Roles of Insulin, Age, and Asymmetric Dimethylarginine on Nitric Oxide Synthesis In Vivo

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We tested the effects of insulin on production of nitrous oxide (NO)-related substances (nitrites and nitrates [NOx]) after ^{15}N-arginine intravenous infusion and on asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) concentrations in conditions reportedly associated with altered NO availability, i.e., aging, hypertension, hypercholesterolemia, and type 2 diabetes mellitus (T2DM). A total of 26 male subjects (age 23–71 years, BMI 23–33 kg/m^2), some of whom were affected by mixed pathologic features, were enrolled. NOx fractional synthesis rate (FSR) was lower in elderly ($P < 0.015$) and T2DM subjects ($P < 0.03$) than in matched control subjects. Hyperinsulinemia generally increased both NOx FSR and absolute synthesis rate (ASR) and reduced NOx, ADMA, and SDMA concentrations. Insulin sensitivity was impaired only in T2DM. With use of simple linear regression analysis across all subjects, age was inversely correlated with both NOx FSR ($R^2 = 0.23$, $P < 0.015$) and ASR ($R^2 = 0.21$, $P < 0.02$). NOx FSR inversely correlated with both ADMA and SDMA. With use of multiple regression analysis and various models, NOx FSR remained inversely associated with age and ADMA, whereas ASR was inversely associated with age and diabetes. No association with insulin sensitivity was found. We conclude that whole-body NOx production is decreased in aging and T2DM. Age, ADMA concentration, and T2DM, but not insulin resistance, appear as negative regulators of whole-body NOx production. ***Diabetes*** **62:2699–2708, 2013**

Nitric oxide (NO) is a molecule with key functions in the cardiovascular, immune, and nervous systems (1). NO production is modified during physical exercise (2) and is altered in aging (3), diabetes (4,5), hypertension (6,7), and hypercholesterolemia (8,9). The understanding of the pathophysiological mechanism(s) underlying the altered NO metabolism in these diseases is important also for the development of therapeutic interventions aimed at improving vascular function.

NO is synthesized from the guanidine group of arginine via the enzyme family NO synthases (NOS), which include three isoforms (10). One of these, the constitutive endothelial NOS (eNOS) enzyme, is stimulated by hormones (insulin and estrogens), physical exercise, and cofactors such as tetrahydrobiopterin (10). Conversely, it is inhibited by the endogenous methylarginines asymmetric dimethylarginine (ADMA), L-monomethylarginine (LMMA), and symmetric dimethylarginine (SDMA) (11,12). ADMA and LMMA inhibit both eNOS and arginine cellular transport, whereas SDMA inhibits arginine transport (11,12). Dimethylarginines are increasingly recognized as important markers or factors of endothelial dysfunction and cardiovascular disease (11). ADMA concentration is increased in diabetes, hypertension, hypercholesterolemia, and aging (11,13).

Insulin is an important regulator of NO production, and insulin resistance is frequently associated with endothelial dysfunction (14). Insulin mediates both glucose entry into insulin-sensitive tissues and NO production via stimulation of protein kinase B/Akt (15), translocation of GLUT4 on cell membrane, and stimulation of eNOS (16). Since in insulin-resistant states insulin signaling is altered at the Akt level (17), any pathway downstream of Akt (including glucose metabolism and NOS activity) should be concomitantly affected. Furthermore, in many insulin-resistant states, ADMA levels are increased, too (18), and they may thus interfere with the insulin signaling on NOS activity and NO production.

The relative roles of insulin sensitivity and of ADMA and SDMA concentrations, as well as of other potential interfering factors such as age, on NO production in vivo have never been comprehensively investigated. Therefore, this study was designed to measure whole-body insulin sensitivity (i.e., the insulin-stimulated glucose disposal), ADMA and SDMA concentrations, and basal and insulin-stimulated NO production (19) in human subjects over a wide range of insulin sensitivity and age either healthy or affected by hypertension, hypercholesterolemia, or type 2 diabetes mellitus (T2DM). NO production was determined by a precursor product, isotope dilution technique (5). A key target of this study was also to examine the possible correlates between production of nitrites and nitrates (NOx) and ADMA, SDMA, insulin sensitivity, and age.

RESEARCH DESIGN AND METHODS

The clinical and biochemical characteristics of the 26 enrolled subjects are reported in Table 1. Age ranged between 23 and 71 years and BMI between ~23 and ~33 kg/m^2. Six subjects were healthy, normotensive, and normolipidemic with normal glucose homeostasis. Three additional normoglycemic subjects were affected by familial hypercholesterolemia, and a further nine subjects were affected by hypertension, four of whom were hypercholesterolemic too. Finally, eight patients were affected by T2DM, hypertension, and diabetic nephropathy (three of whom had microalbuminuria and the remaining five macroproteinuria). Five of these diabetic subjects also had hypercholesterolemia. The data on NOx production rate in the T2DM patients had previously been reported (5), with the exception of those of one control subject, who was replaced by another subject for a better age matching. Patients' treatment consisted of ACE inhibitors ($n = 6$), angiotensin receptor blockers ($n = 7$), antiadrenergic agents ($n = 6$), calcium antagonists ($n = 4$), diuretics ($n = 7$), aspirin ($n = 5$), statins ($n = 7$), fibrates ($n = 1$), allopurinol ($n = 1$), oral hypoglycemic agents ($n = 5$), and insulin ($n = 3$). All drugs were suspended the night before the study day.

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See accompanying commentary, p. 2645.

