion after short-term pretreatment with either placebo or a CCB. Manidipine (MANI) was the CCB used because of its action on the L-type CCs in the afferent renal arteriole and T-type CCs in the efferent arteriole (24, 25, 47). The effects of MANI pretreatment were also compared with those of AT1R blockade with losartan (LOS), which, under the same experimental conditions, does not prevent renal hemodynamic and excretory effects of NOS inhibition in man (7, 33, 37, 38, 40).

METHODS

Participants. Fourteen healthy volunteers, 10 males and four females, were included in the study. All participants provided written, informed consent according to the ethics protocols of the Parma University Medical School. Participants had no history of heart, liver, kidney, or endocrine disease, had never smoked or abused drugs and alcohol, and did not take any medication. Obesity, diabetes, lipid disturbances, liver or kidney disease, and atherosclerotic diseases were excluded on the basis of clinical examination, laboratory screening electrocardiogram, and abdominal, vascular, and heart ultrasonography studies. All participants had normal blood pressure (BP), with mean sitting systolic pressure (SAP) <130 mmHg and diastolic pressure (DAP) <80 mmHg, as measured on three separate visits. Anthropometric measurements, BP values, and laboratory results at baseline are summarized in Table 1.

Experimental design and procedures. Participants underwent in a randomized order three infusion studies, with a washout period of ~2 wk for men and 4 wk for women, who were studied during the follicular phase of the menstrual cycle. Each subject was trained by a dietitian to maintain for 7 days before each infusion a detailed, written dietary regimen that contained 250 ± 10 mmol Na^+, 80 ± 6 mmol K^+, and 2,450 ± 70 kcal daily (55% carbohydrates, 15% protein and 30% lipids) and a fixed intake of antioxidants and $NO_2 + NO_3$ (NO_x) based on content in vegetables, fruit, and processed meats (26). The adherence to dietary requirements was estimated at the baseline of each infusion, based on written food record, and measured urinary excretion of sodium (UNaV), urea nitrogen (UUN), and $NO_2 + NO_3$ (UNO_xV) (38). To avoid any confounding disturbance on prostaglandin production (40), over-the-counter, nonsteroidal, anti-inflammatory drugs were not permitted during the study.

Table 1. *Demography, clinical data, and baseline laboratory results of 14 healthy subjects included in the study*

Sex, M/F	10/4
Age, yr	33 ± 1
Body weight, kg	69.7 ± 2.0
Body surface area, m^2	1.77 ± 0.03
Systolic BP, mmHg	111.2 ± 0.9
Diastolic BP, mmHg	65.8 ± 0.7
Plasma creatinine, mmol/l	0.081 ± 0.002
Plasma Na^+, mmol/l	141.9 ± 1.0
Plasma K^+, mmol/l	4.4 ± 0.1
Plasma Cl^-, mmol/l	104.9 ± 0.7
Plasma uric acid, mmol/l	0.32 ± 0.02
Plasma cholesterol, mmol/l	4.5 ± 0.3
Plasma triglycerides, mg/dl	83.9 ± 7.5
Fractional hematocrit	0.431 ± 0.015
Blood glucose, mmol/l	4.6 ± 0.1
Glycated hemoglobin, %	5.3 ± 0.3
Urine albumin excretion, μg/min	5.4 ± 1.2
UNaV, μmol/min*	144 ± 14
Recumbent PRA, ng Ang I·ml^{-1}·h^{-1}*	1.48 ± 0.31

Values are means ± SE. M, males; F, females; BP, blood pressure; UNaV, urinary sodium; PRA, plasma renin activity; Ang I, angiotensin I. *On habitual diet.

In the 3 days before each study, participants received either PL, 50 mg of LOS, or 10 mg of MANI at 10 PM, thus preventing substantial systemic and renal hemodynamic changes related to an acute AT1R or CC blockade. In particular, such doses of LOS counteract effectively in humans the BP effects of exogenous AII (39) and also prevent the low-dose L-NAME-induced BP increases even 10–12 h after administration (37, 38, 40).

After an overnight fast, experiments were initiated at 7 AM, with the participant on a seated position that was maintained for the entire study also receiving 300 ml of tap water hourly. Two plastic indwelling catheters patented with 1.0 ml/h 0.9% NaCl were immediately placed in both arms, the left for infusions and the right for blood sampling. After 2 h, priming doses of 800 mg/1.73 m^2 para-aminohippurate (PAH) and 3,000 mg/1.73 m^2 inulin (INU) were injected. Then, a constant rate infusion of 7–9 mg/min PAH and 18–22 mg/min INU was immediately initiated and continued throughout the entire study, using a 50-ml syringe precision pump to maintain steady plasma levels of ~0.015 and 0.2 mg/ml, respectively. After 1 h of PAH and INU infusion and voiding, a 45-min baseline clearance period (−45 min to *time 0*) was performed. At *time 0*, after voiding again, a pump infusion of 3.0 μg·kg^{-1}·min^{-1} L-NAME was initiated, and two additional 45-min clearance periods were performed (L-NAME P1 from *time 0* to +45 min and L-NAME P2 from +45 to + 90 min, respectively); then the experiment was stopped (37, 38, 40). At the end of each 45-min period, subjects voided, and samples of urine volume were taken for Na^+ excretion, whereas UNO_xV and excretion of 8-isoprostane ($U8\text{-}iso\text{-}PGF_{2\alpha}V$), the latter used as an in vivo index of oxidative stress (OS), were measured at baseline and in the urine volume from the entire L-NAME infusion (38). Heparinized blood samples were drawn for PAH and INU every 15 min and PRA and Na^+ at the end of the baseline and L-NAME P2 periods. Systolic (SAP) and diastolic BP (DAP) and heart rate (HR) were measured every 5 min in each period using an automated, oscillometric device (TM 2421; A & D, Tokyo, Japan).

