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Acute Consumption of Flavanol-Rich Cocoa and the Reversal of Endothelial Dysfunction in Smokers

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OBJECTIVES This study was designed to assess the effect of flavanol-rich food on the circulating pool of bioactive nitric oxide (NO) and endothelial dysfunction in smokers.

BACKGROUND Studies suggest that smoking-related vascular disease is caused by impaired NO synthesis and that diets rich in flavanols can increase bioactive NO in plasma.

METHODS In smokers (n = 11), the effects of flavanol-rich cocoa on circulating NO species in plasma (RXNO) measured by reductive gas-phase chemiluminescence and endothelial function as assessed by flow-mediated dilation (FMD) were characterized in a dose-finding study orally administering cocoa containing 88 to 370 mg flavanols and in a randomized double-blind crossover study using 100 ml cocoa drink with high (176 to 185 mg) or low (<11 mg) flavanol content on two separate days. In addition to cocoa drink, ascorbic acid and NO-synthase inhibitor L-NMMA (n = 4) were applied.

RESULTS There were significant increases in RXNO (21 ± 3 nmol/l to 29 ± 5 nmol/l) and FMD (4.5 ± 0.8% to 6.9 ± 0.9%, each $p < 0.05$) at 2 h after ingestion of 176 to 185 mg flavanols, a dose potentially exerting maximal effects. These changes correlated with increases in flavanol metabolites. Cocoa-associated increases in RXNO and FMD were reversed by L-NMMA. Ascorbic acid had no effect.

CONCLUSIONS The circulating pool of bioactive NO and endothelium-dependent vasodilation is acutely increased in smokers following the oral ingestion of a flavanol-rich cocoa drink. The increase in circulating NO pool may contribute to beneficial vascular health effects of flavanol-rich food. (J Am Coll Cardiol 2005;46:1276–83) © 2005 by the American College of Cardiology Foundation

Cigarette smoking leads to atherosclerosis, with the severe clinical end points of myocardial infarction and stroke. Long before morphologic alterations of arterial walls become apparent, an impairment of endothelium-dependent flow-mediated vasodilation (FMD) can be detected (1). Endothelial dysfunction is an early stage of atherosclerosis and has been attributed to impaired nitric oxide (NO) bioactivity and enhanced formation of oxygen-derived free radicals (2,3). Endothelium-derived NO is an essential short-lived signaling molecule important for vascular homeostasis, including vasodilation, inhibition of platelet adhesion, and inhibition of smooth muscle proliferation in the vessel wall (4,5). Until recently, it was supposed that the bioactivity of NO was limited to close temporal and spatial proximity of the endothelium and that NO could travel only short distances in the bloodstream before being oxidized to nitrite and nitrate (6). Current studies challenge this concept by suggesting that NO exists as a pool of various NO-containing plasma components (S- and N-nitrosoproteins, iron-nitrosyl complexes) (7–9) with bioactivity resembling that of authentic NO (10,11); thus, these species together comprise the circulating NO pool (RXNO). Up to the present study, it was unknown whether chronic smoking-related impairment of endothelium-dependent vasodilation measured as FMD extended to the circulating NO pool was reflected in decreased concentrations of bioactive NO species in plasma.

Dietary interventions have suggested that intake of plant-derived foods and beverages is inversely associated with the risk of cardiovascular disease (12). These beneficial effects have been frequently ascribed to flavanols, a subgroup of flavonoids, the polyphenolic family of antioxidant chemicals abundantly present in fruits and vegetables (13–15). Studies in humans and animal models suggested that vascular effects of flavanols are due, at least in part, to an increased nitric oxide synthase activity and thus an augmented supply of bioactive NO (16–18). We have recently reported that in individuals with cardiovascular risk factors or coronary artery disease, the consumption of a flavanol-rich cocoa drink can acutely restore endothelium-dependent vasodilation paralleled by an increase in the circulating NO pool

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Abbreviations and Acronyms

FMD = flow-mediated dilation
GTN = glycerol trinitrate
HFCD = high-flavanol cocoa drink
LFCD = low-flavanol cocoa drink
NOS = nitric oxide synthase
RXNO = sum of circulating NO species

(19). The present study is a full report on the subgroup of smokers from our original study (19). We address the question whether a dietary intervention using a flavanol-rich cocoa drink can acutely reverse endothelial dysfunction in smokers and elucidate mechanisms involved.