TABLE 1
Clinical and biochemical characteristics of the subject studied

Subject number	Age (years)	Group	BMI (kg/m^2)	Coexisting diseases	BG (mmol/L)/ basal clamp	Total cholesterol (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	TG (mmol/L)
1	33	Yc, NTc, NCc, NDc*	29.75	None	4.28/4.97	3.39	1.34	1.74	0.65
2	57	E, NTc, NCc, NDc*	24.00	None	4.89/5.39	4.71	1.81	2.54	0.78
3	26	Yc, NTc, NDc*	30.08	None	4.78/4.50	4.80	1.3	1.56	0.60
4	23	Yc, NTc	22.92	None	5.16/5.11	4.60	1.25	1.42	0.50
5	23	Yc, NTc, NCc	25.85	None	5.44/5.00	3.25	1.65	1.35	0.84
6	38	NTc, NCc	27.40	None	4.78/5.22	4.99	1.16	1.00	3.37
7	47	E, NTc, HC	28.24	HC	5.00/4.33	8.15	1.27	6.44	0.96
8	57	E, NTc, HC	23.53	HC	4.28/4.72	5.12	1.40	3.10	1.37
9	23	Yc, HC	22.72	HC	5.17/4.25	5.97	1.24	4.35	0.82
10	61	E, HT, NCc, NDc*	29.03	HT	5.00/5.28	4.40	1.09	2.73	1.28
11	23	Yc, HT, NCc, NDc*	26.12	HT	5.06/4.83	4.00	1.20	1.40	1.00
12	61	E, HT, NCc, NDc*	25.47	HT	4.78/5.28	5.30	1.01	3.62	1.48
13	58	HT, NCc, NDc*	25.82	HT	4.61/4.56	5.02	0.96	1.39	2.15
14	67	E, HT, HC, NDc	29.40	HT, HC	5.33/5.00	3.47	0.93	1.13	1.00
15	71	E, HT, HC, NDc*	25.48	HT, HC	4.61/4.89	5.90	1.50	3.83	1.24
16	74	HT, HC, NDc*	25.43	HT, HC	4.78/5.22	5.82	1.66	3.83	0.73
17	43	Yc, HT, HC	26.87	HT, HC	5.50/4.28	7.19	1.01	4.63	2.08
18	33	Yc, HT, HC	33.46	HT, HC	4.83/4.93	6.03	0.83	3.97	2.69
19	67	T2*	28.72	HT, T2	14.11/5.06	6.49	1.22	3.23	4.47
20	63	T2*	24.82	HT, T2, HC	12.17/4.67	4.45	1.22	2.87	0.79
21	69	T2*	27.47	HT, T2, HC	10.28/4.72	3.49	1.53	1.04	2.01
22	49	T2*	29.53	HT, T2	11.00/5.00	4.68	0.91	3.08	1.52
23	71	T2*	29.07	HT, T2, HC	8.00/4.89	4.81	1.22	2.96	1.39
24	67	T2*	28.40	HT, T2, HC	18.22/4.78	5.17	1.01	3.08	2.36
25	68	T2*	30.86	HT, T2, HC	9.61/4.94	4.65	1.06	3.21	0.84
26	51	T2*	32.55	HT, T2	13.22/4.61	5.04	1.53	2.98	1.17

E, elderly; HC, hypercholesterolemia; HDL-C, HDL cholesterol; HT, hypertension; LDL-C, LDL cholesterol; NCc, normocholesterolemic control subject for hypercholesterolemic subject; NDc, nondiabetic control subject for T2DM subject; NTc, normotensive control subject for hypertensive subject; T2, T2DM; TG, triglyceride; Yc, younger control subject for elderly subject. *Subjects previously included in the study of ref. 5 either as a T2DM or control subject.

The nondiabetic subjects were rearranged and analyzed separately (both those belonging to the test groups and those to the corresponding matched control subjects) according to age, hypertension, or hypercholesterolemia. The diabetic subjects were separately compared with nondiabetic control subjects, chosen as the best matches for age, hypertension, and hypercholesterolemia. (See Table 1 for patients' allocation.) When the effect of age was tested, we arbitrarily set the threshold value for the nondiabetic elderly group at >55 years and that of the younger nondiabetic group at <43 years (Table 1). Resulting ages were means ± SD 63 ± 7 years (n = 8) in the elderly group vs. 26 ± 5 years in the younger control subjects (n = 9) ($P < 0.0001$ between the two groups). BMI was similar in these elderly (26.3 ± 2.3 kg/m^2) and younger (27.1 ± 3.5 kg/m^2) subjects (P = NS between the two groups) (Table 1). Four and three individuals in the elderly group and three and four individuals in the younger group also had either increase blood pressure or hypercholesterolemia, respectively (Table 1).

When the effect of hypertension was tested, nondiabetic subjects were allocated either to the hypertensive group (n = 9, age 55 ± 18 years) or to the normotensive group (n = 8, age 38 ± 15 years, $P < 0.05$ vs. the hypertensive group). BMI was not different between these hypertensive (26.6 ± 1.7 kg/m^2) and normotensive (26.5 ± 2.2 kg/m^2) subjects. Four subjects in the hypertensive group and two subjects in the normotensive controls also had increased cholesterol levels.

When the effects of hypercholesterolemia were studied, eight subjects with hypercholesterolemia (age 52 ± 19 years, BMI 27.3 ± 2.3 kg/m^2) and eight subjects with normal cholesterol levels (age 45 ± 16 years, BMI 26.8 ± 3.5 kg/m^2; P = NS for both parameters vs. hypercholesterolemic group) were selected. Four subjects in each group also had high blood pressure (Table 1).

Finally, nine nondiabetic subjects (age 53 ± 19 years, BMI 26.9 ± 2.4 kg/m^2) were selected as the nondiabetic controls for the eight T2DM patients (age 63 ± 8 years, BMI 28.9 ± 2.3 kg/m^2; NS for both parameters vs. the diabetic group). The data of all of these diabetic as well as nondiabetic subjects, with the exception of one nondiabetic subject newly recruited in this study (no. 14 in Table 1), had previously been published (5). All of the T2DM patients had increased blood pressure, whereas five also had hypercholesterolemia compared with six and two nondiabetic control subjects, respectively. Experimental design, analytical measurements (20–22), calculations, and statistical analyses are available in the Supplementary Data.

RESULTS

Basal glucose concentrations were increased only in the T2DM subjects (Table 1). Basal plasma insulin concentration ranged between ~50 and ~60 nmol/L in all of the nondiabetic groups, but it was approximately doubled in the T2DM subjects (100 ± 53 nmol/L, $P < 0.02$ vs. nondiabetic controls). During the clamp, plasma insulin was rapidly raised and maintained at ~1,000–1,400 nmol/L (young subjects 1,090 ± 274 nmol/L, older subjects 1,282 ± 418 nmol/L, hypertensive subjects 1,226 ± 456 nmol/L, hypercholesterolemic subjects 1,402 ± 382 nmol/L, and diabetic subjects 1,240 ± 222 nmol/L) without differences among groups. Blood glucose concentrations were brought to or maintained between 4.2 and 5.1 mmol/L throughout the study and were not different among the four subjects' groups in the last 60 min of the clamp.

Elderly versus younger subjects. NOx fractional synthesis rate (FSR) (Fig. 1) was lower in elderly than in younger subjects, whereas NOx absolute synthesis rate (ASR) was slightly albeit insignificantly decreased in the elderly group (Table 2). Arginine concentration, R_a, the fraction of arginine R_a converted to NOx, as well as NOx, ADMA, and SDMA concentrations and insulin-mediated glucose disposal, were similar between the two groups (Tables 2 and 3). Hyperinsulinemia increased NOx FSR (Fig. 1), NOx ASR, and arginine conversion to NOx, whereas it decreased arginine concentration and R_a, NOx,