Calculations and analytical methods. Mean arterial pressure (MAP) was calculated as [SAP + (2 × DAP)/3]. Steady-state plasma levels of PAH and INU were maintained throughout the infusion, with a variability in the baseline period (1.8% PAH, 2.5% INU) close to that in duplicate analysis of single plasma samples (1.5 and 2.0%, respectively). Effective renal plasma flow (ERPF) and glomerular filtration rate (GFR) were estimated without urine PAH and INU being measured on the basis of the steady-state infusion clearance technique (37, 38, 40), thus avoiding an unethical bladder catheterization. For this purpose, the PAH and INU infusion rates (mg/min) that were calculated by multiplying the measured PAH and INU concentrations in infused solution (mg/ml) for the infusion rate (ml/min) were divided for each measured plasma PAH and INU concentration (mg/ml). Therefore, the results of four clearance measurements (as ml/min) for the baseline period and three measurements for each L-NAME period were averaged to express data that were referred to as 1.73 m^2. Based on the fixed renal PAH extraction at low plasma levels, no correction was made for PAH clearance, which, therefore, was considered equal to the ERPF. Filtration fraction (FF), RBF, renal vascular resistance (RVR), and Na^+ fractional excretion rate (FENa) were then calculated as GFR/ERPF, ERPF/(1-fractional

hematocrit), MAP/RBF, and urinary Na^+ clearance/GFR, respectively.

Standard methods were used for routine clinical and biochemical measurements. Methods for Na^+, UUN, PAH, INU, PRA, UNO_xV, UAE, and U8-iso-$PGF_{2\alpha}V$ were described elsewhere (37, 38, 40).

Study drugs. PAH and INU (20 and 10% solution, respectively) were purchased from J. Monico, Venice, Italy. Commercially available 50 mg of LOS and 10-mg MANI tablets were used, whereas pharmaceutical-grade L-NAME (10-mg ampoules) was obtained from Clinalfa.

Statistics. Data were expressed as means ± SE. Paired Student's *t*-test was used for differences at baseline between different phases of the study, for changes in variables from baseline to the end of each L-NAME infusion, and for those in UNO_xV from baseline to the pooled urine collection during L-NAME infusions, whereas for U8-iso-PGF2αV analysis the nonparametric Wilcoxon signed-rank test was required because of a nonnormal distribution according to a Kolmogoroff-Smirnoff test. For the comparison of changes in MAP, GFR, ERPF, RBF, RVR, and Na^+ urinary excretion vs. time in response to L-NAME infusions after the three different pretreatments, analysis of variance (ANOVA), followed by Bonferroni's test for multiple comparisons, was performed (SPSS 20.0 software package; IBM, Armonk, NY). Time and type of treatment (PL, MANI, or LOS) were considered as the factors exerting an interaction in the analysis of data. Two-tailed $P \leq 0.05$ was considered to indicate statistical significance.

RESULTS

Effects of L-NAME on BP and renal hemodynamic function. The baseline MAP, similar between PL+L-NAME and LOS+L-NAME, was slightly lower after MANI ($P < 0.05$ vs. both PL and LOS), with a mean −2.7 to −2.0 mmHg difference vs. PL and LOS, respectively (Table 2). As a consequence, RVR also was slightly lower ($P < 0.05$) after MANI vs. both PL and LOS, whereas neither previous LOS nor MANI significantly affected the baseline GFR (Fig. 1), RBF (Fig. 2), or FF ($P > 0.05$ vs. PL).

With L-NAME infusion, HR decreased approximately by 5 beats/min ($P < 0.005$ vs. baseline), a variation that was essentially equal in each study phase ($P > 0.05$).

During PL + L-NAME, MAP did not change during the L-NAME P1 time period ($P > 0.05$ vs. baseline), although the overall time course analysis showed a significant increase ($P < 0.005$ vs. time) due to a late 6% rise ($P < 0.005$ vs. baseline) in the L-NAME P2 only. In contrast, during both LOS + L-NAME and MANI + L-NAME, MAP remained unchanged either in L-NAME P1 ($P > 0.05$ vs. baseline) or in the overall time course ($P > 0.05$ vs. time), with a significant interaction (ANOVA $P < 0.005$ vs. PL + L-NAME) exerted by either pretreatment on MAP time course.

With PL+ L-NAME, GFR (−10%; Fig. 1) and RBF (−20%; Fig. 2) decreased substantially, and FF (+13%) and RVR (+33%) increased ($P < 0.001$ vs. time). These renal hemodynamic changes were significant even when MAP was not changed vs. baseline at L-NAME P1 [$P < 0.05$ GFR (Fig. 1), $P < 0.01$ RBF (Fig. 2), $P < 0.05$ FF, and $P < 0.01$ RVR]. With LOS + L-NAME, the time course for renal hemodynamic parameters was similar vs. PL + L-NAME (ANOVA interaction by type of treatment: $P > 0.05$; Figs. 1 and 2). In contrast, with MANI + L-NAME, these changes, although still significant at L-NAME P1 vs. baseline as well as vs. time, were markedly blunted vs. both PL + L-NAME and LOS + L-NAME [−6% GFR (Fig. 1), −12% RBF (Fig. 2), +7% FF, and +16% RVR; ANOVA interaction by type of treatment: $P < 0.005$, except $P < 0.001$ for RVR].

