

# Nitrite Infusion in Humans and Nonhuman Primates

## Endocrine Effects, Pharmacokinetics, and Tolerance Formation

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**Background**—The recent discovery that nitrite is an intrinsic vasodilator and signaling molecule at near-physiological concentrations has raised the possibility that nitrite contributes to hypoxic vasodilation and to the bioactivity of nitroglycerin and mediates the cardiovascular protective effects of nitrate in the Mediterranean diet. However, important questions of potency, kinetics, mechanism of action, and possible induction of tolerance remain unanswered.

**Methods and Results**—In the present study, we performed biochemical, physiological, and pharmacological studies using nitrite infusion protocols in 20 normal human volunteers and in nonhuman primates to answer these questions, and we specifically tested 3 proposed mechanisms of bioactivation: reduction to nitric oxide by xanthine oxidoreductase, nonenzymatic disproportionation, and reduction by deoxyhemoglobin. We found that (1) nitrite is a relatively potent and fast vasodilator at near-physiological concentrations; (2) nitrite functions as an endocrine reservoir of nitric oxide, producing remote vasodilation during first-pass perfusion of the opposite limb; (3) nitrite is reduced to nitric oxide by intravascular reactions with hemoglobin and with intravascular reductants (ie, ascorbate); (4) inhibition of xanthine oxidoreductase with oxypurinol does not inhibit nitrite-dependent vasodilation but potentiates it; and (5) nitrite does not induce tolerance as observed with the organic nitrates.

**Conclusions**—We propose that nitrite functions as a physiological regulator of vascular function and endocrine nitric oxide homeostasis and suggest that it is an active metabolite of the organic nitrates that can be used therapeutically to bypass enzymatic tolerance. (*Circulation*. 2007;116:1821-1831.)

**Key Words:** endothelium-derived factors ■ free radicals ■ hemoglobin ■ nitroglycerin ■ nitric oxide

Although conventional wisdom held that nitrite was an inert end product of nitric oxide (NO) oxidation with no physiological bioactivity,<sup>1-3</sup> recent data suggest that nitrite is a relatively potent vasodilator *in vivo*.<sup>4</sup> Despite initial controversy, nitrite has now been shown to possess vasodilatory activity in humans, primates, sheep, dogs, rats, and mice.<sup>4-11</sup> Nitrite is generated both from the conversion of dietary nitrate into nitrite by commensal symbiotic oral flora, which possess nitrate reductase enzyme systems lacking in mammals,<sup>12-14</sup> and by ceruloplasmin-dependent oxidation of intravascular NO produced by endothelial NO synthase.<sup>15</sup> Nitrite levels are higher in arterial plasma and erythrocytes, and these levels decrease with blood deoxygenation from artery to vein.<sup>16,17</sup>

### Clinical Perspective p 1831

In addition to vasodilatory effects, nitrite has now been shown to potentially inhibit cytotoxicity and apoptosis after ischemia-reperfusion injury of the heart, liver, and brain at physiological concentrations and at treatment doses comparable to those obtained by consumption of a leafy green salad.<sup>10,11,18-20</sup> This has raised the interesting speculation that nitrate conversion to nitrite could underlie the cardiovascular protective effects of the Mediterranean diet.<sup>21,22</sup> These studies also suggest that nitrite could represent a bioactive metabolite of the organic nitrate class of drugs. If true, and nitrite could bypass enzymatic tolerance, nitrite could provide a more direct therapy for cardiovascular disease.

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Many pressing questions about the kinetics, potency, and mechanisms of bioactivation of nitrite remain to be addressed. In the present study, we address these questions using nitrite-infusion protocols in normal human volunteers and nonhuman primates.

## Methods

### Human Subjects and Sampling Methodology

Twenty healthy volunteers were enrolled in an institutional review board–approved forearm blood flow study. Exclusion criteria included the presence of cardiovascular risk factors, liver disease, renal failure, acute and chronic infections, glucose-6-phosphate dehydrogenase deficiency, and cytochrome B5 deficiency. Volunteers fasted overnight before studies began. All participants provided written informed consent. Human protocols were approved by the Scientific and Institutional Review Boards of the Intramural Division of the National Heart, Lung, and Blood Institute, National Institutes of Health. Sodium nitrite was prepared in the National Institutes of Health Pharmaceutical Development Section and administered under Investigational New Drug No. 70,411.

Studies were performed in a quiet hospital room and began at 7 AM. Venous angi catheters were inserted into the antecubital veins of both arms, and an arterial angi catheter was inserted into the right brachial artery after anesthesia with 1% lidocaine. Saline was infused via the brachial artery at a rate of 120 mL/h for 20 minutes. Baseline measurements were then taken, and blood was drawn for biochemical analyses. Blood pressure was measured via the arterial angi catheter, and heart rate was determined by electrocardiography and pulse oximetry. Forearm blood flow was determined in both arms by venous occlusion plethysmography.<sup>23</sup>

### Study Protocols

The human study protocol was divided into 2 parts. Part A was performed on 5 healthy volunteers. Part B was performed on a study cohort that comprised 15 volunteers.

