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Increased Blood Flow Induces Oxidative Stress Through an Endothelium- and Nitric Oxide-Independent Mechanism

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Abstract

Bradykinin, a potent vasodilator, stimulates the formation of reactive oxygen species and F₂-isoprostanes *in vitro*. The effect of bradykinin on oxidative stress in humans is not known. This study tested the hypothesis that bradykinin induces oxidative stress through a nitric oxide (NO)-dependent mechanism in the human vasculature. We measured forearm blood flow (FBF) and net F₂-isoprostane release in response to intra-arterial bradykinin (50–200ng/min), nitroprusside (1.6–6.4μg/min) or diltiazem (3.6–14.4μg/min) in the absence and presence of the NO synthase inhibitor N^ω-monomethyl-L-arginine (L-NMMA) in normotensive and hypertensive subjects pre-treated with aspirin. L-NMMA significantly decreased basal FBF and basal net F₂-isoprostane release (from 28.7±5.2 to 13.4±3.5 pg/min/100ml, P=0.01) in all subjects. Bradykinin caused a significant increase in FBF and net F₂-isoprostane release in both normotensive and hypertensive subjects. During NO synthase inhibition, bradykinin significantly increased net F₂-isoprostane release in both groups (P=0.001) and there was no effect of L-NMMA on bradykinin-stimulated F₂-isoprostane release (P=0.46). Nitroprusside also significantly increased net F₂-isoprostane release in hypertensive subjects (P=0.01) and this response was not affected by L-NMMA (P=0.50). Diltiazem increased FBF as well as net F₂-isoprostane release (from 44.5±12.5 to 61.2±14.7 pg/min/100ml at the highest dose, P=0.05). Increasing blood flow induces oxidative stress through a NO- and endothelium-independent mechanism.

Keywords

bradykinin; oxidative stress; isoprostanes; blood flow; nitric oxide

Introduction

The endothelium regulates vascular tone, cell adhesiveness, inflammation, coagulation and permeability.¹ Endothelial dysfunction, a precursor of atherosclerosis, predicts the development of cardiovascular events in patients at risk for coronary artery disease.^{2, 3} Coronary endothelial dysfunction has been associated with enhanced endogenous oxidative

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stress as measured by F₂-isoprostane release.⁴ F₂-isoprostanes are sensitive and specific markers of oxidative stress and are not only elevated in patients with cardiovascular disease but also present in atherosclerotic lesions.^{5, 6} However, the mechanism(s) governing F₂-isoprostane release and the role of nitric oxide (NO) in this process is incompletely understood.

Bradykinin, an endothelium-dependent agonist, causes vasodilation in part by increasing NO, prostacyclin and endothelium-derived hyperpolarizing factor.⁷ In addition, bradykinin and several other activators of phospholipase A₂ have been shown to stimulate the formation of F₂-isoprostanes in isolated human umbilical vein.⁸ Nitroprusside, an endothelium-independent agonist, increases isoprostane concentrations in human cavernosal cultured cells.⁹ Inhibition of NO synthase reduces oxidative stress in human pulmonary artery further suggesting a role for NO in F₂-isoprostane generation.¹⁰ The effect of bradykinin on F₂-isoprostane release has not been studied in humans.

This study set out to test the hypothesis that bradykinin induces oxidative stress, as measured by net F₂-isoprostane release, through a NO-dependent mechanism in the human vasculature.

Methods

Subjects

Twenty-six hypertensive subjects and thirty-three normotensive subjects were studied. After written informed consent was obtained, all subjects underwent a complete history and physical examination. Subjects with cardiovascular (except hypertension), renal, pulmonary, endocrine, or hematological disease were excluded. Pregnancy was excluded in women of childbearing potential by measurement of urine β -HCG. Subjects were defined as hypertensive if they had more than one documented pressure greater than 140 mmHg systolic or greater than 90 mmHg diastolic or had been treated for hypertension for more than 6 months. All antihypertensive medications were withdrawn for 3 weeks prior to study. Subjects with fasting cholesterol greater than 5.7 mmol/L (220 mg/dl) and smokers were excluded.