Table 2. *Baseline urinary excretion rate of urea nitrogen and time course of blood pressure and renal hemodynamic function in 14 healthy volunteers during 3 infusion studies, with each divided into 2 consecutive 45-min periods (L-NAME P1 and L-NAME P2) of 3 $\mu g \cdot kg^{-1} \cdot min^{-1}$ L-NAME and preceded by 3 days of treatment with either PL + L-NAME, LOS + L-NAME, or MANI + L-NAME*

Time Period	PL + L-NAME	LOS + L-NAME	MANI + L-NAME
UUN, mg/min			
Baseline	11.0 ± 2.1	10.3 ± 2.0	10.8 ± 1.8
SAP, mmHg			
Baseline	108.2 ± 0.4	107.2 ± 0.5	104.0 ± 0.3*
L-NAME P1	109.0 ± 0.3	108.5 ± 0.5	104.8 ± 0.2
L-NAME P2	112.3 ± 0.3†§	108.9 ± 0.5	105.2 ± 0.3
DAP, mmHg			
Baseline	62.9 ± 0.4	62.3 ± 0.5	61.0 ± 0.2
L-NAME P1	63.6 ± 0.3	62.9 ± 0.4	61.4 ± 0.3
L-NAME P2	68.3 ± 0.4†§	63.9 ± 0.5	61.9 ± 0.4
MAP, mmHg			
Baseline	78.0 ± 0.4	77.3 ± 0.7	75.3 ± 0.4*
L-NAME P1	78.7 ± 0.3	78.1 ± 0.4	75.9 ± 0.4
L-NAME P2	83.0 ± 0.4†§	78.9 ± 0.5	76.3 ± 0.3
HR, beats/min			
Baseline	65 ± 0.4	66 ± 0.6	65 ± 0.4
L-NAME P1	62 ± 0.6	62 ± 0.6	63 ± 0.6
L-NAME P2	60 ± 0.7†	60 ± 0.6†	59 ± 0.7†
GFR, $ml \cdot min^{-1} \cdot 0.1.73\ m^{-2}$			
Baseline	101.4 ± 2.6	100.7 ± 3.4	103.6 ± 2.6
L-NAME P1	95.3 ± 2.9#	94.2 ± 3.2#	99.3 ± 2.6#
L-NAME P2	90.7 ± 2.9‡	89.3 ± 3.3‡	96.9 ± 2.6†‖
ERPF, $ml \cdot min^{-1} \cdot 0.1.73\ m^{-2}$			
Baseline	549.8 ± 27.0	555.6 ± 30.1	578.8 ± 24.2
L-NAME P1	479.6 ± 29.4**	489.0 ± 33.2**	529.6 ± 31.0**
L-NAME P2	441.3 ± 35.0‡	440.4 ± 39.0‡	506.3 ± 32.7†‖
FF			
Baseline	0.185 ± 0.014	0.181 ± 0.012	0.179 ± 0.016
L-NAME P1	0.199 ± 0.017**	0.193 ± 0.016**	0.187 ± 0.018**
L-NAME P2	0.210 ± 0.019‡	0.203 ± 0.018‡	0.191 ± 0.017†‖
RBF, $ml \cdot min^{-1} \cdot 0.1.73\ m^2$			
Baseline	948.9 ± 27.1	957.9 ± 28.2	997.9 ± 29.3
L-NAME P1	826.9 ± 28.4**	843.1 ± 31.8**	913.1 ± 30.4**
L-NAMEP2	760.3 ± 30.0‡	759.3 ± 32.6‡	872.9 ± 32.5†‖
RVR, $mmHg \cdot min^{-1} \cdot l^{-1}$			
Baseline	82.2 ± 2.2	80.7 ± 2.9	75.6 ± 3.1*
L-NAMEP1	95.2 ± 2.5**	92.6 ± 3.2**	83.1 ± 3.4**
L-NAMEP2	109.2 ± 3.1‡	103.9 ± 3.5‡	87.4 ± 3.6‡¶

Values are means ± SE. L-NAME, N^G-nitro-L-arginine-methyl-ester; PL + L-NAME, placebo; LOS + L-NAME, 50 mg of losartan; MANI + L-NAME, 10 mg of manidipine; UUN, urinary excretion rate of urea nitrogen; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; MAP, mean arterial pressure; HR, heart rate; GFR, glomerular filtration rate; FF, filtration fraction; ERPF, effective renal plasma flow; RBF, renal blood flow; RVR, renal vascular resistance. *$P < 0.05$ for MANI + L-NAME vs. PL + L-NAME and LOS + L-NAME at baseline; †$P < 0.005$; ‡$P < 0.001$, ANOVA for time-dependent changes; §$P < 0.005$, ANOVA comparison of time-dependent changes during PL + L-NAME vs. MANI + L-NAME and LOS + L-NAME (interaction by type of treatment). ‖$P < 0.005$; ¶$P < 0.001$, ANOVA comparison of time-dependent changes during MANI + L-NAME vs. PL + L-NAME and LOS + L-NAME (interaction by type of treatment); #$P < 0.05$; **$P < 0.01$, L-NAME P1 vs. baseline.

To summarize, L-NAME infusion lowered GFR (Fig. 1) and RBF (Fig. 2) and increased FF and RVR. These effects, which developed to a significant extent before any BP change, were not influenced by AT1R blockade, whereas MANI administration led to blunted responses.

L-NAME effects on PRA and urinary excretion of Na^+ and metabolites. As predicted, baseline PRA was markedly elevated after LOS vs. PL and MANI ($P < 0.001$; Table 3). However, the expected absolute decrease (30) in PRA with