In part A of the protocol (n=5), the regional response to dose escalations of nitrite was measured. After baseline measurements were obtained, saline was infused for 30 minutes, and physiological measurements were repeated. Arterial and venous blood samples were then drawn before nitrite infusion began. Sodium nitrite, along with saline to maintain a total infusion volume of 120 mL/h, was infused at doses from 0, 7, 14, 28, and 55 to 110  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ . Each dose was infused for 5 minutes, and during the infusion, blood flow was measured at 15-second intervals in both arms, ie, ipsilateral and contralateral to the infusion site. After each dose was infused, blood was drawn from both veins to measure plasma and whole blood nitrite, and the infusion was paused briefly to determine blood pressure and heart rate. Then, the next dose of nitrite was initiated.

After the last dose of nitrite was administered (5 minutes at 110  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  as described above), the metabolism of nitrite in blood was measured. Saline was infused for 180 minutes while venous blood samples were drawn from the arm contralateral to the infusion site after 0, 5, 10, 20, 30, 60, 90, 120, and 180 minutes. Forearm blood flow, blood pressure, and heart rate were measured as described above.

After completion of the saline infusion for 180 minutes, a final nitrite infusion was initiated at a dose of 28  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ; during this infusion, we measured blood flow ipsilateral and contralateral to the infusion site, along with heart rate and blood pressure over 5 minutes. After 5 minutes, we drew venous blood on the contralateral site of the infusion arm.

Part B of the protocol (n=15) was designed to elucidate the mechanisms of nitrite bioactivation in the human peripheral circulation and to evaluate the lowest dose of nitrite that would produce detectable vasodilation in the forearm circulation. We infused nitrite at increasing doses of 0.07, 0.140, 0.350, 0.700, 1.400, 3.500, 7, 14, and 28  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  into the brachial artery of 15 healthy individuals. These subjects were randomized into 3 groups of 5 subjects to receive a coinfusion (with the nitrite infusion) of either

saline (0.9%, n=5), oxypurinol (600  $\mu\text{g}/\text{min}$ , n=5), or ascorbic acid (24 mg/min, n=5).<sup>24</sup> With coinfusion of nitrite, ascorbic acid, or oxypurinol, the infusion rate of saline was tapered such that the total infusion rate of solutions was always maintained at 120 mL/h. The order of coinfusions with nitrite was randomized. Analyses of the forearm blood flow tracings were done by an investigator who was blinded to allocation of coinfusion (saline, ascorbic acid, or oxypurinol) and who was not present during the study day.

### Nonhuman Primate Protocol to Evaluate Tolerance to Nitrite

The animal protocol was reviewed by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke and met the National Institutes of Health guidelines for animal care. To evaluate whether long-term nitrite infusion induces tolerance to nitrite-dependent vasodilation, we continuously infused nitrite 12.5  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  over a period of 24 hours for 14 days in 3 cynomolgus primates. We administered an additional morning nitrite dose of 12 000  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  daily (with 1.5 mg/kg ketamine anesthesia for blood collection) and measured whole blood nitrite and mean arterial blood pressure before and after the boluses. This protocol was performed as part of a treatment study of nitrite in the setting of experimental middle cerebral artery hemorrhage.<sup>5</sup>

### Pharmacokinetic Analysis

Because of the intra-arterial infusion of nitrite in the present study, a 2-compartment model with absorption was fitted to the whole-blood-nitrite-concentration-versus-time curves, and pharmacokinetic parameter values were estimated with the software SAAM (SAAM Institute, Inc, Seattle, Wash). Half-life for plasma nitrate, methemoglobin, and iron nitrosyl hemoglobin was estimated by linear regression of the logarithm concentration-versus-time curves during part C of the study with the software Prism V4.0a (GraphPad Software, Inc, San Diego, Calif).

### Laboratory Assays

Acquisition and processing of blood samples and measurement of iron nitrosyl hemoglobin, plasma nitrite and nitrate, and whole blood nitrite are described in detail elsewhere.<sup>25</sup> These parameters were measured at all time points in part A of the study. Methemoglobin levels were measured by cooximetry at bedside.

### Statistical Analysis

Data processing was performed with Prism software (GraphPad, version 4). Original tracings were analyzed by Origin V7 (Origin-Lab, Northampton, Mass). A 2-way ANOVA coupled with a Bonferroni post hoc test was done to compare effects of ascorbic acid and oxypurinol on nitrite-induced vasodilation. Data are given as mean  $\pm$  SEM, and significance was accepted with  $P < 0.05$ .