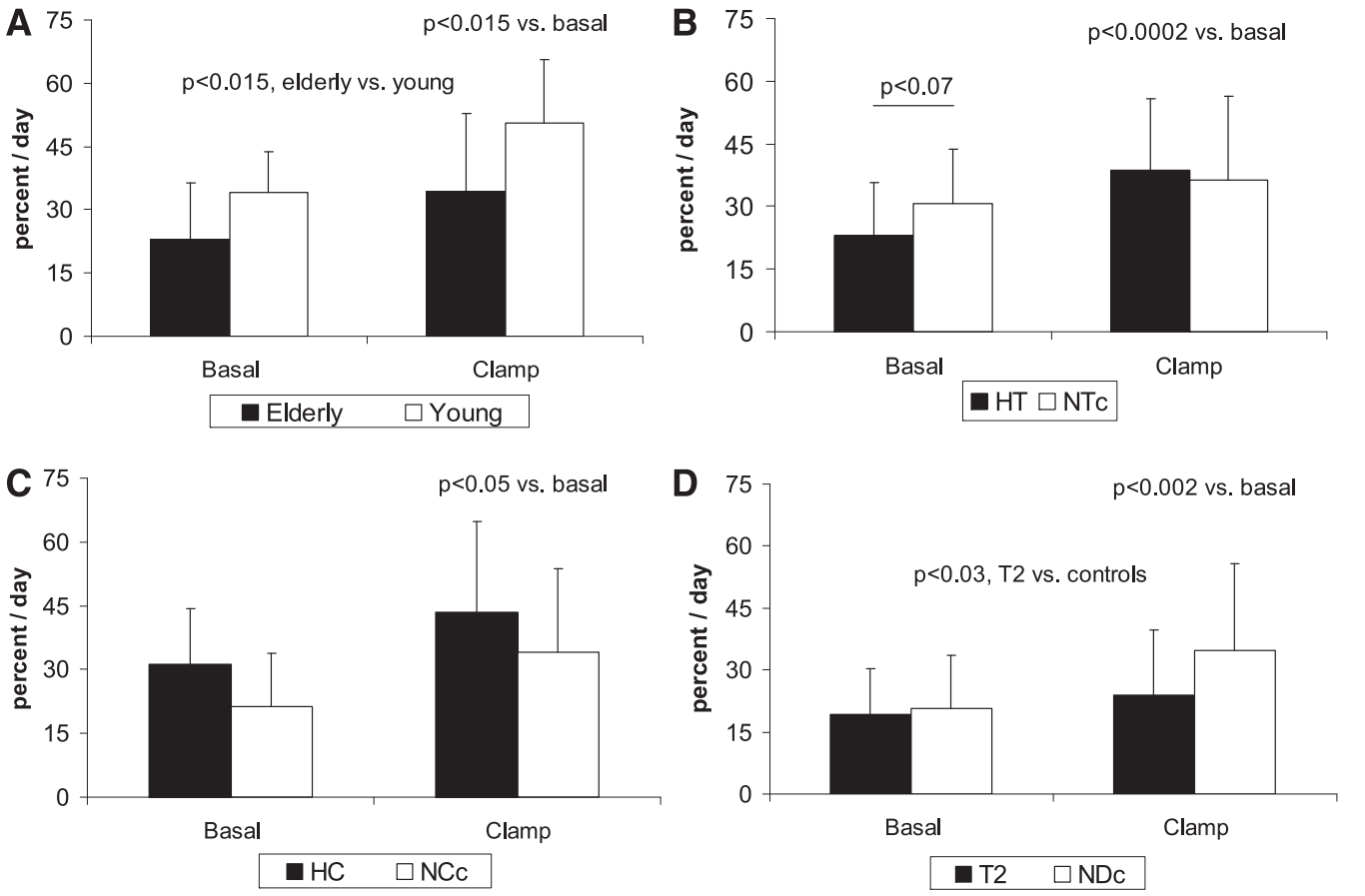

FIG. 1. NOx FSR in the basal and the clamp periods in the subjects selected by age (elderly vs. young) (*A*), by presence or absence of hypertension (HT) vs. normotensive control subjects (NTc) (*B*) and of hypercholesterolemia (HC) or normocholesterolemia (NCc) (*C*), and by T2DM (T2) vs. nondiabetic control subjects (NDc) (*D*). The significance values reported indicate an overall difference between test group and control subjects by the two-way ANOVA for repeated measurements (either group or interaction effect). The *P* values for significant differences between the clamp and the basal values, by the two-way ANOVA for repeated measurements (treatment effect), are also reported.

ADMA, and SDMA concentrations without differences between the groups (Tables 2 and 3). Plasma creatinine was similar in older (76 ± 6 μmol/L) and younger (85 ± 14 μmol/L) subjects.

Hypertensive versus normotensive subjects. Basal NOx FSR in the hypertensive subjects was ~25% lower than in normotensive control subjects ($P < 0.07$ by the t test), but these insignificant differences were abolished by hyperinsulinemia (Fig. 1). There was no overall difference in NOx FSR (Fig. 1), NOx ASR, or arginine concentration or R_a, the fraction of arginine converted to NOx, or NOx concentrations (Tables 2 and 3) between the two groups (by ANOVA). ADMA and SDMA concentrations were greater in the hypertensive than in the normotensive subjects (Table 3). Hyperinsulinemia increased NOx FSR (Fig. 1), NOx ASR, and arginine conversion to NOx, whereas it decreased arginine concentration, R_a, and NOx, ADMA, and SDMA concentrations without differences between the groups (Tables 2 and 3). The relative response of NOx FSR to insulin was even greater in the hypertensive (95 ± 92%) than in the normotensive (21 ± 45%, $P < 0.04$) subjects (Fig. 1). NOx ASR followed the same pattern (Table 2). In the hypertensive subjects, insulin-mediated glucose disposal was ~20% lower than in control subjects, though not significantly ($P = 0.13$) (Table 2). Plasma creatinine was not different between the hypertensive (78 ± 12 μmol/L) and the normotensive (79 ± 11 μmol/L) subjects.

Hypercholesterolemic versus normocholesterolemic subjects. NOx FSR tended to be greater in the subjects with than in those without hypercholesterolemia ($P = 0.11$ by ANOVA, group effect) (Fig. 1). NOx concentrations were lower ($P < 0.04$ by ANOVA, group effect) and those of SDMA greater ($P < 0.05$ by ANOVA, group effect) in hypercholesterolemia (Table 3). Also, NOx ASR tended to be greater in the hypercholesterolemic subjects ($P < 0.08$) (Table 2). Arginine concentration and R_a, the fraction of arginine R_a converted to NOx, ADMA concentrations, and insulin-mediated glucose disposal were similar between the two groups (Tables 2 and 3). Hyperinsulinemia increased NOx FSR, NOx ASR, and arginine conversion to NOx, whereas it decreased arginine concentration, R_a, and NOx, ADMA, and SDMA concentrations without differences between the groups (Fig. 1 and Tables 2 and 3). Plasma creatinine was normal in both groups (hypercholesterolemic subjects 82 ± 12 μmol/L, normocholesterolemic control subjects 75 ± 5 μmol/L).