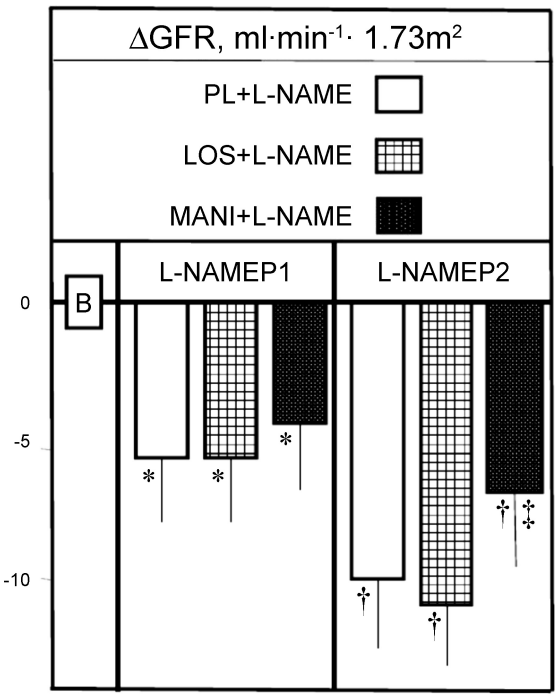

Fig. 1. Decreases from the baseline values (B) in glomerular filtration rate (ΔGFR) in 14 healthy individuals during 3 infusion studies of 3 $\mu g \cdot kg^{-1} \cdot min^{-1}$ N^G-nitro-L-arginine-methyl-ester (L-NAME), with each divided into 2 consecutive 45-min periods (L-NAME P1 and L-NAME P2) and preceded by 3-day treatment with either placebo (PL + L-NAME), 50 mg of losartan (LOS + L-NAME), or 10 mg of manidipine (MANI + L-NAME) (means ± SE). *ΔGFR at this time period already was significant ($P < 0.05$); †significance of ΔGFR in the whole time course ($P < 0.001$ vs. time; see Table 2); ‡ΔGFR in the whole time course was significantly blunted vs. both PL + L-NAME and LOS + L-NAME (ANOVA $P < 0.005$, interaction by type of treatment), with $P > 0.05$ between PL + L-NAME and LOS + L-NAME.

L-NAME ($P < 0.01$ vs. baseline) was approximately the same during the PL, MANI, and LOS phases of the study ($P > 0.05$).

At the baseline of each L-NAME infusion, UNaV and FENa averaged ~160 μmol/min and 1.15%, respectively, thereby matching remarkably the high Na^+ content of the recommended diet. UUN, UNO_xV, and U8-iso-PGF2αV also were similar.

In response to PL+ L-NAME, both UNaV and FENa declined (−48 and −41%, respectively, $P < 0.001$ vs. time), which was consistent with the well-known action of NOS inhibition of lowering filtered Na^+ load and increasing tubular reabsorption as well (21, 37, 38, 40). Such variations were already significant during the PL + L-NAME P1 period ($P < 0.05$ vs. baseline; Fig. 3). The declines in UNaV and FENa in response to LOS + L-NAME did not differ significantly from those following PL + L-NAME ($P > 0.05$, ANOVA interaction by treatment; Fig. 3). In contrast, during MANI + L-NAME, although there was still a significant decrease in UNaV (−34%, $P < 0.001$) and FENa (−29%, $P < 0.001$), it was attenuated vs. PL+ L-NAME and LOS + L-NAME (ANOVA interaction by treatment: $P < 0.01$; Fig. 3). To summarize, the expected reduction in Na^+ excretion during PL + L-NAME was not affected by LOS administration, but it was substantially blunted by CC blockade.

With PL + L-NAME, UNO_xV decreased significantly and U8-iso-PGF2αV increased (both $P < 0.01$; Fig. 4), with similar changes during LOS + L-NAME ($P > 0.05$ vs. PL + L-NAME). In contrast, with MANI + L-NAME, although UNO_xV still declined ($P < 0.05$), such a decrease was blunted vs. PL + L-NAME and LOS + L-NAME ($P < 0.05$), and the change in U8-iso-PGF2αV was not significant ($P > 0.05$ vs. baseline, $P < 0.005$ vs. PL + L-NAME and LOS + L-NAME). To summarize, L-NAME infusion lowered UNO_xV and increased U8-iso-PGF2αV. Although AT1R-blockade did not affect these changes, CC blockade blunted the fall in UNO_xV and abolished the rise in U8-iso-PGF2αV.

DISCUSSION

The first goal of our study was to determine whether and to what extent blockade of either CC or AT1R alters renal hemodynamic responses to acute NOS inhibition in healthy volunteers on a Na^+-replete diet independently of changes in BP. Because of the substantial BP rise that invariably follows the systemic infusion of standard-dose L-NMMA or high-dose L-NAME (8, 33, 35, 42), an accentuated RBF decrease is expected in response to systemic NOS inhibition, and as a consequence the BP-dependent mechanisms of renal hemody-

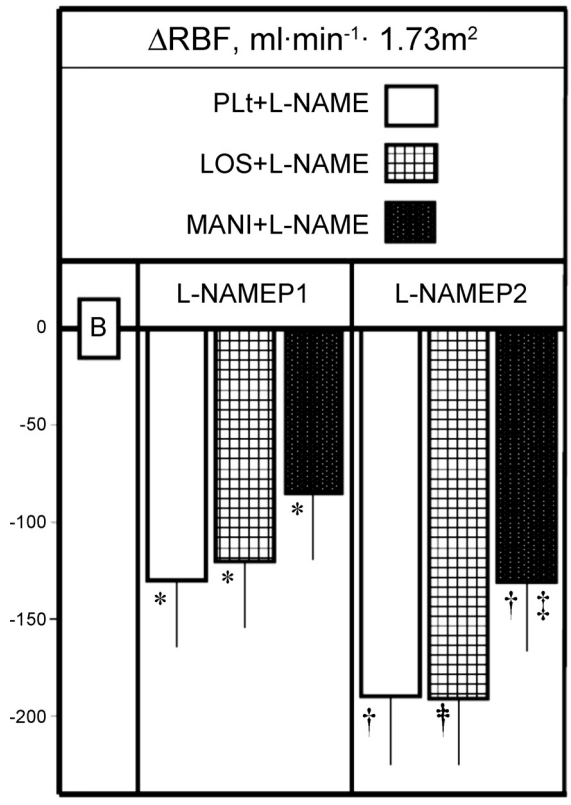

Fig. 2. Decreases in renal blood flow (ΔRBF) from the baseline values (B) in 14 healthy individuals during 3 infusion studies of 3 $\mu g \cdot kg^{-1} \cdot min^{-1}$ N^G-nitro-L-arginine-methyl-ester (L-NAME), with each divided into 2 consecutive 45-min periods (L-NAME P1 and L-NAME P2) and preceded by 3-day treatment with either placebo (PL + L-NAME), 50 mg of losartan (LOS + L-NAME), or 10 mg of manidipine (MANI + L-NAME) (means ± SE). *Already significant ΔRBF ($P < 0.01$) at this time period in each infusion; †$P < 0.001$ significance of ΔRBF in the whole time course of each infusion; ‡ΔRBF in the whole time course of infusion was significantly reduced vs. both PL + L-NAME and LOS + L-NAME (ANOVA, $P < 0.005$, interaction by type of treatment), with $P > 0.05$ between PL + L-NAME and LOS + L-NAME.