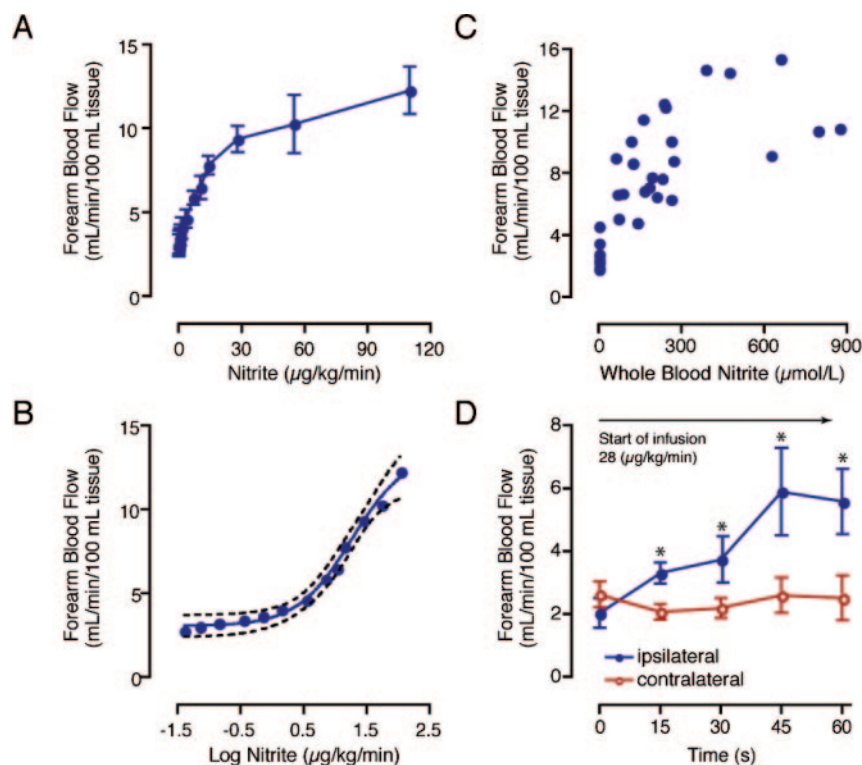
The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

## Results

Twenty healthy normal human volunteers (8 males and 12 females) were recruited with a mean age of  $28 \pm 3$  years, a height of  $172 \pm 7$  cm, and a weight of  $71 \pm 7$  kg. Baseline methemoglobin levels averaged  $0.6 \pm 0.2\%$  of total hemoglobin. Routine blood studies and measurements of glucose-6-phosphate dehydrogenase levels were in the normal range.

### Vasodilation of Regional Vasculature During Nitrite Infusion

To measure the full range of blood flow responses to all doses of nitrite, we evaluated the 5 normal volunteers from part A of the study together with the 5 normal volunteers from part B of the protocol who received nitrite alone (without oxy-



**Figure 1.** Effects of nitrite on regional vascular bed. A, Intra-arterial nitrite infusions (0, 0.07, 0.14, 0.35, 0.7, 1.4, 3.5, 7, 14, 28, 55, and 110  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) caused a dose-dependent increase in forearm blood flow. B, Dose response of forearm blood flow to nitrite infusions plotted in semi-logarithmic plot ( $\text{ED}_{50} = 18.6 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ,  $r = 0.85$ ,  $P < 0.001$ ). C, Relationship between forearm blood flow and measured whole blood nitrite levels in the draining vein of the arm where nitrite was infused intra-arterially. D, Nitrite-induced effects were immediate; forearm blood flow increased within 15 seconds when 28  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  was infused intra-arterially.

purinol or ascorbic acid). This analysis of the stepwise infusions of nitrite from 0 to 110  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  into the ipsilateral forearm brachial artery of 10 volunteers is shown in Figure 1A. Blood flow increased in a dose-dependent manner from  $2.8 \pm 0.2$  to  $12.3 \pm 1.4 \text{ mL} \cdot \text{min}^{-1} \cdot 100 \text{ mL}^{-1}$  tissue ( $P < 0.001$ ;  $n = 10$ ; Figure 1A and 1B). The  $\text{ED}_{50}$  of nitrite-induced vasodilation of the human forearm was 18.6  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  ( $r = 0.85$ ,  $P < 0.001$ ; Figure 1B). Whole-blood nitrite levels were measured at all time points in the 5 volunteers in part A of the study, and these levels measured from the ipsilateral arm correlated significantly with ipsilateral forearm blood flow but exhibited saturation of the vasodilatory effect above  $\approx 300 \mu\text{mol/L}$  ( $r = 0.88$ ,  $P < 0.001$ ;  $n = 5$ ; Figure 1C).

To determine the response time for nitrite-induced vasodilation, blood flow in both the ipsilateral and contralateral forearms was analyzed simultaneously at 15-second intervals during nitrite infusions at 7  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  (the first infusion dose in the 5 subjects from part B treated with nitrite alone) and 28  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  (in the 5 subjects given this dose after the 180-minute saline infusion in part A). In the 5 individuals in whom nitrite was infused with a starting dose of 7  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ , forearm blood flow increased significantly from  $2.4 \pm 0.2$  to  $3.7 \pm 0.6 \text{ mL} \cdot \text{min}^{-1} \cdot 100 \text{ mL}^{-1}$  tissue after 60 seconds ( $P < 0.05$ ). At the higher dose of 28  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ , a significant increase in ipsilateral blood flow was observed after only 15 seconds (from  $2.2 \pm 0.5$  to  $3.3 \pm 0.3 \text{ mL} \cdot \text{min}^{-1} \cdot 100 \text{ mL}^{-1}$  tissue;  $P = 0.03$ ) and after 60 seconds ( $5.6 \pm 1.0 \text{ mL} \cdot \text{min}^{-1} \cdot 100 \text{ mL}^{-1}$  tissue;  $P < 0.01$ ; Figure 1D).