T2DM versus nondiabetic subjects. In the T2DM patients, NOx FSR (Fig. 1), NOx ASR, arginine conversion to NOx, insulin-mediated glucose disposal (Table 2), and NOx concentrations (Table 3) were lower than in nondiabetic control subjects, as previously reported (5). Arginine concentration, R_a, and ADMA concentrations were, however, similar between the two groups, whereas SDMA

TABLE 2
NOx ASR (in mmol/day), arginine R_a (in μmol/kg × min), the fraction of arginine flux converted to NOx (%), and the insulin-mediated glucose disposal (M) (in mg/kg × min) in the postabsorptive and hyperinsulinemic conditions in the four studied groups and matched control subjects

	NOx ASR		Arg R_a		% of Arg R_a to NOx		M
	Basal	Clamp	Basal	Clamp	Basal	Clamp	clamp
E	0.64 ± 0.47	0.78 ± 0.46[e]	1.35 ± 0.16	1.01 ± 0.19[c,f]	0.43 ± 0.32	0.72 ± 0.46[f]	7.0 ± 2.2
Yc	0.75 ± 0.39	1.01 ± 0.53[a,e]	1.23 ± 0.14	0.92 ± 0.09[c,f]	0.49 ± 0.26	0.86 ± 0.44[d,f]	7.9 ± 2.7
HT	0.62 ± 0.39	0.92 ± 0.47[b,e]	1.26 ± 0.21	0.93 ± 0.19[c,f]	0.41 ± 0.25	0.82 ± 0.40[c,f]	6.2 ± 2.1
NTc	0.75 ± 0.40	0.78 ± 0.51[e]	1.25 ± 0.13	0.95 ± 0.12[c,f]	0.52 ± 0.28	0.73 ± 0.48[e,f]	8.0 ± 1.6
HC	0.70 ± 0.38	0.88 ± 0.48[f]	1.29 ± 0.15	0.96 ± 0.17[c,f]	0.43 ± 0.23	0.76 ± 0.44[c,f]	6.9 ± 1.9
NCc	0.64 ± 0.44	0.84 ± 0.54[f]	1.25 ± 0.22	0.95 ± 0.16[c,f]	0.46 ± 0.32	0.78 ± 0.47[f]	7.4 ± 1.9
T2	0.32 ± 0.18[g]	0.35 ± 0.20[f,g]	1.13 ± 0.16	0.84 ± 0.17[c,f]	0.22 ± 0.13[g]	0.32 ± 0.16[a,f,g]	4.4 ± 1.6[h]
NDc	0.57 ± 0.42	0.87 ± 0.54[f,b]	1.29 ± 0.14	0.96 ± 0.16[c,f]	0.40 ± 0.32	0.82 ± 0.52[c,f]	6.5 ± 2.4

Data are reported as means ± SD. E, elderly; HC, hypercholesterolemia; HT, hypertension; NCc, normocholesterolemic control subject for hypercholesterolemic subject; NDc, nondiabetic control subject for T2DM subject; NTc, normotensive control subject for hypertensive subject; T2, T2DM; Yc, younger control subject for elderly subject. [a]$P < 0.04$, [b]$P < 0.01$, [c]$P < 0.01$, [d]$P < 0.005$, clamp vs. basal, by the two-tailed paired t test within each group. [e]$P < 0.05$ and [f]$P < 0.015$, clamp vs. basal, by the two-way ANOVA (treatment effect for test and control groups). [g]$P < 0.04$, vs. corresponding control group, by the two-way ANOVA (sample and/or interaction effect). [h]$P < 0.02$, T2DM vs. the nondiabetic control subjects, by the two-tailed Student t test.

tended to be 25–30% greater in the T2DM group ($P = 0.058$ by ANOVA) (Tables 2 and 3). In the T2DM group, hyperinsulinemia did not increase NOx FSR (Fig. 1), NOx ASR, or the fraction of arginine converted to NOx (P = NS, by paired t test) as opposed to control subjects (Table 2). In contrast, hyperinsulinemia decreased arginine concentration, R_a, and ADMA and SDMA concentrations to the same extent in both the diabetic and the control subjects (Tables 2 and 3). Insulin decreased NOx concentration in the control ($P < 0.03$ by the paired t test) but not in the diabetic (P = NS) group, although an overall effect to decrease NOx concentration was detected by ANOVA (treatment effect) (Table 2). Plasma creatinine in T2DM (105 ± 35 μmol/L) tended to be greater ($P > 0.1$, <0.05) than in the control subjects (75 ± 6 μmol/L), due to the presence of three subjects with mild to moderate renal insufficiency.

Correlations. In the whole patients' group, we found significant, inverse simple correlations between either ADMA or SDMA and NOx FSR either including both the basal and the hyperinsulinemic periods (ADMA vs. NOx FSR, $R^2 = -0.19$, $P < 0.005$; SDMA vs. NOx FSR, $R^2 = 0.12$, $P < 0.05$) or only during hyperinsulinemia (Fig. 2). An inverse correlation between ADMA and insulin sensitivity was also detected (Fig. 2). Within individual groups (with the exception of the T2DM subjects), similar trends of inverse correlation (although not always significant) between NOx FSR and ADMA were found (healthy control subjects $R^2 = 0.36$, $P < 0.05$; hypertensive subjects $R^2 = 0.20$, $P < 0.1$; and hypercholesterolemic subjects $R^2 = 0.10$, $P < 0.25$). NOx FSR and SDMA were inversely correlated in subjects with hypercholesterolemia ($R^2 = 0.22$, $P < 0.05$) and diabetes ($R^2 = 0.24$, $P < 0.05$) but not in the other groups. Age was inversely correlated with both insulin sensitivity (M) and NOx FSR and directly correlated with both ADMA and SDMA during the clamp (Fig. 3). ASR, too, was inversely correlated with age ($R^2 = 0.46$, $P < 0.02$). Age and NOx FSR were inversely correlated with each other also in the basal state ($R^2 = 0.26$, $P < 0.005$). In contrast, no correlation was found between insulin sensitivity and NOx FSR either within each group (data not reported) or when all subjects were analyzed together (Fig. 4). No correlations were found between plasma creatinine and either ADMA ($P > 0.3$) or SDMA ($0.1 < P > 0.05$),

TABLE 3
Plasma arginine, ADMA, SDMA, and blood NOx concentrations in the postabsorptive and hyperinsulinemic conditions in the groups studied and matched control subjects