Table 3. *Changes in plasma renin activity, plasma Na^+, and urinary excretion of Na^+, $NO_2 + NO_3$ and 8-isoprostane in 14 healthy volunteers during 3 infusion studies, with each divided into 2 consecutive 45-min periods (L-NAME P1 and L-NAME P2), of 3 $\mu g \cdot kg^{-1} \cdot min^{-1}$ L-NAME, preceded by 3-day treatment with either PL + L-NAME, LOS + L-NAME, or MANI + L-NAME*

Time Period	PL + L-NAME	LOS + L-NAME	MANI + L-NAME
PRA, ng Ang $I \cdot ml^{-1} \cdot h^{-1}$			
Baseline	1.21 ± 0.13	2.88 ± 0.30*	1.12 ± 0.15
End L-NAME	0.85 ± 0.15†	2.35 ± 0.27†	0.89 ± 0.17†
Plasma Na^+, mmol/l			
Baseline	140.0 ± 0.4	140.3 ± 0.5	140.0 ± 0.5
End L-NAME	139.7 ± 0.3	139.8 ± 0.6	139.9 ± 0.4
UNaV, μmol/min			
Baseline	158 ± 12	164 ± 13	166 ± 14
L-NAME P1	104 ± 11††	100 ± 13††	135 ± 17††
L-NAME P2	81 ± 18‡	76 ± 14‡	110 ± 14‡§
FENa × 10^2			
Baseline	1.11 ± 0.10	1.16 ± 0.12	1.14 ± 0.12
L-NAME P1	0.78 ± 0.09††	0.76 ± 0.12††	0.97 ± 0.12††
L-NAME P2	0.64 ± 0.11‡	0.62 ± 0.09‡	0.81 ± 0.11‡§
UNO_xV, μmol/min			
Baseline	1.43 ± 0.20	1.57 ± 0.28	1.64 ± 0.25
L-NAME P1+ P2	0.57 ± 0.17‖	0.68 ± 0.44‖	1.24 ± 0.34¶#
U8-iso-PGF2αV, pg/min			
Baseline	187 ± 34	166 ± 36	171 ± 30
L-NAME P1+ P2	385 ± 40‖	417 ± 49‖	240 ± 47**

Values are means ± SE. L-NAME, N^G-nitro-L-arginine-methyl-ester; PL + L-NAME, placebo; LOS + L-NAME, 50 mg of losartan; MANI + L-NAME, 10 mg of manidipine; PRA, plasma renin activity; UNaV, Na^+ excretion rate; FENa, Na^+ fractional excretion rate; UNO_xV, urinary excretion rate of $NO_2 + NO_3$; U8-iso-PGF2αV, urinary excretion rate of 8-isoprostane (8-iso-$PGF_{2\alpha}$). *$P < 0.001$, LOS + L-NAME vs. PL + L-NAME or MANI + L-NAME at baseline; ‡$P < 0.001$, ANOVA for time-dependent changes; †$P < 0.01$, end L-NAME vs. baseline; §$P < 0.01$, ANOVA comparison of time-dependent changes during MANI + L-NAME vs. PL + L-NAME and LOS + L-NAME (interaction by type of treatment); ‖$P < 0.01$; ¶$P < 0.05$, L-NAME P1+ L-NAME P2 vs. baseline; #$P < 0.05$; **$P < 0.01$, comparison of changes from baseline during MANI + L-NAME vs. PL + L-NAME and LOS + L-NAME; ††$P < 0.05$, L-NAME P1 vs. baseline.

namic autoregulation act on the afferent arteriole (28, 29). Therefore, in an effort to minimize the nonspecific BP-related renal vasoconstriction, participants in each phase of the present study received a systemic infusion of L-NAME at a dose as low as 3 $\mu g \cdot kg^{-1} \cdot min^{-1}$. Consistent with observations using similar L-NAME doses (8, 37, 38, 40), NOS inhibitor infusion resulted in decreased ERPF, RBF, GFR, and UNaV and increased FF and RVR early in the PL + L-NAME P1 time period. As intended with the low L-NAME dose, however, during PL + L-NAME P1 there was no change in MAP, which in contrast increased only (+ 6%) during the subsequent PL + L-NAME P2 accompanied by more pronounced renal hemodynamic and Na^+ excretory responses vs. PL + L-NAME P1. Consistent with acute NOS inhibition studies (5–8, 31, 32, 42) and with PL + L-NAME (5–8, 31, 32, 42), HR showed an immediate and subsequently maintained 8% decrease, perhaps reflecting attenuated chronotropic effects of NO independent of sympathetic activity (32, 42) or changes in baroreflex activation in response to systemic vasoconstriction, leading to overall sympathetic inhibition (5–8, 31, 32, 42). Since BP was left unchanged during PL + L-NAME P1, such an autonomic response was likely sufficient to prevent any rise in BP. Therefore, in the early phases of our protocol, despite the