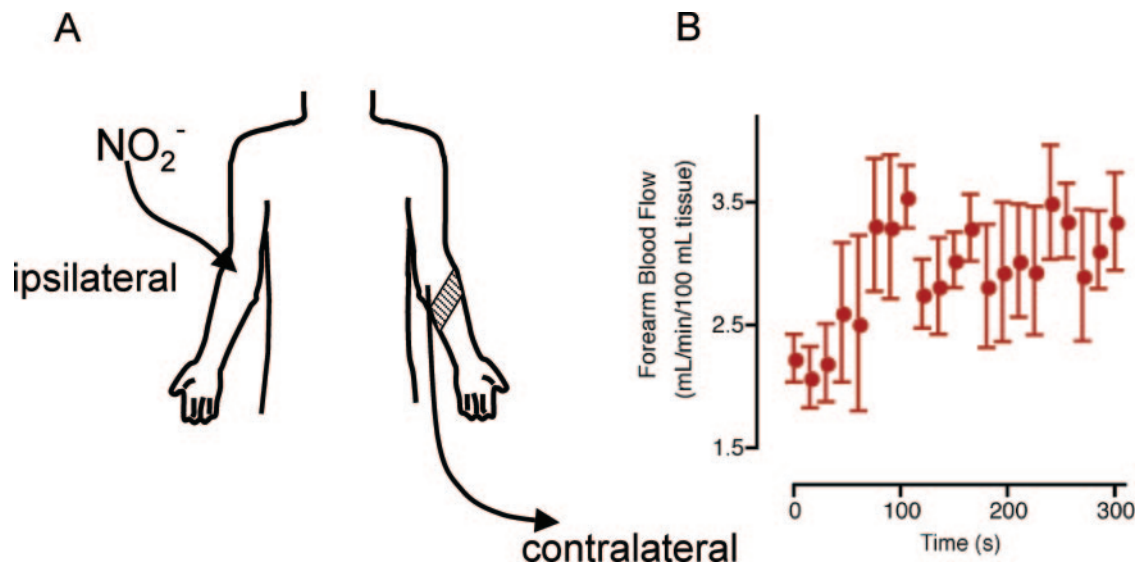
### Endocrine Vasodilator Effects of Nitrite

As mentioned in Methods, in part A of the protocol, which comprised 5 healthy volunteers, after completion of the nitrite

dose-escalation studies followed by a 180-minute infusion of saline, a second 28- $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  nitrite infusion was started for 20 minutes to measure the effects of nitrite on the contralateral forearm after 1 full circulatory cycle (Figure 2A). This protocol minimized the apparent compensatory systemic constriction of the contralateral arm evident in the slower dose-escalation studies done earlier in part A (data not shown). Also note that these data represent the evaluation of a longer time interval of blood flow measurements in the contralateral arm of results shown in Figure 1D. Consistent with endocrine nitrite transport of NO bioactivity in blood, forearm blood flow contralateral to the infusion site increased significantly after the first minute of nitrite infusion, a vasodilation consistent with the full circulatory time in humans ( $\approx 1$  minute). Flow increased quantitatively from  $2.2 \pm 0.2$  to  $3.3 \pm 0.5 \text{ mL} \cdot \text{min}^{-1} \cdot 100 \text{ mL}^{-1}$  tissue ( $P < 0.001$  by 1-way ANOVA; Figure 2B). After 5 minutes of the 28- $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  infusion, whole blood nitrite levels increased in the contralateral arm from  $0.65 \pm 0.15$  to  $4.6 \pm 1.1 \mu\text{mol/L}$  (data not shown). Our pharmacokinetic calculations suggested that at 60 seconds, when blood flow significantly increased (Figure 2B), the contralateral nitrite “first-pass” concentration would be  $\approx 1.0 \mu\text{mol/L}$ . These data suggest that nitrite is transported in human circulation and exerts vasodilatory effects distant to its site of infusion. Interestingly, previous observations of such an effect were ascribed to S-nitrosated or N-nitrosated species.<sup>26</sup>

### Redox Disposition of Nitrite in the Human Circulation

During infusion of nitrite in part A of the study, plasma nitrite increased from  $0.13 \pm 0.049$  to  $26.1 \pm 6.1 \mu\text{mol/L}$ , and systemic intraerythrocytic nitrite concentration increased in con-



**Figure 2.** Nitrite is an endocrine delivery source of NO bioactivity. A, Schematic of protocol. Nitrite was infused at a dose of  $28 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ , and forearm blood flow was measured in the opposite arm. B, In line with the circulatory time, nitrite increased forearm blood flow significantly within 40 to 60 seconds of the start of nitrite infusion ( $P < 0.001$ ). At that time, nitrite concentration in the contralateral arm was  $\approx 1 \mu\text{mol/L}$ .