	Arginine		ADMA		SDMA		NOx	
	Basal	Clamp	Basal	Clamp	Basal	Clamp	Basal	Clamp
E	94 ± 13	66 ± 12[a]	0.50 ± 0.07	0.42 ± 0.04[a]	0.49 ± 0.09	0.42 ± 0.06[a]	136 ± 90	111 ± 60[a]
Yc	85 ± 12	56 ± 9[a]	0.52 ± 0.05	0.39 ± 0.06[a]	0.48 ± 0.07	0.39 ± 0.05[a]	96 ± 22	87 ± 22[a]
HT	88 ± 29	60 ± 19[a]	0.53 ± 0.06[c]	0.42 ± 0.05[a,c]	0.53 ± 0.09[c]	0.45 ± 0.05[a,c]	131 ± 81	112 ± 55[a]
NTc	87 ± 16	62 ± 8[a]	0.46 ± 0.04	0.37 ± 0.03[a]	0.42 ± 0.05	0.37 ± 0.03[a]	109 ± 38	97 ± 28[a]
HC	96 ± 20	62 ± 12[a]	0.51 ± 0.05	0.39 ± 0.04[a]	0.54 ± 0.07[b]	0.44 ± 0.04[a,b]	95 ± 15[b]	87 ± 16[a,b]
NCc	83 ± 21	59 ± 17[a]	0.50 ± 0.08	0.42 ± 0.06[a]	0.43 ± 0.08	0.38 ± 0.07[a]	146 ± 87	122 ± 59[a]
T2	74 ± 17	57 ± 12[a]	0.51 ± 0.06	0.42 ± 0.05[a]	0.60 ± 0.19	0.55 ± 0.17[a,b]	81 ± 50[b]	65 ± 27[a,b]
NDc	86 ± 27	62 ± 17[a]	0.50 ± 0.07	0.41 ± 0.06[a]	0.48 ± 0.11	0.42 ± 0.07[a]	132 ± 82	109 ± 56[a]

Data are means ± SD. E, elderly; HC, hypercholesterolemia; HT, hypertension; NCc, normocholesterolemic control subject for hypercholesterolemic subject; NDc, nondiabetic control subject for T2DM subject; NTc, normotensive control subject for hypertensive subject; T2, T2DM; Yc, younger control subject for elderly subject. [a]$P < 0.01$, clamp vs. basal by two-way ANOVA (treatment effect for the test group and the corresponding controls). [b]$P < 0.05$ and [c]$P < 0.01$, group difference vs. the corresponding control group by two-way ANOVA (sample or interaction effect).

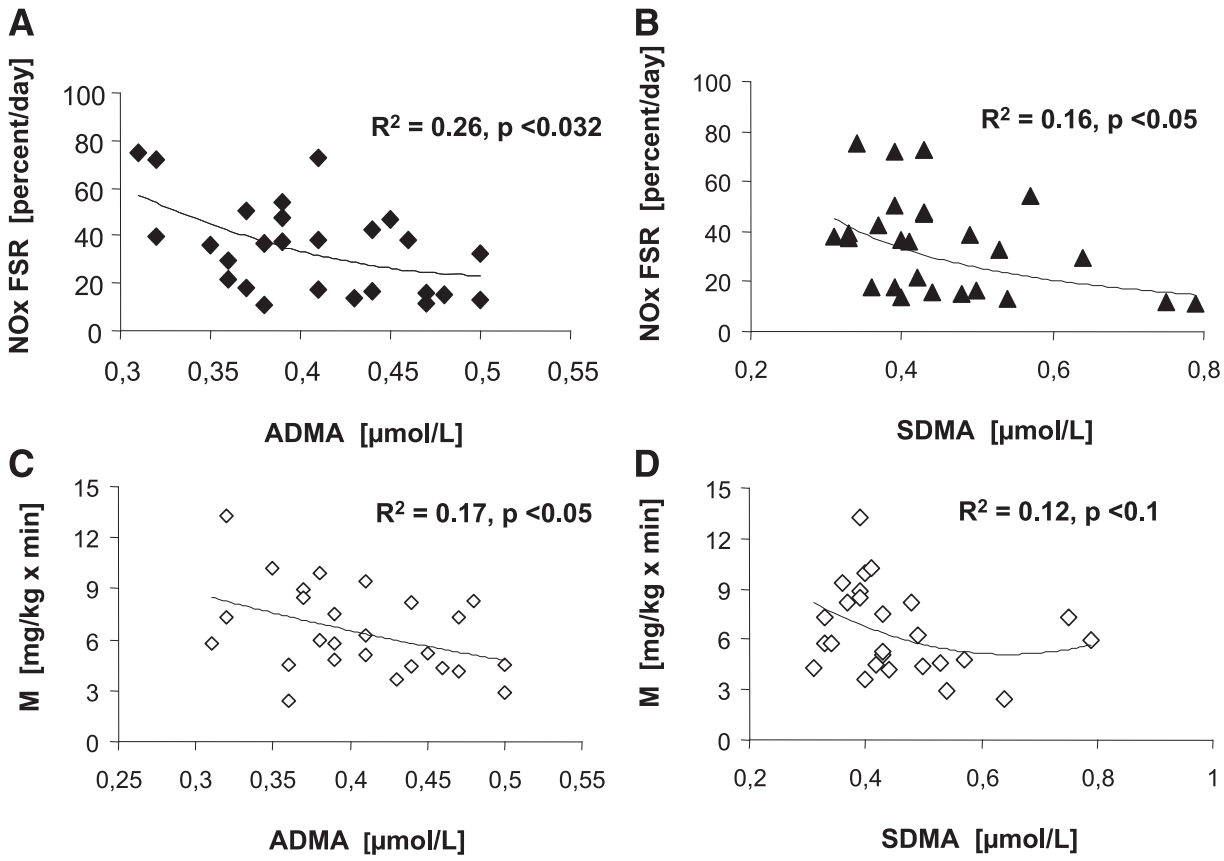

FIG. 2. Inverse correlations between either plasma ADMA (*A*) or plasma SDMA (*B*) concentrations and NOx FSR, as well as between either plasma ADMA (*C*) or plasma SDMA (*D*) concentrations and the insulin sensitivity index (*M*). A two-order polynomial model was used for all the regression analysis and the linear fit. The multiple R^2 regression values are reported. The R^2 value of the correlation between age and NOx FSR (*top-right panel*) is slightly different from that calculated with a simple linear regression model ($R^2 = 0.23$, $P < 0.015$). (The two highest SDMA values in the *bottom-right panel* belong to two diabetic subjects.) The *P* values for significance are also reported.

between NOx FSR and total, LDL, or HDL cholesterol or BMI within each individual group, or in all subjects together (data not reported).

We also performed multiple regression analysis using various models (Table 4). Both NOx FSR and ASR were significantly and inversely associated with age both before and after stepwise addition of BMI, hypertension, and hypercholesterolemia. NOx FSR was inversely associated with ADMA, an association that remained after stepwise addition of BMI, hypertension, hypercholesterolemia, and T2DM (Table 4). Conversely, NOx ASR was inversely associated with the presence of T2DM and was also associated after addition of BMI, hypertension, hypercholesterolemia, and ADMA. No association with insulin sensitivity of either the FSR or ASR of NOx, using any model, was detected (Table 4 and Fig. 4).

DISCUSSION

The purpose of this study was to investigate whole-body NO kinetics (through its oxidation products, NOx), as well as their response to hyperinsulinemia, in conditions reportedly associated with alterations of NO availability, such as aging, hypertension, hypercholesterolemia, and T2DM. Secondly, we sought for relationships between NOx production and some factors known to affect NO synthesis, among them insulin sensitivity, dimethylarginine concentrations, and age.