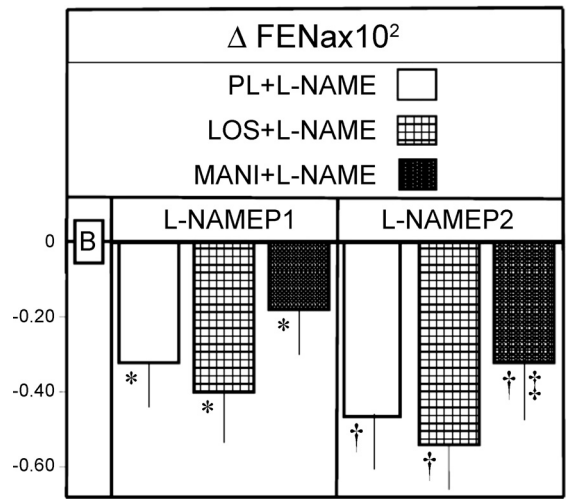

Fig. 3. Decreases from the baseline values (B) in fractional excretion of sodium (ΔFENa × 10^2) in 14 healthy individuals during 3 infusion studies of 3 $\mu g \cdot kg^{-1} \cdot min^{-1}$ N^G-nitro-L-arginine-methyl-ester (L-NAME), with each divided into 2 consecutive 45-min periods (L-NAME P1 and L-NAME P2) and preceded by 3-day treatment with either placebo (PL + L-NAME), 50 mg of losartan (LOS + L-NAME), or 10 mg of manidipine (MANI + L-NAME) (means ± SE). *Already significant ΔFENa ($P < 0.05$) at this time period in each infusion; †$P < 0.001$ significance of ΔFENa × 10^2 in the whole time course of each infusion; ‡ΔFENa in the whole time course was significantly reduced vs. both PL + L-NAME and LOS + L-NAME (ANOVA, $P < 0.005$, interaction by type of treatment), with $P > 0.05$ between PL + L-NAME and LOS + L-NAME.

generalized NO withdrawal due to systemic L-NAME infusion, renal changes took place dissociated from systemic BP (2, 5–8), thus approaching a selective intrarenal infusion of NOS inhibitors to the extent possible in humans (23).

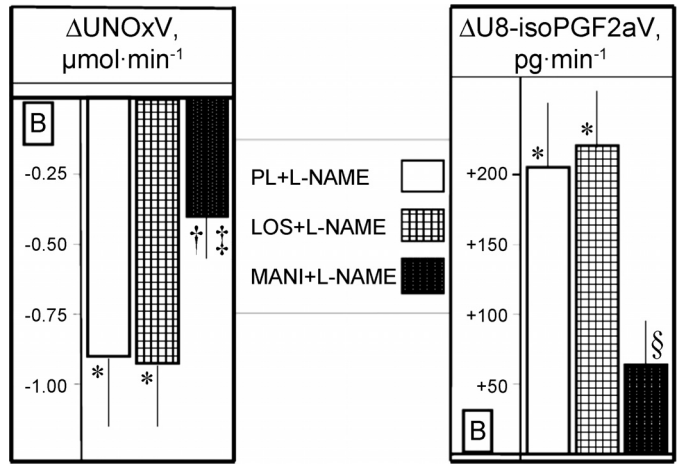

Fig. 4. Decreases from the baseline values (B) in urinary $NO_2 + NO_3$ (ΔUNO_xV) and increases in urinary 8-isoprostane (ΔU8-iso-PGF2αV) in 14 healthy individuals during 3 infusion studies of 3 $\mu g \cdot kg^{-1} \cdot min^{-1}$ N^G-nitro-L-arginine-methyl-ester (L-NAME) for 90 min, with each preceded by 3-day treatment with either placebo (PL + L-NAME), 50 mg of losartan (LOS + L-NAME), or 10 mg of manidipine (MANI + L-NAME) (means ± SE). *Both ΔUNOxV and ΔU8-iso-PGF2αV were significant ($P < 0.01$), with similar values ($P > 0.05$) between the 2 infusions; ‡ΔUNOxV, although still significant (†$P < 0.05$), was reduced ($P < 0.05$) vs. PL + L-NAME and LOS + L-NAME; §ΔU8-iso-PGF2αV, which was still not significant ($P > 0.05$), was also reduced vs. PL + L-NAME and LOS + L-NAME ($P < 0.01$).

Our first major observation relates just to effects in the systemic circulation, because previous administration of LOS or MANI fully prevented the significant increase in MAP observed in the PL+ L-NAME P2 time period, which is consistent with the BP-lowering effects of ARBs (37, 38, 40) and CCBs (2, 17). MANI pretreatment was associated with a 2.7-mmHg lower baseline MAP vs. PL and LOS, a difference, however largely within the limits of renal autoregulation, that would have prevented this change in baseline MAP from impacting the renal hemodynamic responses to L-NAME. Since BP levels also were similar during the L-NAME infusion with MANI vs. LOS, therefore, it is unlikely that any between-drug differential renal effects after L-NAME were BP related. Furthermore, it is also unlikely that hemodynamic differences with LOS vs. MANI were based on incomplete AT1R blockade, since LOS counteracts the effects of AII infusion even when given as a single 50-mg dose 12 h before physiological measurements (39), thus indicating that effective AT1R blockade was achieved at the time of L-NAME infusion. This is related to the prolonged AT1R blockade exerted by the active metabolites of LOS, mainly EXP 3174, the half-life of which exceeds 12 h (37–39). Since the highly lipophilic CCBs such as MANI bind strongly to CCs, resulting in a long-lasting CC blockade (16, 22, 47), our protocol, including LOS and MANI treatments, was appropriate to effectively compare blockade of AT1R vs. CCs.

In the present analysis, consistent with previous observations (7, 33, 37, 38, 40), the renal responses to L-NAME after AT1R blockade were similar to those after PL. This indicates that there was no significant AT1R-mediated interaction with endogenous AII in the renal effects of NOS inhibition in healthy humans, likely reflecting the minimal role of intrarenal NO-AII interactions when RAAS activity was suppressed. Similar results have also been shown in unstressed animals (2) and in patients with diuretic-induced moderate RAAS activation (33).