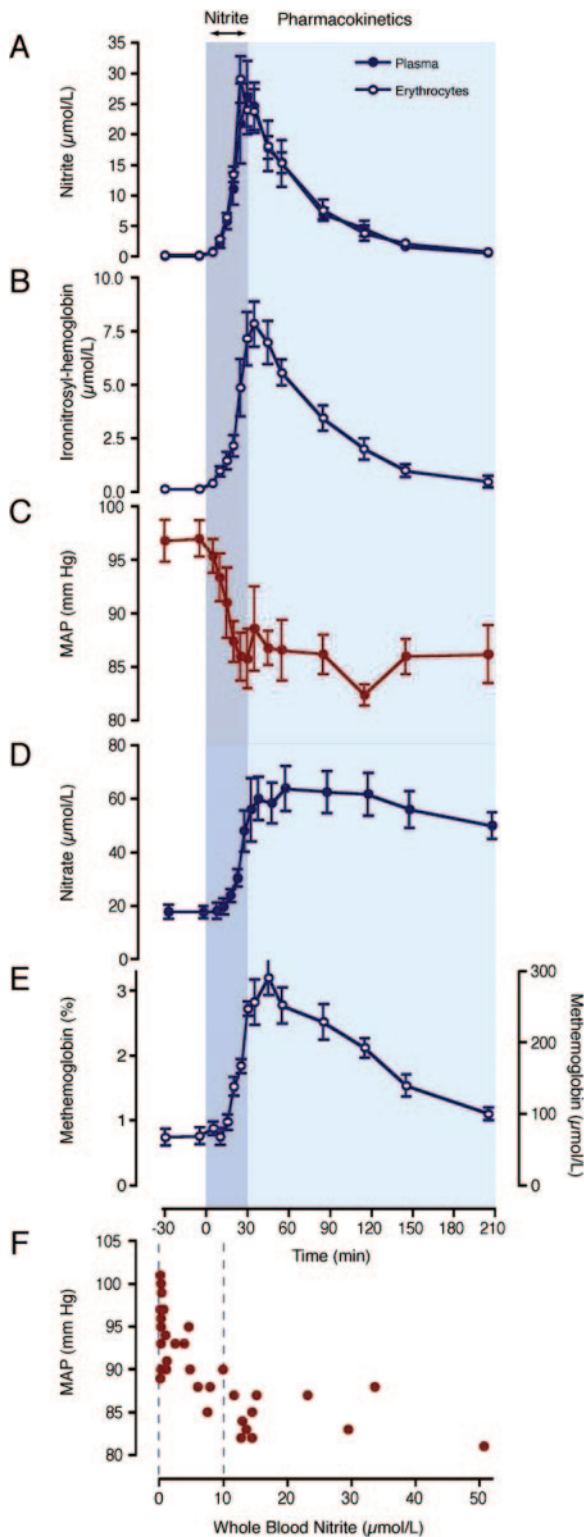
cert with plasma nitrite from  $0.29 \pm 0.06$  to  $34.9 \pm 6.7 \mu\text{mol/L}$  during nitrite infusion (Figure 3A). At baseline, before the nitrite infusion, approximately two thirds of the total nitrite in blood was located within the erythrocytes; however, as nitrite was infused, the ratio between plasma and erythrocyte approached 1:1. Fitting of the compartmental model to the whole blood nitrite concentrations revealed that the volume of distribution in the central compartment was  $5.17 \pm 3.53 \text{ L}$ , and the volume of distribution at steady state was  $25.2 \pm 10.3 \text{ L}$ . Mean clearance was  $0.948 \pm 0.423 \text{ L/min}$ , and intercompartmental clearance was  $0.665 \pm 0.424 \text{ L/min}$ . Terminal half-life was  $42.1 \pm 10.2$  minutes.

Increases in the levels of erythrocytic nitrite were coupled with intraerythrocytic reduction of nitrite to form NO, which binds to deoxyhemoglobin, forming iron nitrosyl hemoglobin (Figure 3B). Systemic iron nitrosyl hemoglobin levels increased from  $0.14 \pm 0.02$  to  $7.9 \pm 1.1 \mu\text{mol/L}$  after the highest dose of nitrite was infused in part A of the study. The apparent half-life of iron nitrosyl hemoglobin under these conditions was  $53 \pm 2$  minutes (Figure 3B). Increases in blood nitrite and NO formation in the red blood cell were associated with decreases in mean arterial blood pressure (from  $97 \pm 1.7$  to  $86 \pm 2.2 \text{ mm Hg}$ ,  $P < 0.001$ ; Figure 3C). Heart rate increased slightly but nonsignificantly from  $68 \pm 3$  to  $76 \pm 4$  bpm at the highest rate of nitrite infusion. A close relationship existed between amount of nitrite infused per minute and mean arterial blood pressure ( $r = -0.47$ ,  $P = 0.0088$ ;  $n = 30$  measurements in 5 subjects; Figure 3D). Interestingly, mean arterial blood pressure remained significantly lower than baseline levels for 120 minutes after nitrite infusions were discontinued and then gradually returned to baseline levels. Ninety minutes after nitrite infusion was terminated, blood pressure reached its lowest level of  $82 \pm 1 \text{ mm Hg}$ , with a change in blood pressure of  $15.0 \pm 1.1 \text{ mm Hg}$ .