Effects of age. In elderly subjects (mean age 63 years), NOx FSR was lower than in younger control subjects (mean age 27 years) in the basal and the hyperinsulinemic state. To our knowledge, this is the first report on in vivo NO kinetics in relation to age in humans. Previous studies, largely based on in vitro experimental models, have reported data compatible with our findings, showing an age-related decrease of NO availability (3,23). Such a decrease might be due to superoxide-mediated NO utilization and scavenging to form peroxynitrite, to decreased NOS expression (both of the constitutive and the inducible isoforms), or to upregulation of arginase. These mechanisms, either alone or in combination, likely result in a gradual decline of endothelial function, as shown also by forearm blood flow measurements (24–26). Such a decrease cannot likely be attributed to insulin resistance, since the effect of insulin to stimulate both NOx FSR and ASR was not affected by aging (Fig. 1, Table 2).

The elderly subjects had at least one coexisting condition (hypertension or lipid abnormalities) that could affect NO metabolism per se; therefore, a pure effect of age cannot be strictly established. Nevertheless, an effect of these confounders would be excluded by the stepwise multiple regression analysis (Table 4), showing an independent effect of age in the reduction of both NOx FSR and ASR. The reduced NO availability in aged people may be enhanced by antioxidants and by tetrahydrobiopterin, which prevent NOS uncoupling as well as NO use as a scavenger of oxygen radicals (26–28). Although the measurement of circulating antioxidants was beyond the purpose of our study, it would be interesting to test their effects in vivo on NOx kinetics.

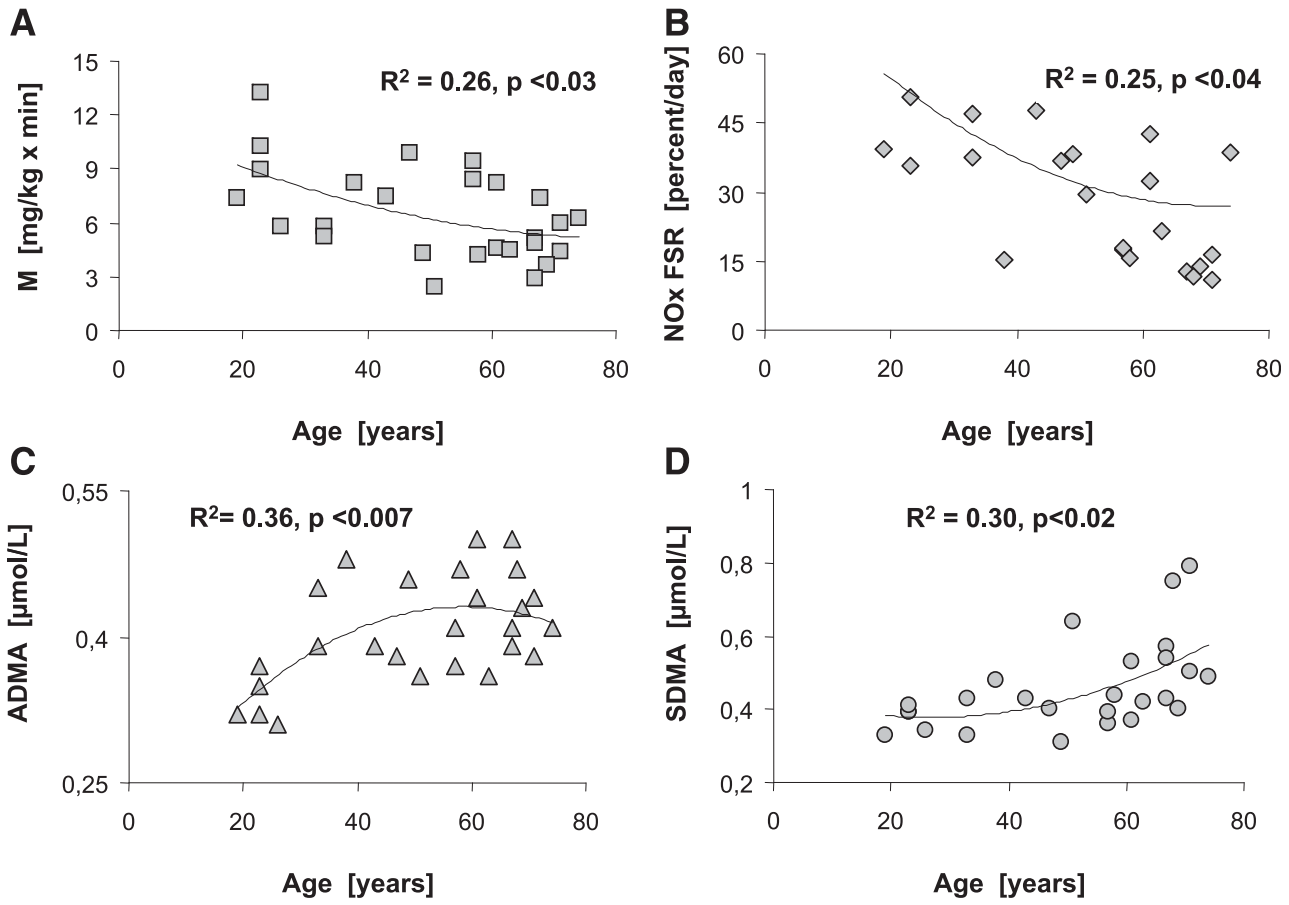

FIG. 3. Inverse correlations between age and the *M* value (*A*), between age and NOx FSR (*B*), and between age and either ADMA (*C*) or SDMA (*D*) concentrations. A two-order polynomial model was used for the correlation analysis and the linear fit. The multiple R^2 regression values are reported. The *P* values for significance are also reported.

The failure to attain a significant difference between the two groups in NOx ASR (as opposed to FSR) might be explained by the slightly greater NOx concentrations observed in the elderly, which partly offset the lower FSR values (Table 3). FSR is the primary output measurement in these precursor-pool approaches. It indicates the fractional production (and/or release into plasma) of a given substrate, calculated from the progressive incorporation of the tracer moving from the precursor pool into the product. FSR is also equal to the fractional disposal rate under steady-state conditions, such as those chosen/assumed in this study. Conversely, ASR is a complex parameter derived by multiplying FSR times the NOx pool, in turn calculated as the product of NOx concentrations and its estimated volume of distribution. Since NOx concentrations tended to be increased in the elderly, ASR was also increased. The reason(s) why NOx concentrations tended to be greater in the elderly than in the younger subjects cannot be easily understood from our data.