Experimental Protocol

The study protocol was approved by the Vanderbilt University Institutional Review Board and conducted according to the Declaration of Helsinki. Studies were performed in the morning in a temperature-controlled room. Subjects were studied in the supine position after an overnight fast. All subjects received 325mg of acetylsalicylic acid (ASA, non-selective COX inhibitor) prior to placement of the catheters. ASA was administered to exclude a contribution of prostacyclin to bradykinin-mediated vasodilation. An intravenous catheter was placed in the antecubital vein in the non-dominant arm. After subdermal administration of 1% lidocaine, a 20 gauge polyurethane catheter (Cook Inc., Bloomington, IN) was inserted into the brachial artery of the non-dominant arm allowing direct intra-arterial administration of drugs. Prior to the infusion of vasoactive drugs arterial catheter patency was maintained by infusion of 0.9% sodium chloride at a rate of 1 ml/minute. Following placement of catheters, subjects were allowed to rest 30 minutes before baseline measurements were made. Forearm blood flow (FBF) was measured by silastic-in-mercury strain-gauge plethysmography. After measurement of basal FBF and blood sampling, graded doses of bradykinin (an endothelium-dependent vasodilator, Clinalfa AG, Lufelfingen, Switzerland), or sodium nitroprusside (SNP, a NO donor, Major Pharmaceuticals Inc, Livonia MI) were infused. Bradykinin was infused at 50, 100, and 200ng/min and SNP was infused at 1.6, 3.2 and 6.4g/min. Each dose of agonist was infused for 5 minutes and FBF

was measured during the last 2 minutes. A twenty minute rest period was allowed between infusion of bradykinin and nitroprusside. Thirty minutes after administration of bradykinin or nitroprusside a continuous intra-arterial infusion of N^ω-monomethyl-L-arginine (L-NMMA, NO synthase inhibitor; Bachem, Torrance, CA) at 4–12 μmol/min was started. While continuing the infusion of L-NMMA, baseline measurements and infusion of bradykinin or nitroprusside were repeated. Fifty-eight subjects received bradykinin (25 hypertensive and 33 normotensive), 30 subjects received nitroprusside (20 hypertensive and 10 normotensive), 33 subjects received bradykinin + L-NMMA (10 hypertensive and 23 normotensive) and 5 hypertensive subjects received nitroprusside + L-NMMA. To measure the effect of vasodilation on vascular F₂-isoprostane release in the absence of endogenous or exogenous NO, we also studied the effect of intra-arterial diltiazem at 3.6, 7.2 and 14.4 μg/min in six additional hypertensive subjects (3 men and 3 women).

Forearm Perfusion Measurements

FBF was measured by silastic-in-mercury strain-gauge plethysmography.¹¹ The wrist was supported in a sling to raise the forearm to above the level of the atrium, and the strain gauge was placed at the widest part of the forearm. The strain gauge was connected to a plethysmograph (model EX-5, D. E. Hokanson, Inc., Bellevue, WA), which was calibrated to measure the percent change in volume and connected to a chart recorder. For each measurement, a cuff placed around the upper arm was inflated to 40 mm Hg with a rapid cuff inflator (model E-10, D. E. Hokanson, Inc., Bellevue, WA) to occlude venous outflow from the extremity. The hand was excluded from the measurement of blood flow by inflation of a pediatric sphygmomanometer cuff around the wrist to 200 mm Hg before and during measurements of FBF. Flow measurements were recorded for ~7 of 15 seconds, and the slope was derived from the first 3 or 4 pulses; 5 to 7 such readings were obtained for each mean value.

Blood Sampling and Biochemical Assays

After measurement of FBF, simultaneous arterial and venous samples were obtained from the infused arm before and after baseline and highest dose of bradykinin and nitroprusside. Blood samples were collected on ice and centrifuged immediately, and plasma was stored at – 80°C until the time of assay. Free F₂-isoprostane (non-esterified) concentrations were determined by gas chromatography-mass spectrometry as previously described.¹² Ateriovenous concentration gradients were calculated by subtracting the plasma level measured in simultaneously collected venous and arterial blood. Forearm plasma flow was calculated from the FBF and arterial hematocrit corrected for 1% trapped plasma. Thus, individual net release or uptake rates at each time point were calculated by the following formula: net release = $(C_v - C_a) \times \{FBF \times [101 - \text{hematocrit}/100]\}$, where C_v and C_a represent the concentration of F₂-isoprostanes in the brachial vein and artery, respectively. Net F₂-isoprostane release is expressed as pg/min/100ml. Venous platelet-activating factor (PAF) acetylhydrolase activity was measured as previously described.¹³