METHODS

Study protocols. In order to assess the potential of dietary intervention using flavanol-rich foods to reverse endothelial dysfunction, plasma RXNO and endothelium-dependent dilation (FMD) were measured in a subgroup of 11 healthy volunteers, with smoking as the only major cardiovascular risk factor (Table 1) (19). Exclusion criteria were hypertension, diabetes mellitus, hyperlipidemia, malignancies, terminal renal failure, and acute inflammation (C-reactive protein >0.5 mg/dl).

To assess the optimal flavanol dose for the larger crossover study (see the following text), a preliminary dose-finding study was conducted. The RXNO as well as FMD were measured in four smokers before and 2 h after (19–22) ingestion of tap water (100 ml) or 50, 100, and 200 ml of high-flavanol cocoa drink (HFCD) (CocoaVia, Mars Inc., Slough, United Kingdom) on four separate days. The HFCD contained 176 to 185 mg of flavan-3-ols per 100 ml (40% epicatechin/catechin, 60% procyanidins). The variability of flavanol content was due to several factors, including natural variations of the flavanol content in plant-based foods, normal variability produced as a result of commercial-scale food manufacturing, and the variability of the analytic methods employed. Selected flavanol metabolites were estimated from plasma samples.

Table 1. Characteristics of Study Population

	Smokers
n	11
Age, yrs	31 ± 1
Gender, M/F	6/5
BMI, kg/m^2	21.8 ± 0.8
Mean arterial pressure, mm Hg	87 ± 2
Total cholesterol, mg/dl	192 ± 5
LDL, mg/dl	116 ± 5
HDL, mg/dl	67 ± 6
Glucose, mg/dl	86 ± 1
Pack-years	12 ± 2
Cigarettes per day	17 ± 2
RXNO, nmol/l	21 ± 3
Brachial artery diameter, mm	4.2 ± 0.2
Flow-mediated dilation, %	4.5 ± 0.8
Glycerol trinitrate-mediated dilation, %	14.7 ± 2.1

Data given as mean ± SE.
BMI = body mass index; HDL = high-density lipoprotein; LDL = low-density lipoprotein; RXNO = sum of circulating species.

Table 2. Composition of Cocoa Drinks

Per 100 ml	High Flavanol	Control
Total flavanols, mg	176–185	<11.5
Monomers, mg	70–74	<1
Epicatechin, mg	20–22	<1
Procyanidins, mg	106–111	<11.4
Caffeine, mg	8.6	4.3
Theobromine, mg	128.9	125.8
Energy, kJ (kcal)	307 (73)	277 (66)
Total protein, g	3	3.1
Total carbohydrate, g	13.2	12.1
Dietary fibers, g	0.87	0.24
Sugars, g	12.4	11.8
Total fat, g	0.89	0.57

On the basis of the results of our dose-finding study, we designed a larger randomized double-blind crossover study (19). On two days, RXNO and FMD were measured before and 2 h after double-blind ingestion of 100 ml HFCD or control low-flavanol cocoa drink (LFCD) (100 ml cocoa drink with <11 mg of flavanols [CocoaVia, Mars Inc.]). In our original study (19), we used a balanced crossover design with 10 individuals receiving flavanol-rich drinks on their first study day and 10 on the second day. Six of each group were included in the subgroup of smokers (n = 12 total). One female subject (HFCD on day 1) was excluded because of hypercholesterolemia after completion of the study, resulting in 11 smokers (n = 5 HFCD and n = 6 LFCD on day 1). Both drinks were similar in taste and indistinguishable by color and packaging; furthermore, these products were matched on the basis of macronutrients, calories, theobromine, and caffeine (Table 2).