Effects of essential hypertension. Both NOx FSR and ASR were not significantly different between hypertensive and normotensive subjects, although basal NOx FSR was ~25% lower ($P < 0.07$) than in normotensive subjects. In contrast, ADMA and SDMA concentrations were greater in the hypertensive subjects (Table 3). Hyperinsulinemia elicited an appropriate or even enhanced increase in both NOx FSR and ASR in the hypertensive subjects (Fig. 1 and Table 2). The lower basal NOx FSR in hypertension might have been due, at least in part, to their older age (see RESEARCH DESIGN AND METHODS); however, a decreased NO availability was previously reported in hypertension (6,7). Forte et al. (6) found an inverse correlation between urinary nitrate excretion and mean ambulatory blood pressure in untreated patients with essential hypertension. The patients of our study were not newly diagnosed, and they had been treated for months or even years with hypotensive drugs, which were withdrawn just the evening before the study. For these reasons, we did not look for correlates between NOx FSR and ambulatory blood pressure. In the hypertensive subjects, the normal or even enhanced response of NOx FSR to hyperinsulinemia is interesting, and it excludes any insulin resistance at this site. Notably, in our patients also the insulin-stimulated glucose disposal was marginally and insignificantly lower than in normotensive control subjects. As a matter of fact, not all subjects with essential hypertension are insulin resistant (29).

We confirm increased ADMA and SDMA concentrations in hypertension, in agreement with previous reports (7). Such an increase cannot be accounted for by a defective renal removal of the dimethylarginines due to impaired renal function, which was actually normal (see RESULTS). The increased dimethylarginine levels may originate from an increased posttranslational protein methylation rate, from a decreased expression or activity of the dimethylarginine dimethylaminohydrolase (DDAH) enzyme(s), or from an increased arginase activity, as reported in experimental models of pulmonary hypertension (30).

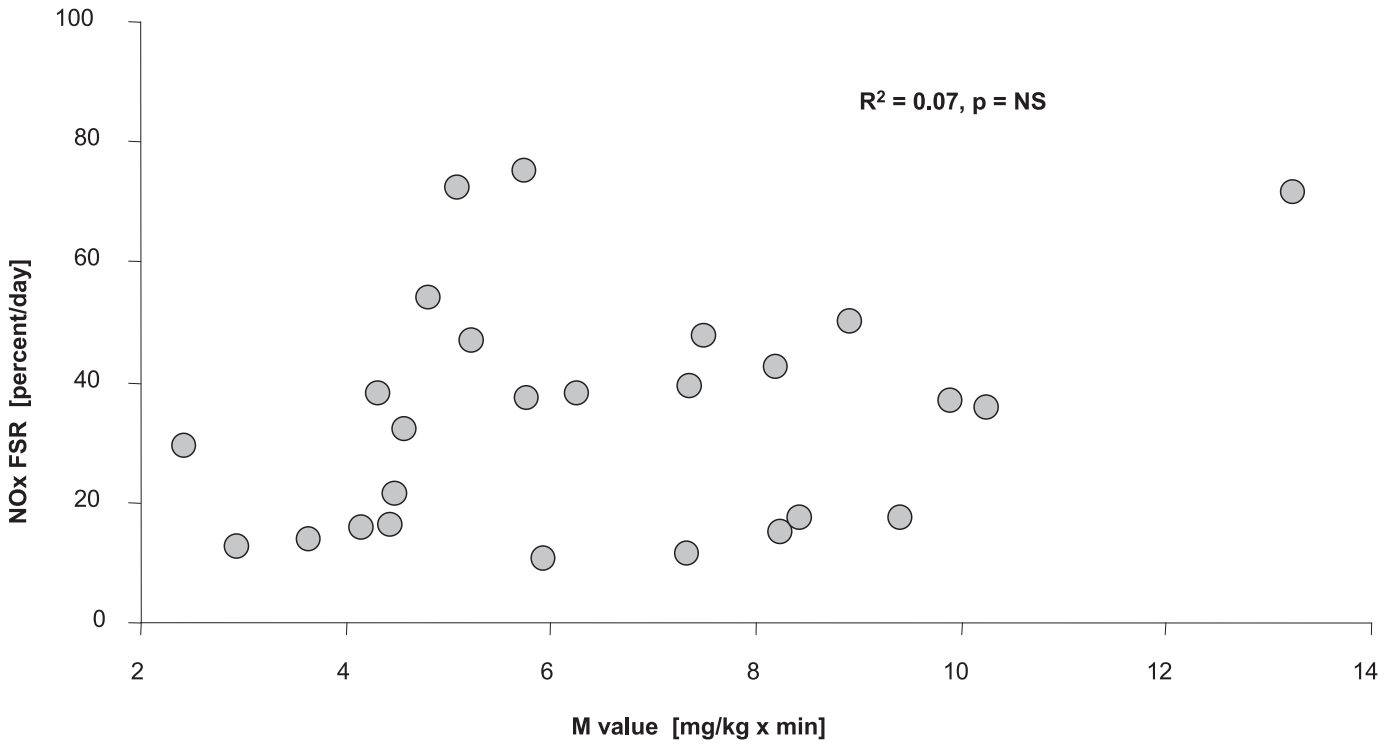

FIG. 4. Absence of relationship between the insulin sensitivity index (*M*) and NOx FSR.

Effects of hypercholesterolemia. In the patients with hypercholesterolemia, both NOx FSR and ASR were normal, NOx concentrations were decreased, and those of SDMA increased under both basal and hyperinsulinemic conditions (Fig. 1 and Tables 2 and 3). Decreased NOx concentration, despite a normal or even slightly greater NOx FSR (Table 2), suggests an increased NO consumption or removal from the circulation. To our knowledge, no data exist on the rate of NO turnover or consumption in hypercholesterolemia. A possible increased NO removal may be operated by superoxide anion(s) working as scavengers of NO to produce peroxynitrites. Increased superoxide anion levels were actually reported in hypercholesterolemia, with their production being associated with NO consumption and vascular dysfunction (31).

We found increased SDMA concentrations in hypercholesterolemia (Table 3), in partial agreement with the previously reported increased ADMA levels in this condition (11). Also, in these patients the increase in SDMA cannot be attributed to renal dysfunction, since plasma creatinine was normal in the patients (see RESULTS). Therefore, other potential causes, as discussed above also for aging and hypertension, could be involved.

Effects of diabetes. We have previously reported, in T2DM patients with diabetic nephropathy, a decreased NOx production as well as a reduced response to hyperinsulinemia (5). In this study, we also show that SDMA levels tended to be greater in T2DM. ADMA and SDMA concentrations were previously reported to be increased in diabetic subjects with microvascular complications (32). The presence of a mild-to-moderate degree of renal insufficiency in three of the eight diabetic subjects may in part explain such an increase.

The multiple regression analysis unveiled interesting associations. Age was an inverse, independent, and rather strong determinant of both NOx FSR and ASR across all subjects and remained so after correction for a number of variables (Table 4). The inclusion of subjects with various pathologic features actually should be viewed as an enrichment rather than a drawback of the study, expanding the overall significance of our findings. This finding is new, and it may stimulate further research on the causal mechanisms and on the effects of a decreased NO availability in older people, as well as of possible treatments aimed at improving vascular function.