Our second major observation was that, in contrast to AT1R blockade with LOS, which left unaltered renal vasoconstriction to L-NAME, previous CC blockade with MANI blunted the renal responses to NOS inhibition, consistent with a BP-independent interaction between NO and CCs, which impacts renal hemodynamic and Na^+ excretory function in healthy humans. Baseline production of NO in the endothelium of vascular tissues is known to attenuate physiologically the activity of CCs in the adjacent smooth muscle cells (20, 29). Therefore, the markedly blunted renal vasoconstrictor response to L-NAME with MANI may reflect a significant participation of NO-dependent inhibition of CCs (20, 24, 25, 29, 47) in the baseline vasodepressor tone. In the kidney, CCs are represented primarily by high-voltage-activated (L-type) CCs in the afferent arteriole and the low-voltage-activated (T-type) CCs in the efferent arteriole (20, 24, 25, 47). In vitro studies with CCBs known to inhibit L- or T-type CCs (20, 24) have shown blunted vasoconstrictor responses to NOS inhibition at afferent and efferent renal arterioles, indicating that intrarenal NO withdrawal activates both L- and T-type CCs. NO production is generally acknowledged as the main physiological factor responsible for baseline vasodepressor tone at the afferent arteriole (14, 28, 36, 44, 49). Accordingly, the selective L-type CCB nifedipine (16, 24, 25, 29, 47), when coinfused with L-NMMA in healthy humans (17), reversed the reduction in GFR but left the elevated FF unaltered compared with L-NMMA alone, consistent with a blunted vasoconstriction response to NOS inhibition in the afferent arteriole only. However, since the simultaneous BP increase was also fully abolished by nifedipine, the selective blunting of afferent vasoconstriction could also reflect the withdrawal of some BP-related effect of RBF autoregulation, which is known to become even more efficient under NOS inhibition and normalized by CC blockade (20, 28, 29). Renal vasoconstriction in response to NOS inhibition, however, is also significant at the efferent arteriole, leading to a lesser reduction in GFR relative to ERPF with increased FF (5–8, 12–18, 27, 33, 35–38, 40, 41, 43–45), which could reflect a participation of activated efferent T-type CCs (20, 24, 25, 47). In our study, consistent with the ability of MANI to block both L- and T-type CCs (24, 25, 47) and thus to affect hemodynamic function in both afferent and efferent renal arterioles, the reduction in GFR and ERPF and elevation in FF in response to L-NAME infusion were attenuated to approximately the same extent with MANI vs. PL without any concurrent BP change (24, 25, 47). Therefore, these findings suggest a contribution of MANI-sensitive efferent T-type CCs to L-NAME-induced renal hemodynamic changes.

Based on the assumption that a decreased RBF response to systemic 1–4 $mg \cdot kg^{-1} \cdot min^{-1}$ L-NMMA infusion reflects loss of renal NO bioactivity, such responses have been proposed as a measure of renal endothelial function in human clinical conditions of systemic endothelial dysfunction (6, 13–15, 27, 41, 43–45). However, in these patients, the vasoconstrictor response to L-NMMA in the renal circulation may be either basally normal, as in untreated hypertension (27, 35), type 2 diabetes (41), and heart failure (6), reduced, as in chronic glomerular diseases (43), or even augmented, as in type 1 diabetic subjects with baseline renal hyperfiltration (12, 40).

Studies of repeated L-NMMA infusions in hypertensive patients with or without type 2 diabetes (13, 41, 45) showed that medium- to long-term treatment with RAAS inhibitors led to substantial increases in RBF and decreases in MAP, RVR, and FF, which were associated with and directly related to enhanced on-treatment renal vasoconstriction responses to the NOS inhibitor (13). Therefore, it was suggested that RAAS blockade, similar to what is known for the systemic circulation (10, 19, 22, 48), could also restore NO bioactivity in the renal microcirculation (13, 15, 19, 41, 45). In other studies (14, 15), however, whereas baseline RBF remained unchanged after treatment with valsartan or amlodipine, the RBF response to L-NMMA was blunted by the CCB and not affected by the ARB, consistent with our present results in healthy volunteers. Taken together, the L-NMMA-based data (13, 14, 15, 41, 45) suggest that a significant RAAS blockade-induced chronic renal vasodilation is likely required to reveal an accentuated on-treatment renal hemodynamic sensitivity to NOS inhibition. Therefore, it cannot be excluded that the enhanced renal vasoconstriction to L-NMMA was a nonspecific consequence of the RAAS blockade-related condition of substantial renal vasodilation instead of a recovered NO dependency in the renal circulation. Additionally, in those studies (13–15, 41, 43–45), since the acute BP action of L-NMMA was not prevented by a previous RAAS blockade, RBF autoregulation may have contributed to the enhanced renal hemodynamic response to NOS inhibition. Interestingly, in an early study involving hyperten-

sive patients before and after enalapril or nifedipine treatment (18), L-NMMA-induced BP increases were enhanced to the same extent by either drug, whereas the degree of simultaneously measured renal vasoconstriction remained essentially the same vs. the off-treatment study. Such findings, which were considered to reflect a recovered NO dependency in the systemic vasculature, but not in the kidney (18), could still have been influenced by the accentuated on-treatment BP responses to L-NMMA, thereby obscuring any between-drug differential effect on the renal response.

To summarize, although improved renal endothelial function can contribute to the beneficial effects of RAAS or CC blockade, studies of L-NMMA-based NOS inhibition (6, 12–15, 27, 35, 40, 41, 43–45) do not uniformly confirm this hypothesis.