To gain more insight into the potential mechanisms underlying the observed vascular responses, HFCD was administered to a randomly chosen subset of the 11 smokers described earlier on two separate days (n = 4). After the 2-h time point, nitric oxide synthase (NOS) was inhibited by intravenous infusion of L-N^G-monomethyl-arginine (L-NMMA) (Clinalfa, Laeufelfingen, Switzerland) over 30 min and measurements were repeated. Infusion rates were 1 mg·kg$^{\cdot -1}$·min^{-1} for the first 3 min followed by 0.2 mg·kg$^{\cdot -1}$·min^{-1} for 27 min sufficient to abolish NO-dependent FMD in healthy nonsmoking volunteers (data not shown). Ascorbic acid was reported to increase NO synthesis in smokers (23). To elucidate whether ascorbic acid further increases FMD and RXNO, a group of smokers (n = 4) received 2 g intravenous L-ascorbic acid (20 ml 0.57 mol/l; Cebion N forte, Merck, Darmstadt, Germany) in addition to the HFCD (administered at 0 h) at the 2-h time point.

Volunteers fasted overnight and were required to refrain from smoking 12 h before beginning and during the entire assessment period. The study was performed at the Heinrich-

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Heine University, Duesseldorf, Germany, after formal approval of the protocol by the local ethics committee.

Measurement of vascular parameters. Flow-mediated dilation was measured as previously described (24,25). Briefly, the diameter of the brachial artery was measured by a 15 MHz transducer (Sonos 5500, Philips Medizin Systeme, Hamburg, Germany) and automatic edge-detection software (Brachial Analyzer, Medical Imaging Applications, Iowa City, Iowa) yielding a coefficient of variation of <1%. Reactive hyperemia was induced by 5 min of distal lower arm occlusion. After 60 s, the diameter was assessed and FMD calculated as relative diameter gain compared to baseline. In our laboratory, reference values are 7.1 ± 0.6% in a comparable nonsmoking control group without hypertension, diabetes, or hypercholesterolemia (26). Endothelium-independent dilation was measured 4 min after sublingual application of 400 μg glycerol trinitrate. Mean arterial pressure was calculated as 1/3 (systolic-diastolic blood pressure) plus diastolic blood pressure.

Biochemical parameters. Plasma levels of circulating NO pool (RXNO: sum of S-, N-nitrosothiols, and iron-nitrosyl complexes) were determined using a gas-phase chemiluminescence assay (27). Using the identical methodology, we have measured 36 ± 2 nmol/l plasma RXNO in a comparable nonsmoking control group without hypertension, diabetes, or hypercholesterolemia. Whole blood was diluted 1:5 in ice-cold 0.9% saline containing N-ethylmaleimide (5 mmol/l) and EDTA (2 mmol/l) and centrifuged at 750 × g for 5 min. Plasma was treated with 1/10 volume of 5% sulfanilamide in 1M HCl for 10 min and then injected into a tri-iodide–containing vessel actively purged with a helium stream in line with an NO chemiluminescence analyzer (88NOe, EcoPhysics, Duernten, Switzerland). Flavanol (epicatechin and catechin) and select flavanol metabolites (epicatechin-7-beta-D-glucuronide, 4′-*O*-methyl-epicatechin, 4′-*O*-methyl-epicatechin-β-D-glucuronide) present in plasma were analyzed by an HPLC with an FLD detection system (Hewlett Packard 1100 Series, Mississauga, Ontario, Canada); HPLC column: Phenomenex C18(2) 150 × 4.8 mm, 3 μm) under utilization of authentic standards as detailed elsewhere (28,29).

Statistical analyses. Results are expressed as means ± SE. Comparisons between groups were analyzed by Student *t* test. Repeated measurements two-way analysis of variance was used to estimate intra-individual effects. Pairwise comparisons were corrected by the Bonferroni confidence interval. Correlations were determined with two-tailed Pearson's r value. Normal distribution was estimated using Kolmogorov-Smirnov test. Statistical significance was assumed if a null hypothesis could be rejected at p = 0.05. Increases in RXNO, FMD, and flavanol metabolites were calculated as data at given time points minus baseline. All analyses were performed with SPSS 11.0.1 (SPSS Inc, Chicago, Illinois).