Hemoglobin concentration and arterial oxygen saturation remained constant throughout the study protocol, and plasma nitrate ( $\text{NO}_3^-$ ) increased from  $17.8 \pm 2.7$  to  $63.9 \pm 8.3 \mu\text{mol/L}$  30 minutes after the nitrite infusion was stopped and then decayed to  $50.8 \pm 5 \mu\text{mol/L}$  over the next 3 hours, consistent with a half-life of  $362 \pm 21$  minutes (Figure 3D). Whole blood methemoglobin levels increased during nitrite infusion from  $0.7 \pm 0.1\%$  ( $\approx 70 \mu\text{mol/L}$ ) to  $3.2 \pm 0.3\%$  ( $\approx 320 \mu\text{mol/L}$ ) of total hemoglobin. Highest levels were reached 20 minutes after discontinuation of nitrite. Thereafter, methemoglobin levels decayed with an apparent half-life of  $78 \pm 2$  minutes (Figure 3E).

### Alternative Mechanisms of Nitrite Bioactivation

To test whether xanthine oxidase, electron donation by other reducing agents, or nitrite disproportionation contributes to nitrite bioactivation in vivo, we infused nitrite at doses ranging from  $0.07$  to  $28 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  into the brachial arteries of 15 individuals, along with coinfections of oxypurinol, saline, or ascorbic acid in 3 subgroups of 5 individuals (protocol part B; Figure 4A). Nitrite was infused, and forearm blood flow was measured with venous occlusion plethysmography. Forearm blood flow increased dose-dependently from  $3.1 \pm 0.9$  to  $9.2 \pm 1.4$ , from  $2.7 \pm 0.7$  to  $9.8 \pm 0.5$ , and from  $2.5 \pm 0.3$  to  $10.8 \pm 1.2 \text{ mL} \cdot \text{min}^{-1} \cdot 100 \text{ mL}^{-1} \text{ tissue}$  for saline, oxypurinol, and ascorbic acid, respectively (Figure 4B and 4C). A 2-way ANOVA comparing the vasodilatory effects of nitrite in all 3 treatment groups showed that nitrite significantly increased blood flow in all groups ( $P < 0.001$ ; repeated-measures ANOVA) and that coinfections of ascorbic acid and oxypurinol (to a lesser extent) augmented nitrite-dependent vasodilation ( $P = 0.004$  and  $P = 0.01$ , respectively, by repeated-measures ANOVA). Although ascorbic acid and oxypurinol increased nitrite-dependent vasodilation by  $\approx 10\%$  over all time points, no significant difference existed



**Figure 3.** Pharmacokinetics and redox disposition of nitrite infusions in humans. A, Nitrite levels increased both in plasma and erythrocytes to micromolar levels and then decayed with an apparent biological half-life of 42 minutes. At baseline, two thirds of nitrite was located in the erythrocytes, whereas under nitrite infusions, the distribution of nitrite between plasma and erythrocytes was equal. B, Iron nitrosyl hemoglobin levels increased to micromolar levels and decayed with a half-life of 50 minutes. C, Nitrite infusions decreased mean arterial blood pressure (MAP) by 10 mm Hg. Nitrite-induced hypotension lasted for 3 hours. D, Plasma nitrate levels rose from 20  $\mu\text{mol/L}$

in blood flow between the coinfusion regimens at any of the individual nitrite doses. Oxypurinol alone did not change baseline forearm blood flow compared with forearm blood flow during saline infusion (saline  $2.8 \pm 0.3 \text{ mL} \cdot \text{min}^{-1} \cdot 100 \text{ mL}^{-1}$  tissue versus oxypurinol  $2.7 \pm 0.7 \text{ mL} \cdot \text{min}^{-1} \cdot 100 \text{ mL}^{-1}$  tissue).

### Assessment of Nitrite Potency

Because no significant difference existed between the effects of saline, ascorbic acid, and oxypurinol on nitrite-dependent vasodilation at any given nitrite dose, we combined all 15 individuals' data to explore the effect of low nitrite concentration on forearm blood flow. Nitrite vasodilated the human vasculature at the lowest dose infused ( $0.07 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ); at this dose, forearm blood flow increased from  $3.0 \pm 0.3$  to  $3.4 \pm 0.3 \text{ mL} \cdot \text{min}^{-1} \cdot 100 \text{ mL}^{-1}$  tissue ( $P=0.044$ ,  $n=15$ ; Figure 5A and 5B). This lowest dose of nitrite increased ipsilateral antecubital vein nitrite concentration to  $350 \text{ nmol/L}$ , a level well within the physiological concentration range of nitrite in mammalian species.<sup>27,28</sup>