Statistical Analysis

Data are presented as means ± standard error of the mean. Baseline characteristics between hypertensive and normotensive groups were compared using independent Student's *t*-test. Categorical data were compared using Chi-squared or Fischer's exact tests, as appropriate. The effect of L-NMMA on basal vasodilation and oxidative stress response was determined by a paired *t*-test. The effect of bradykinin, nitroprusside and diltiazem on F₂-isoprostane release was determined using general linear model-repeated measures ANOVA in which the between-subject variable was hypertension status, and the within-subjects variables were drug (control, +L-NMMA) and dose of agonist. F₂-isoprostane and FBF data that were not normally distributed were log transformed prior to analyses. A 2-tailed P value less than

0.05 was considered statistically significant. Statistical analyses were performed with the statistical package SPSS for Windows (Version 17.0, SPSS, Chicago, IL).

Results

Baseline Subject Characteristics

Table 1 provides the baseline characteristics of the subjects. Hypertensive subjects were significantly older, had higher serum cholesterol, and by definition had higher systolic, diastolic and mean arterial blood pressure compared to normotensive subjects. Basal FBF, FVR, basal venous F₂-isoprostane concentration, basal arterial-venous F₂-isoprostane gradient and net F₂-isoprostane release were similar in the two groups. Basal net F₂-isoprostane release did not correlate with basal FBF in either hypertensive or normotensive subjects ($P=0.97$, $P=0.90$ respectively). Basal net F₂-isoprostane release correlated significantly with age ($r^2=0.20$, $P=0.009$) and correlated inversely with BMI ($r^2=0.13$, $P=0.047$) in normotensive subjects but not in hypertensive subjects. There was no relationship between basal F₂-isoprostane release and gender, race, blood pressure or cholesterol in either group.

Effect of NO synthase inhibition on basal vascular function and oxidative stress response

L-NMMA significantly decreased basal FBF and increased FVR in all subjects combined (Table 2). L-NMMA significantly decreased basal FBF in normotensive but not hypertensive subjects. L-NMMA significantly decreased basal net F₂-isoprostane release. The decrease in basal net F₂-isoprostane release during L-NMMA was statistically similar in normotensive and hypertensive subjects.

Stimulated forearm blood flow response

Bradykinin caused a significant dose-dependent increase in FBF in both hypertensive ($F=70.81$, $P<0.001$) and normotensive subjects ($F=65.0$, $P<0.001$; Figure) with no significant difference between the two groups ($P=0.06$). L-NMMA significantly blunted bradykinin-stimulated FBF in both groups. Nitroprusside significantly and dose-dependently increased FBF in both hypertensive (from 4.9 ± 0.4 to 16.4 ± 1.4 ml/min/100ml at highest dose; $F=74.5$, $P<0.001$) and normotensive subjects (from 9.4 ± 2.3 to 18.7 ± 2.6 ml/min/100ml at highest dose, $F=8.9$, $P=0.01$). There was no effect of L-NMMA on nitroprusside-stimulated FBF in hypertensive subjects ($P=0.43$ for effect of drug).

Stimulated net F₂-isoprostane release

Bradykinin caused a significant increase in net F₂-isoprostane release in all subjects ($F=8.8$, $P=0.005$, Figure) and the effect was similar in normotensive and hypertensive subjects. During NO synthase inhibition, bradykinin significantly increased net F₂-isoprostane release in all subjects ($F=15.1$, $P=0.001$ for effect of dose) and this response was not significantly different compared to control ($P=0.46$ for effect of drug), even though baseline net release was decreased. Nitroprusside significantly increased net F₂-isoprostane release in hypertensive subjects (from 28.4 ± 6.3 to 60.2 ± 49.0 pg/ml/min at highest dose, $P=0.01$) and this response was not significantly affected by L-NMMA ($P=0.50$).

To determine whether a NO-independent agonist would increase F₂-isoprostane release, we measured net release of F₂-isoprostane in response to intra-arterial diltiazem in six additional hypertensive subjects. Diltiazem increased FBF in a dose-dependent fashion (from 3.0 ± 0.5 to 6.2 ± 1.1 ml/min/100ml at the highest dose, $P=0.048$). Diltiazem increased the net F₂-isoprostane release (from 44.5 ± 12.5 to 61.2 ± 14.7 pg/min/100ml at the highest dose, $P=0.05$).