RESULTS

Baseline characteristics of study population. The clinical baseline characteristics (age, gender, body mass index, mean arterial pressure, total cholesterol, low-density lipoprotein and high-density lipoprotein cholesterol, and fasting plasma glucose) were within normal limits (Table 1). Smokers had a history of 12 ± 2 pack-years and smoked 17 ± 2 cigarettes per day as assessed by the reported number of cigarettes smoked on the day before the first study day. The baseline plasma concentration of RXNO was 21 ± 3 nmol/l and FMD was 4.5 ± 0.8%. No significant correlations between baseline smoking behavior (pack years, cigarettes per day) and FMD or plasma RXNO at baseline were seen.

Dose-dependence of flavanols on circulating NO pool in smokers. In order to assess optimal flavanol dose to be used in a larger crossover study, increasing volumes of HFCD were administered. Dose-dependent increases in RXNO (50 ml [88 to 92 mg total flavanols]: 13 ± 1 nmol/l to 17 ± 1 nmol/l, p = 0.023; 100 ml [176 to 185 mg]: 14 ± 1 nmol/l to 23 ± 1 nmol/l, p = 0.001; 200 ml [352 to 370 mg]: 13 ± 1 nmol/l to 23 ± 1 nmol/l, p = 0.001) and FMD (50 ml: 3.3 ± 0.8% to 4.1 ± 0.6%, p = 0.444; 100 ml: 2.2 ± 1.0% to 5.9 ± 0.6%, p = 0.030; 200 ml: 3.8 ± 1.2% to 7.5 ± 0.8%, p = 0.039) were observed 2 h after consumption of HFCD (p values corrected for three pairwise comparisons). Figure 1 depicts absolute increases in RXNO and FMD over baseline. Neither ingestion of 100 ml tap water nor LFCD (<11 mg) had a significant effect on

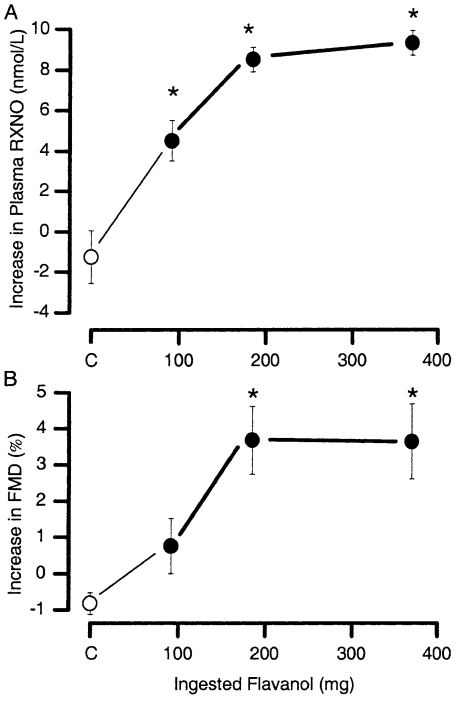

Figure 1. Dose-finding. Flavanol-rich cocoa dose-dependently increased the circulating nitric oxide (NO) pool (RXNO) **(A)** and flow-mediated dilation (FMD) **(B)** at 2 h **(filled circles)**. RXNO and FMD did not significantly change after water control (C, **open circles**). Data represent means ± SE. (n = 4 smokers) *p < 0.05.

Table 3. Plasma Flavanol Metabolites at Baseline and Following 2 h After Consumption of Cocoa Beverage

	Flavanol Content of Drinks, mg					
	88–92		176–185		352–370	
	Baseline	2 h	Baseline	2 h	Baseline	2 h
Epicatechin, nmol/l	3 ± 0	3 ± 0	3 ± 0	11 ± 3*	6 ± 3	19 ± 6*
Catechin, nmol/l	10 ± 2	9 ± 3	7 ± 2	19 ± 7	8 ± 2	18 ± 3*
Epicatechin-7-β-D-glucuronide, nmol/l	11 ± 7	9 ± 3	9 ± 6	38 ± 19	14 ± 4	39 ± 13*
4′-*O*-methyl-epicatechin, nmol/l	22 ± 8	25 ± 5	23 ± 9	38 ± 12	26 ± 9	41 ± 10*
4′-*O*-methyl-epicatechin-β-D-glucuronide, nmol/l	157 ± 31	151 ± 46	156 ± 32	202 ± 40*	184 ± 59	287 ± 58*

*$p < 0.05$ (baseline vs. 2 h).