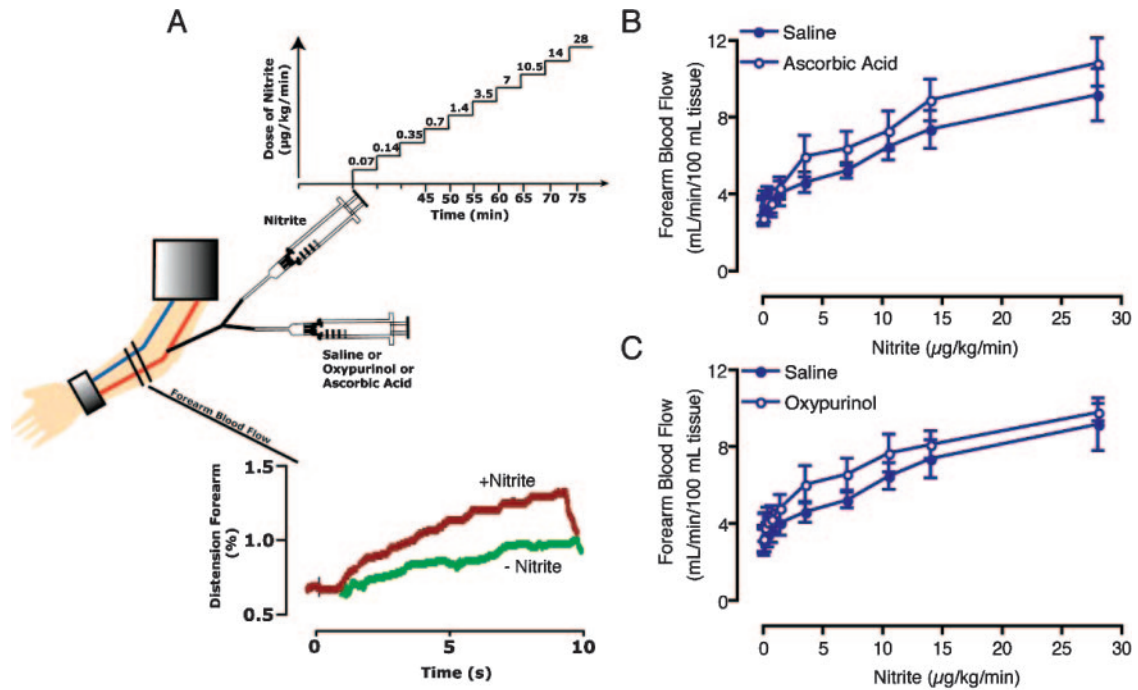
### Assessment of Tolerance Induction to Nitrite-Dependent Vasodilation

To evaluate the potential for induction of tolerance to nitrite, we continuously infused nitrite for 14 days, with daily bolus delivery of additional nitrite in cynomolgus primates (Figure 6A). During the continuous infusion, the animals appeared to compensate for high continuous blood levels of  $11.5 \pm 0.9 \mu\text{mol/L}$  nitrite and maintained their blood pressures (baseline  $95 \pm 3 \text{ mm Hg}$ , on infusion  $98 \pm 2 \text{ mm Hg}$ ,  $P=0.81$ ). However, a daily nitrite bolus of  $12\,000 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  dropped mean arterial blood pressure from  $98 \pm 2$  to  $80 \pm 2 \text{ mm Hg}$  ( $P<0.0001$ ; Figure 6B), even in the setting of ketamine anesthesia, which can induce hypertension. No apparent change occurred in the extent of this nitrite-induced hypotension during a repeated daily nitrite bolus of  $12\,000 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  over the 14 days of nitrite infusion, which revealed a lack of tolerance to nitrite in primates.

### Discussion

We studied the vasodilator activity of nitrite using nitrite-infusion protocols in normal human volunteers and tested tolerance in nonhuman primates to answer questions about the kinetics of vasodilation, the potency of nitrite, the endocrine functionality of nitrite in blood, mechanisms of its activation, and its potential for induction of tolerance. These experiments reveal that nitrite is a rapid vasodilator at physiological concentrations; that nitrite functions as an endocrine reservoir of NO, producing vasodilation in the arm contralateral to the infusion site during first-pass perfusion; and that nitrite is reduced to NO by intravascular reactions

**Figure 3 (Continued).** at baseline to  $60 \mu\text{mol/L}$  and remained constant over the investigation period, consistent with its long half-life of 6 hours. E, Methemoglobin levels increased slightly above the normal range ( $<2.0\%$  [ $200 \mu\text{mol/L}$ ]) during nitrite infusion and decayed with a half-life of 80 minutes. F, Correlation of systemic nitrite concentration in whole blood and mean arterial blood pressure. The majority of nitrite-induced vasodilation occurred at nitrite levels that ranged between 0 and  $10 \mu\text{mol/L}$ , indicated as dotted line.