To determine whether the increase in F₂-isoprostanes is due to release of F₂-isoprostanes from esterified lipids or the formation of F₂-isoprostanes from arachidonic acid due to increased oxidative stress (e.g. increased ROS production) we measured venous plasma PAF acetylhydrolase activity in 10 subjects at baseline and after maximum dose of bradykinin (200ng/min). PAF acetylhydrolase activity did not change (from a baseline of 0.80±0.07 to 0.78±0.07 nmol/min/mg at maximum dose of bradykinin, P=0.47) suggesting that enhanced oxidative stress, rather than increased PAF acetylhydrolase activity, increased F₂-isoprostane formation.

Discussion

This study examined the effect of increased blood flow and the contribution of NO to the oxidative stress response, as measured by F₂-isoprostanes, in normotensive and hypertensive subjects. Bradykinin, an endothelium-dependent agonist, as well as the NO-donor nitroprusside increased the net release of F₂-isoprostanes. NO synthase inhibition decreased blood flow without affecting endothelium-dependent or -independent F₂-isoprostane release, suggesting a NO synthase-independent mechanism. The finding that the calcium channel blocker diltiazem also increased the vascular release of F₂-isoprostanes further suggests that increased vascular F₂-isoprostane release may be a non-specific effect of increased blood flow.

Although various enzyme systems participate in superoxide formation within the vasculature including NO synthase, NAD(P)H oxidase, lipoxygenase, cyclooxygenase, and xanthine oxidase, NAD(P)H oxidase is considered the predominant source of reactive oxygen species (ROS).¹⁴ Under normal conditions endothelial NO synthase produces the vasoprotective molecule NO. Excess production of superoxide, due to risk factors such as smoking and high blood pressure, results in the formation of peroxynitrate when superoxide reacts with NO. Eventually endothelial NO synthase becomes “uncoupled” and instead generates only superoxide.¹⁵ Excessive production of ROS leads to oxidation of various molecules such as lipids, protein, carbohydrates and DNA and this process is commonly referred to as oxidative stress.¹⁶ F₂-isoprostanes, formed by non-enzymatic free radical-induced peroxidation of arachidonic acid, are considered to be reliable markers of oxidative stress *in vivo*.⁵ Increased oxidative stress plays an important role in the pathogenesis of various cardiovascular diseases including atherosclerosis, hypertension and hypercholesterolemia.^{17, 18} However, the mechanism(s) governing the release of F₂-isoprostanes including the role of NO has not been fully elucidated.

In human cavernosal cells, exposure to nitroprusside (a NO donor) increases F₂-isoprostanes concentrations by 2–3 fold.⁹ In addition, the NO synthase inhibitor, L-NAME, attenuates cytokine-stimulated release of F₂-isoprostanes in human pulmonary artery tissue.¹⁰ In DOCA-salt-treated hypertensive mice, NO synthase inhibition significantly decreases ROS formation indicating the important contribution of uncoupled endothelial NO synthase to the oxidative stress response.¹⁹ Lavi et al reported a significant correlation between net coronary isoprostane production and a decrease in coronary artery diameter in response to NO synthase inhibition. Although this prior study suggested that inactivation of NO by oxidative stress to be an important mechanism for endothelial dysfunction, the investigators did not directly examine the effect of NO synthase inhibition on net isoprostane production.⁴ In our study, NO synthase inhibition decreased basal net F₂-isoprostane release as well as flow suggesting that endogenous NO contributes to F₂-isoprostane production as well as basal vascular tone. In contrast, endogenous NO did not contribute to F₂-isoprostane release during pharmacologic vasodilation.

Increased blood flow may enhance F₂-isoprostane release due to an increase in shear stress. Increased blood flow leads to increased shear stress on endothelial cells.²⁰ Both shear stress and mechanical stretch have been previously shown to activate NAD(P)H oxidase (an important source of ROS) in endothelial and smooth muscle cells.²¹ For example, in an animal model of pulmonary hypertension secondary to an increase in blood flow, increased expression and activity of the NADPH oxidase complex increased oxidative stress.²² In addition, in cultured endothelial cells, pulsatile flow induces superoxide production via upregulation of vascular NADPH-dependent oxidase subunits.²³ The finding that PAF acetylhydrolase activity was not significantly increased during bradykinin administration further suggests that enhanced oxidative stress (increased ROS formation), rather than release of F₂-isoprostanes from esterified lipids, increases F₂-isoprostane formation.