RXNO and FMD (data not shown). As there were no significant differences between the effects on RXNO and FMD observed after the consumption of 100 or 200 ml, the 100 ml dosage was used in the crossover trial (30).

Plasma levels of free aglyconic flavanols, as well as those of conjugated flavanol metabolites, which represent only a selected subset of those present in plasma, increased significantly only following the ingestion of >100 ml HFCD containing ≥176 mg of total flavanols. However, following basal level corrections, the ingestion of 50 ml HFCD (88 to 92 mg total flavanols) was without effect. (Table 3) The increases in epicatechin ($r = 0.75$, $p = 0.005$), catechin ($r = 0.76$, $p = 0.004$), epicatechin-7-beta-D-glucuronide ($r = 0.69$, $p = 0.013$), 4′-*O*-methyl-epicatechin ($r = 0.65$, $p = 0.022$), and 4′-*O*-methyl-epicatechin-β-D-glucuronide ($r = 0.67$, $p = 0.018$) correlated significantly with the increase in RXNO. The increases in FMD correlated with the increase in epicatechin ($r = 0.66$, $p = 0.020$) and catechin ($r = 0.62$, $p = 0.031$)

Flavanol-rich cocoa drink acutely increased RXNO and reversed endothelial dysfunction. On the basis of the dose-finding data (Fig. 1), we performed a randomized double-blind crossover study using a cocoa drink high in flavanols (HFCD) and the matched low-flavanol cocoa beverage (LFCD) (Table 2). We performed a repeated-measurements analysis of variance with two within-subject factors (baseline vs. 2 h post-drink and high-flavanol drink vs. control drink) and found a significant two-way interaction (FMD, $p = 0.002$; RXNO, $p = 0.03$). The pairwise comparison showed that RXNO and FMD significantly increased 2 h after ingestion of 100 ml HFCD (4.2 ± 0.9% to 6.9 ± 0.9%, $p < 0.001$, and 20 ± 3 nmol/l to 29 ± 2 nmol/l; $p = 0.001$) (Figs. 2A and 2B), but was unaltered after the LFCD (4.5 ± 0.9% to 3.6 ± 0.9%, $p = 0.141$, and 23 ± 4 nmol/l to 20 ± 3 nmol/l, $p = 0.452$) (Figs. 2C and 2D) (each p corrected for two comparisons), and baseline values were not significantly different on the two study days. Interestingly, we found strong correlations between the number of cigarettes smoked per day and the absolute increases in plasma RXNO ($r = 0.73$, $p = 0.011$) and FMD ($r = 0.69$, $p = 0.020$) 2 h following the ingestion of the high-flavanol drink. There were no significant correlations between the pack-years and the increases in RXNO ($r = 0.40$, $p = 0.218$) or FMD ($r = 0.50$, $p = 0.120$).

NOS dependence of flavanols effects. In order to gain mechanistic insight, we inhibited NOS or alternatively administered ascorbic acid on top of the observed effects. Nitric oxide synthase inhibition via L-NMMA, mimicking acute endothelial dysfunction, abolished cocoa-related improvement of endothelial dysfunction and increase in the circulating NO pool. (Figs. 3A and 3B). Ascorbic acid has been shown to increase endothelial function in smokers by increasing tetrahydrobiopterine, a cofactor of NOS (23). Here, we observed no further increase of improvement in