ADMA, too, emerged as an important negative regulator of whole-body NOx FSR, despite the presence of multiple variables or associated conditions (Table 4). The dimethylated arginines ADMA and SDMA are posttranslationally formed in body proteins through protein methylation at arginine residues (11). After proteolysis, ADMA and SDMA are subsequently released into plasma as free compounds. ADMA has been associated with endothelial dysfunction and vascular disease (11–13), as well as insulin resistance (33). Based on our data, ADMA can interfere with the stimulatory effect of insulin on NO synthesis (5,15).

Interestingly, NOx ASR (although not FSR) was negatively associated with the presence of T2DM, even after correction for age, BMI, hypertension, hypercholesterolemia, dimethylarginine concentrations, and insulin sensitivity (Table 4). Such a negative association indicates that absolute NOx production is decreased in patients with T2DM and nephropathy independently of the coexistence of other conditions. The causes for the decreased NO availability and production in diabetes are largely those discussed above.

Both NO production and glucose disposal are stimulated by insulin action through PKB/Akt phosphorylation (15). Insulin resistance on NO production is considered a cause of endothelial dysfunction in diabetes (4,34,35). Although these metabolic effects of insulin should be correlated to

TABLE 4
Multiple regression analysis for the effects, on either NOx FSR or ASR (as dependent variables), of the independent variables age; BMI; presence/absence of hypertension, hypercholesterolemia, or T2DM; ADMA and SDMA concentrations; and the insulin sensitivity index (M) (in mg/kg × min^{-1})

Model	Age	BMI	HT	HC	T2	M	ADMA	SDMA
Dependent variable: FSR								
Model A								
Age	−0.48*							
Age, BMI	−0.49*	0.119						
Age, BMI, HT	−0.55*	0.09	0.095					
Age, BMI, HT, HC	−0.58*	0.095	0.093	0.087				
Age, BMI, HT, HC, T2DM	−0.48	0.174	0.148	0.084	−0.30			
Model B								
ADMA							−0.49*	
ADMA, SDMA							−0.41*	−0.30
ADMA, SDMA, M						−0.019	−0.41*	−0.30
Model C								
ADMA, BMI		0.187					−0.53*	
ADMA, BMI, HT		0.2	−0.05				−0.51*	
ADMA, BMI, HT, HC		0.188	−0.01	−0.10			−0.52*	
ADMA, BMI, HT, HC, T2DM		0.297	0.19	−0.07	−0.47*		−0.56*	
Dependent variable: ASR								
Model A								
Age	−0.46*							
Age, BMI	−0.47*	0.090						
Age, BMI, HT	−0.56*	0.041	0.159					
Age, BMI, HT, HC	−0.57*	0.043	0.159	0.022				
Age, BMI, HT, HC, T2DM	−0.39	0.184	0.257	0.017	−0.53*			
Model B								
ADMA							−0.28	
ADMA, SDMA							−0.20	−0.30
ADMA, SDMA, M						0.063	−0.18	−0.29
Model C								
ADMA, BMI		0.113					−0.31	
ADMA, BMI, HT		0.140	−0.10				−0.27	
ADMA, BMI, HT, HC		0.123	−0.05	−0.15			−0.28	
ADMA, BMI, HT, HC, T2DM		0.275	−0.232	−0.10	−0.66*		−0.34	

The models tested included the following: model A, age and presence/absence of comorbidities (body weight, hypertension, hypercholesterolemia, and T2DM); model B, the dimethylarginine concentrations and insulin sensitivity; model C, ADMA and presence/absence of comorbidities. The correlation coefficients are reported. HC, hypercholesterolemia; HT, hypertension; M, insulin-mediated glucose disposal; T2, T2DM. *Statistically significant correlation (at $P < 0.05$).

each other or commonly impaired under conditions of insulin resistance (34), our data do not support this view, at least under the present whole-body kinetic approach. As a matter of fact, no correlation was found between NOx FSR and insulin sensitivity both across all the subjects (Fig. 4) and within each group analyzed separately. Also, no correlation was found between either NOx FSR or ASR and insulin sensitivity after the multiple stepwise regression analysis and using various models (Table 4), underlying either the major role in in vivo NOx production of other physiologically relevant mechanisms or the presence of confounding factors inhibiting the insulin effect.

A positive correlation between insulin-mediated forearm glucose disposal and NOS activity was previously reported in T2DM subjects at the skeletal muscle level (34). The reasons for the discrepancy with our findings are not entirely clear. NOS activity in skeletal muscle may not correlate with regional NOx production or it may not reflect whole-body NOx release. In addition, while the tissue origin of NO in whole-body tracer turnover measurement is largely unknown, most of the insulin-mediated glucose disposal occurs in muscle (19). Should the relative contribution of skeletal muscle to whole-body NO production be minor, a correlation between glucose disposal and whole-body NO production would be weak. In addition, the naturally occurring inhibitors of NOS activity (such as ADMA) might have interfered with the insulin effect on NOS, as discussed above.

Whether insulin resistance to glucose disposal is extended to other metabolic pathways is a matter of debate. In T2DM, insulin resistance to amino acid/protein turnover either was (36) or was not (5,37) reported. Contrasting data have been reported also in aging (38,39). In essential hypertension, no insulin resistance on protein turnover was found (40) as opposed to that on glucose metabolism (41). Familial hypercholesterolemia is not an insulin-resistant state, either (42).

An association between endothelial dysfunction and dimethylarginine concentrations was previously reported in aging, although contrasting data also exist (13,43–45). Elderly subjects usually exhibited increased ADMA levels (13,38,46). Age-associated endothelial dysfunction may recognize ADMA as a possible determinant (47). In agreement with these findings, we found both direct simple relationships between age and either ADMA or SDMA concentrations and a correlation between NO FSR and

ADMA levels in the multiple regression analysis. The increased ADMA (and SDMA) concentrations represent the balance between their release from proteolysis and degradation by DDAH (11,48), and they might be increased in conditions of insulin resistance and impaired suppression of proteolysis, as previously reported in obesity and aging (38). It would also be interesting to measure DDAH activity with respect to both insulin sensitivity and circulating ADMA and SDMA concentrations. Interestingly, DDHA overexpression was found to enhance insulin sensitivity in mice (49). A role of ADMA in insulin resistance is also confirmed by the inverse correlation between ADMA and insulin sensitivity (Fig. 2).

In conclusion, our study shows that age, ADMA concentrations, and T2DM are (independently) associated with decreased whole-body NO production in vivo. In contrast, insulin resistance does not appear to play a relevant role on the regulation of NOx production, at least at the whole-body level. Although these conclusions may be hampered by variables frequently coexisting in aging, hypertension, hypercholesterolemia, or diabetes, this study nevertheless provides novel information on the factors regulating whole-body NO production in vivo, focusing the interest on age as well as on circulating inhibitors of NOS activity.

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P.T. wrote the manuscript, designed and performed the study, and researched data. D.C. performed the study and researched data. C.A. researched data and reviewed the manuscript. M.V. and R.M. performed the study and researched data. M.P. reviewed the manuscript and contributed to discussion. L.P. and M.V. performed the study and researched data. P.T. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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