We observed no significant difference in markers of oxidative stress between normotensive and hypertensive subjects in this study. This is in agreement with previous studies,²⁴⁻²⁵ which found no difference in F₂-isoprostanes concentrations, but in contrast to other studies using non-specific markers of oxidative stress.²⁶ These contrasting findings may be explained by the different methodologies used in the assessment of oxidative stress. In addition, endothelium-dependent vasodilation was not significantly decreased in hypertensive patients compared to normotensive subjects which is in agreement with some²⁷ but not other studies.²⁸⁻²⁹

In summary, our study results suggest that increased blood flow enhances oxidative stress independent of NO. The precise role of enhanced shear stress and the activation of NAD(P)H oxidase in vasodilation-induced oxidative stress need to be explored in future studies.

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Non-standard Abbreviations and Acronyms

BMI	Body mass index
FBF	Forearm blood flow
FVR	Forearm vascular resistance
L-NMMA	N ^ω -monomethyl-L-arginine
NO	Nitric oxide
PAF acetylhydrolase	Platelet-activating factor acetylhydrolase
ROS	Reactive oxygen species

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Figure 1. Bradykinin-stimulated forearm blood flow (FBF) and net F₂-isoprostane release in hypertensive and normotensive subjects in the absence and presence the nitric oxide synthase inhibitor N^ω-monomethyl-L-arginine (L-NMMA). *P<0.01, †P<0.001 for effect of drug by ANOVA; ‡P<0.05, §P<0.01 versus control; ‖P<0.05, #P<0.01, **P<0.001 versus baseline.

Table 1**Baseline Subject Characteristics**

	Hypertensive (N=26)	Normotensive (N=33)	P-value
Age (yrs)	45.5±2.0	31.4±1.2	<0.001
Gender (women)	12 (46.2)	15 (45.5)	0.96
Race (white)	15 (57.7)	25 (75.8)	0.14
BMI (kg/m ²)	28.3±1.9	30.1±1.0	0.20
Heart rate (bpm)	69.3±1.9	65.5±1.5	0.12
Systolic blood pressure (mmHg)	135.0±2.5	117.3±1.5	<0.001
Diastolic blood pressure (mmHg)	82.6±1.5	68.4±1.0	<0.001
Mean arterial pressure (mmHg)	100.0±1.7	84.7±0.9	<0.001
Cholesterol (mmol/L)	4.9±0.1	4.4±0.1	0.01
Forearm blood flow (ml/min/100ml)	4.9±0.4	4.1±0.2	0.09
Forearm vascular resistance (mmHg/ml/min/100ml)	24.8±2.3	23.1±1.4	0.51
Venous F ₂ -isoprostane (pg/ml)	46.2±5.1	40.1±4.1	0.25
Arterio-venous F ₂ -isoprostane gradient (pg/ml)	11.4±3.7	13.0±2.6	0.72
Net F ₂ -isoprostane release (pg/min/100ml)	30.8±10.0	27.2±5.0	0.75

Table 2

Effect of NO synthase inhibition on basal blood flow, vascular resistance and oxidative stress response

	Basal	+L-NMMA
<i>All Subjects</i>		
FBF (ml/min/100ml)	4.4±0.2	2.7±0.3 [‡]
FVR (mmHg/ml/min/100ml)	23.8±1.3	39.9±2.5 [‡]
Net F ₂ -isoprostane release (pg/min/100ml)	28.7±5.2	13.4±3.5 [‡]
<i>Hypertensive</i>		
FBF (ml/min/100ml)	4.9±0.4	3.6±0.7
FVR (mmHg/ml/min/100ml)	24.8±2.3	36.4±5.2 [‡]
Net F ₂ -isoprostane release (pg/min/100ml)	30.8±10.0	17.2±16.2 [*]
<i>Normotensive</i>		
FBF (ml/min/100ml)	4.1±0.2	2.3±0.2 [‡]
FVR (mmHg/ml/min/100ml)	23.1±1.4	41.4±2.9 [‡]
Net F ₂ -isoprostane release (pg/min/100ml)	27.2±5.0	12.4±2.0 [*]

FBF indicates forearm blood flow; FVR indicates forearm vascular resistance.

^{*} P<0.05,[‡] P<0.01,[‡] P<0.001 versus basal.