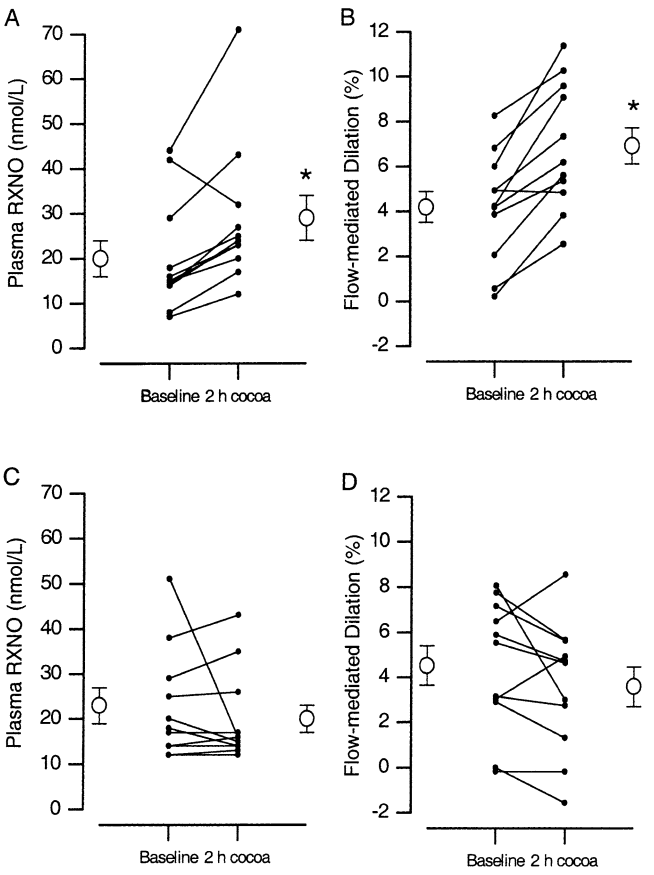

Figure 2. Restoration of circulating nitric oxide (NO) pool by flavanol-rich cocoa drink. Randomized double-blind crossover design, ingestion of 100 ml flavanol-rich cocoa drink (176 to 185 mg flavanols) increased **(A)** circulating NO pool (plasma RXNO) and **(B)** flow-mediated dilation after 2 h (n = 11 smokers). Ingestion of the matched control drink had no significant effect on both parameters **(C, D)**. **Filled circles** = individual values; **open circles** = means ± SE. *$p < 0.05$.

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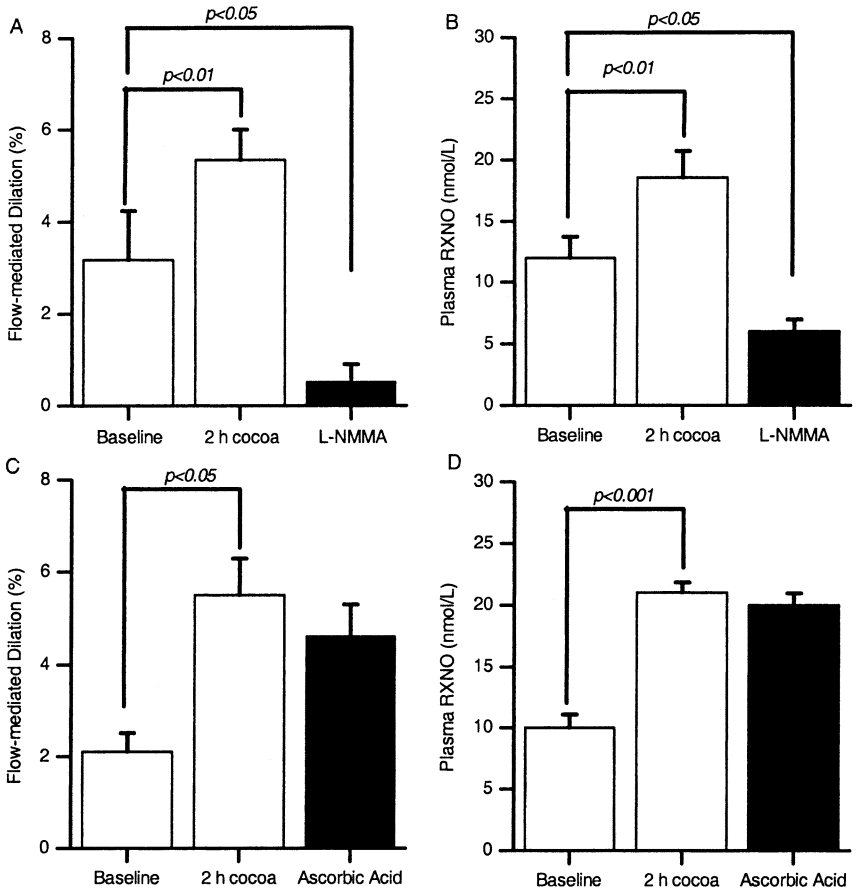