Based on a variety of experimental evidence, reduced systemic and renal NO bioavailability result in a net increase in ROS due to the withdrawal of the "buffering" activity of NO against the formation of O_2^-, which in turn enhances formation of peroxynitrite and further impairs NO bioavailability (10, 19, 22, 34, 43, 46, 48, 50). Therefore, the effects of L-NAME on renal function can be mediated by an acute reduction of baseline NO levels and the altered equilibrium between NO and ROS, leading to renal vasoconstriction and reduced UNaV (34, 38, 46, 50).

AT1R blockade and CC blockade, in addition to the effects on AII-dependent vasoconstriction and regulation, respectively, of Ca^{++} influx into smooth muscle cells may also influence vascular function via interactions with the equilibrium between the NO and OS pathways. The endogenous RAAS activates NADPH oxidase via the AT1R and leads to upregulation of vascular ROS production, and thus RAAS inhibition may suppress oxidative stress. CCBs also may influence OS by acting as antioxidants to scavenge O_2^- (9, 16, 22, 48), thus potentially preserving bioavailability of NO. In addition, L-NAME-sensitive stimulation of vascular NO release was shown as a result of CCBs, suggesting a CCB-mediated activation of endothelial NOS (9, 16). Accordingly, the beneficial effects of both AT1R blockers and CCBs on vascular OS play a key role in the amelioration of endothelial function in the systemic circulation in hypertension and diabetes (10, 19, 22, 48).

In the present human study, our third major observation was that PL + L-NAME acutely reduced UNOxV by 60% (3, 8, 17, 34, 37, 38, 40) and increased U8-iso-PGF2αV by 120% (34, 38). The effect is consistent with an expected reduction in NO bioavailability coupled with enhanced ROS generation (3, 34, 38, 50). To assess whether the observed differential effects of MANI vs. LOS on renal hemodynamic and excretory functions during L-NAME were associated with differential changes in NO bioavailability and ROS generation, variations in UNO_xV and U8-iso-PGF2αV were compared during the LOS and MANI phases of the study. Because LOS did not prevent the L-NAME-induced variations in UNO_xV or U8-iso-PGF2αV, neither renal hemodynamic effects of NOS inhibition nor changes in the NO-ROS equilibrium appear to have been AT1R mediated in our study cohort under RAAS-suppressed conditions. In contrast, in salt-replete type 1 diabetic patients with endogenous RAAS activation and elevated baseline U8-iso-PGF2αV, L-NMMA or L-NAME infusion is associated with greater renal vasoconstriction (12, 38) and an exaggerated increase in U8-iso-PGF2αV (38), both of which are markedly blunted by LOS. Therefore, a combination of endogenously stimulated RAAS and increased ROS production at baseline (34, 46, 50), as found in patients with type 1 diabetes (38), is likely required to show AT1R effects on renal hemodynamic function and NO-ROS pathways in response to NOS inhibition.

In contrast to LOS, MANI blunted the increase in U8-iso-PGF2αV by 70% vs. PL + L-NAME and also attenuated the decrease in UNOxV by 30%. Therefore, MANI counteracted both the enhanced formation of ROS and the impaired NO bioavailability during L-NAME, thereby reducing the influence of NO-derived ROS on renal function. The scavenging of O_2^- by the antioxidant properties of MANI and activation of endothelial NOS both may occur with 1,4 dihydropiridine CCBs (9, 16, 22, 48), thereby contributing to the attenuated response of OS biomarkers to MANI + L-NAME vs. PL + L-NAME or LOS + L-NAME. Since UNOxV, U8-iso-PGF2αV, and PRA at the baseline time point after MANI were unchanged vs. PL, these effects of CC blockade took place independent of baseline ROS production, NO bioavailability, and RAAS activity. Therefore, CCBs, in contrast to ARBs, may influence the renal effects of NOS inhibition in man even in the absence of any baseline disruption of NO-to-ROS equilibrium or Na^+-independent activation of the endogenous RAAS.

Our study has some limitations. First, since the sample size was small, which may have limited our ability to detect some between-infusion or between-drug differences, we attempted to minimize such an effect by a careful prestudy dietary preparation, including a monitoring of Na^+, proteins, antioxidants, and NOx intake. In addition, we also decreased variability by using a study design that allowed each participant to act as her/his own control. Finally, we recognize that our study was performed in a healthy cohort. Although we anticipate that differences observed in this healthy cohort could be exaggerated in patients with RAAS activation or increased OS due to underlying diseases such as diabetes (38) or kidney disease, generalizability to patients suffering from such clinical disease conditions with baseline endothelial dysfunction cannot yet be made, and future studies are required to translate our findings to other disease states.

In conclusion, in the present set of studies in humans, acute NOS inhibition during low-dose systemic infusion of L-NAME produced BP-independent changes in renal function, including vasoconstriction, lowered GFR, and UNaV, with a net increase in OS. These variations were significantly blunted with a CCB but not with an ARB. Although obvious caution should be taken in extending data from acute NOS inhibition studies in healthy individuals to patients with chronic disease, the present results are consistent with the notion that the interaction between CCB and the NO system in the kidney may contribute to a renal-protective physiological profile (24, 25, 47) under clinical conditions of generalized endothelial dysfunction.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.M., A. Cabassi, and D.Z.I.C. conceived and designed research; A.M., G.P., A. Crocamo, Y.L., L.M., A. Cabassi, and D.Z.I.C. analyzed data; A.M.,

G.P., A. Crocamo, Y.L., A. Cabassi, and D.Z.I.C. interpreted results of experiments; A.M., D.L., G.P., A. Cabassi, and D.Z.I.C. edited and revised manuscript; A.M., Y.L., L.M., A. Cabassi, and D.Z.I.C. approved final version of manuscript; D.L., A. Crocamo, and L.M. performed experiments; D.L. and A. Crocamo prepared figures; D.L., A. Crocamo, and L.M. drafted manuscript.

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