Figure 3. Effects of L-NMMA and vitamin C on cocoa effects. Both flow-mediated dilation (FMD) **(A and C)** and the circulating NO pool in plasma (RXNO) **(B and D)** increased 2 h after ingestion of cocoa containing 176 to 185 mg of flavanols. Whereas L-NMMA **(A and B)** reversed both parameters below baseline values, ascorbic acid (2 g, **C and D**) did not cause a further increase when given after measurements at the 2-h time point. Significance levels corrected for 8 pairwise comparisons in RXNO and FMD. Columns represent means ± SE (n = 4 smokers).

endothelial dysfunction and circulating NO pool after application of ascorbic acid in a dose sufficient to reverse endothelial dysfunction in smokers when applied alone (Figs. 3C and 3D).

DISCUSSION

The circulating NO pool and flow-mediated dilation were acutely increased following the consumption of a flavanol-rich cocoa beverage. The increases in RXNO and endothelial function were NOS-dependent, as NOS inhibition abolished the observed effects. Ascorbic acid did not further increase both parameters, suggesting a parallel antioxidant mechanism. The increase in individual flavanol metabolites correlated with the increase in NO.

The RXNOs exhibit biologic vasoprotective actions similar to NO in vivo as well as in vitro (31), including vasodilation (11,27), inhibition of platelet aggregation (32), and interruption of carotid embolization (33). In fact, studies imply that RXNOs in plasma represent a physiologic storage pool for bioactive NO in human blood capable of compensating for local NO deficit. Cannon et al. (10) have shown that inhaled NO can restore blood flow and vascular resistance in the forearm during regional inhibition of NO synthesis. Results from our group suggest that plasma RXNO contributes to systemic vasodilator effects of NO after intravenous and intra-arterial infusion (11).

Endothelial function as measured by FMD is commonly used as a clinical readout of endothelial NO synthesis (1,34). Flow-mediated dilation of the brachial artery is almost entirely NOS-dependent (34) and correlates very well with endothelial function of most other conduit arteries, including the coronary arteries, and can therefore be used as a surrogate for systemic NO synthesis. Several groups have previously shown that smokers show an impaired FMD with normal vasodilation in response to oral glycerol trinitrate (1,35). The baseline values for FMD and plasma RXNO measured in smokers were significantly lower than values we have previously measured in healthy non-smokers (27). Thus, a decreased pool of circulating NO in smokers may be secondary to decreased endothelial function and may potentially contribute to chronic vascular disease.

We show in smokers >12 h after smoking cessation and during abstinence from smoking that the consumption of a flavanol-rich, but not a flavanol-poor, beverage dose-dependently increased the circulating NO pool in plasma

and FMD response by approximately 50% These findings are novel as they were obtained from a study population for which smoking represented the only cardiovascular risk factor. Thus, our findings further support previous epidemiologic investigations and dietary interventions in humans that demonstrated a beneficial role for cocoa flavanols in the general context of cardiovascular health (19,36–38). An increased circulating NO pool may in part compensate for regional deficits, for example, potentially inhibiting platelet adhesion to arteriosclerotic plaques, stabilizing plaques by inhibition of low-density lipoprotein oxidation, and dilating stenosed conduit vessels. Mechanistically, it is interesting to note that the magnitude of increase in RXNO and FMD correlated with the number of cigarettes smoked, but not with the baseline levels of both parameters or the pack-years. This suggests that part of the effect may be due to reversal of acute cigarette smoke-related effects.

We observed that a flavanol-related improvement of RXNO and endothelial function can be reversed by L-NMMA infusion, but not further increased by ascorbic acid. Given the reversal of effects following NOS inhibition, it can be argued that the mechanism of action of flavanol-rich food involves an increase in NOS activity. This is further supported by Fisher et al. (17), who investigated the influence of flavanol-rich cocoa and NO synthase inhibition on blood flow in the finger of otherwise normal healthy subjects. Potential mechanisms leading to acutely increased NOS activity following ingestion of flavanol-rich foods may involve antioxidant properties of flavanols similar to ascorbic acid, either sparing NO from degradation or increasing NO synthesis. Whether one or the other of these potential mechanisms is responsible for the observed effects in this study, or indeed a combination of both, cannot be concluded from the present data because both mechanisms are dependent on each other. Data from investigations in vitro and in vivo demonstrated previously that various factors associated with oxidative stress, including smoking (1), inflammation (39), vitamin deficiency, and others resulted in a decline of NO production and in decreased levels of tetrahydrobiopterin (BH_4), an essential cofactor of NOS. Concordantly, free radicals present in cigarette smoke and increased endogenous radical generation secondary to cigarette smoking (2) were suggested to uncouple NOS by causing a decrease in BH_4, subsequently leading to lower NOS and release (40). In this context, ascorbic acid administration has been shown in vivo (41) and in vitro (42) to attenuate the oxidative stress-related abrogation of NO production by either replenishing BH_4 or by preventing its loss through mechanisms that are causally linked to the antioxidant properties of ascorbic acid. Although ascorbic acid has been shown previously to increase the FMD response in smokers (23,41), the injection of ascorbic acid in the context of flavanol-rich cocoa ingestion did not result in additional increases in FMD as compared with flavanol-rich cocoa alone. The lack of such additive effects implies either that flavanol-rich cocoa consumption resulted in a saturation of ascorbate-mimicking antioxidant effects or that cocoa consumption resulted in a maximal FMD response that is seemingly independent of antioxidant mechanisms as elicited by ascorbate.

Regarding the identification of the bioactive flavanol/metabolite(s), it has to be noted that flavanols are subject to extensive phase I/II metabolism in gut and liver (20,43,44). They also undergo a variety of biotransformations that are related to the activity of enzymes inherent to the gut microflora (45). Thus, the flavanol metabolites present in circulation represent a variety of primary, structurally related as well as secondary metabolites, which exhibit various differences in their physical and chemical properties as compared with the native flavanols ingested. Although various investigators have previously reported on general aspects of flavanol metabolism following the ingestion of certain fruit, wine, tea, and cocoa products, information on flavonoid metabolites in the context of specific pathology-related dietary interventions is scarce. Most investigators use an enzyme hydrolysis to cleave glucuronides and sulphates to aglycones and *O*-methylated flavanol derivatives before analyzing plasma or urinary samples. Although this approach does provide the required evidence that flavanols were absorbed, and thus were present in circulation, any information regarding the identity of specific metabolites, which may be essential in determining the active flavanol derivatives, will be lost. Thus, during this investigation, we have aimed at providing not just evidence of flavanol absorption, but also information regarding the identity of predominant circulating flavanol metabolites. The correlation between increases in RXNO, FMD, and increases in the selected flavanol metabolites in plasma suggests that the observed effects are indeed related to ingested flavanols. Furthermore, the results obtained demonstrate that 4′-*O*-methyl-epicatechin-β-D-glucuronide is likely one of the major metabolites present in circulation following the ingestion of the cocoa beverage used, reaching maximal plasma concentration of up to 287 nmol/l. However, baseline values are relatively high, and it is also possible that there are other major metabolites in plasma that may be responsible for the observed effects, or that flavanols are only indicators of unknown bioactive substances in cocoa.

Conclusions. Taken together, the results herein suggest that the circulating pool of bioactive NO and endothelium-dependent dilation are acutely increased by intake of a flavanol-rich cocoa drink during abstinence from smoking. This supports the concept that dietary flavanols can reverse endothelial dysfunction. The increase in circulating NO species may contribute to the potential health effects of flavanol-rich foods and may have application for the treatment of diseases characterized by impaired regional NO production, such as peripheral and coronary artery disease. However, long-term studies are needed to investigate

whether this promising potential transforms unambiguously into long-term health benefits.

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