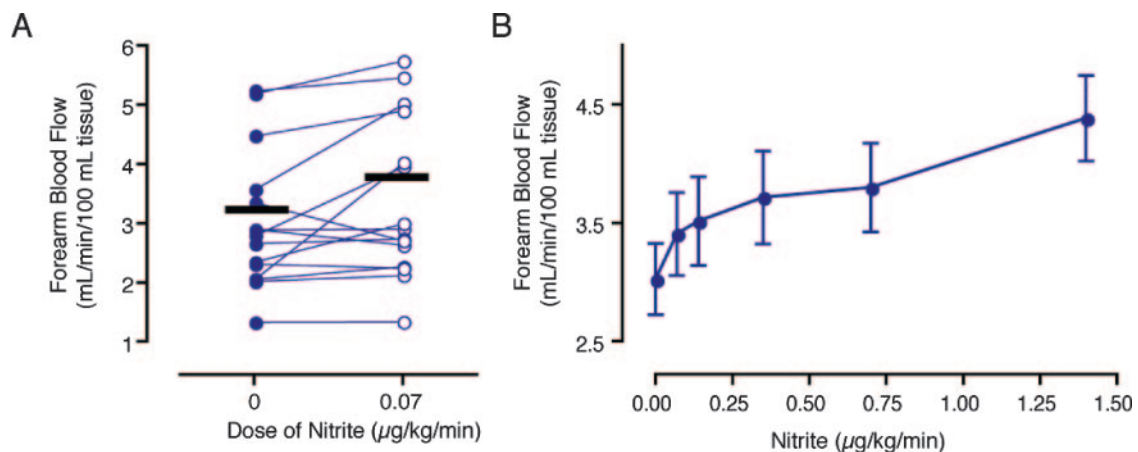
**Figure 4.** Mechanism of nitrite bioactivation. A, Schematic of study protocol. Nitrite was infused intra-arterially at increasing concentrations ranging from 0.07 to 28  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ , and saline, oxypurinol, or ascorbic acid was coin infused. The infusion rate was kept constant for each intervention at 1% of total forearm blood flow. Original data depict the forearm blood flow response to either saline alone or nitrite/saline after 15 seconds of nitrite infusion. B and C, Nitrite dose dependently dilated human vasculature. Coinfusion of ascorbic acid (B) and oxypurinol (C) modestly augmented nitrite-induced vasodilation. This effect was negligible at low doses of infused nitrite ( $<0.7 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ).

with hemoglobin, with apparent potentiation by intravascular reductants (ie, ascorbate). The inhibition of xanthine oxidoreductase with oxypurinol under the experimental conditions used here did not inhibit nitrite-dependent vasodilation but slightly potentiated it, which raises the possibility of a role for xanthine oxidase in superoxide/hydrogen peroxide formation and NO scavenging rather than nitrite reduction. Finally, we found that nitrite does not induce tolerance as observed with the organic nitrate class of drugs. These data support a role for nitrite as a physiological regulator of vascular function and intravascular endocrine NO homeosta-

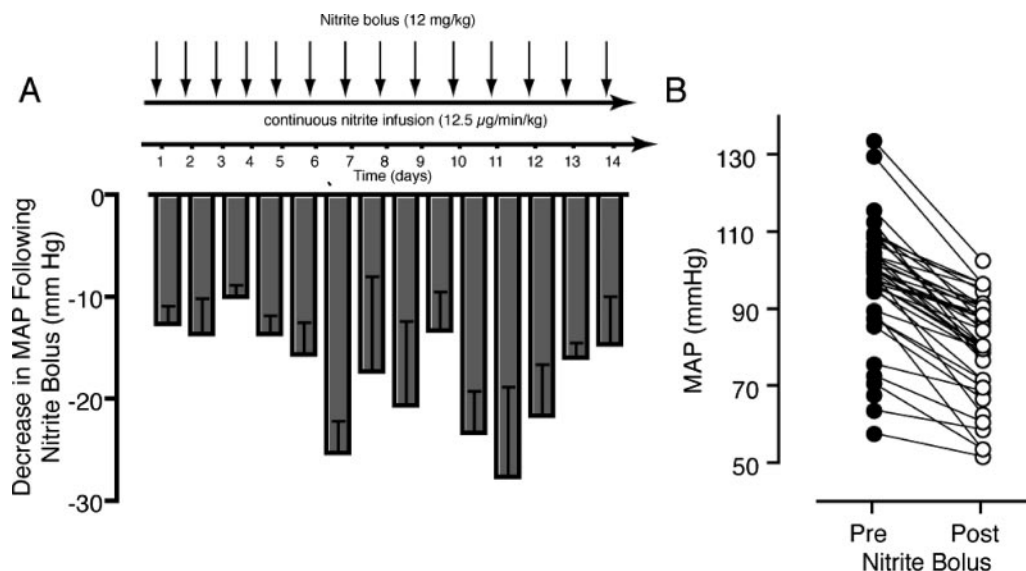
sis and suggest that nitrite may represent an active metabolite of nitroglycerin that may bypass classic enzymatic tolerance.

**Nitrite Is a Regional and Systemic Endocrine Vasodilator**

Considerable recent interest and controversy have focused on the role of intravascular NO-derived molecules that conserve and stabilize NO bioactivity and may contribute to the regulation of blood flow and oxygen delivery. A prominent role for circulating NO species in the regulation of vascular tone is supported by animal and human studies using NO gas



**Figure 5.** Nitrite vasodilates at physiological concentrations. The lowest dose of nitrite infused ( $0.07 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) induced vasodilation of human forearm vasculature. Blood flow response of all 15 individuals was analyzed. The horizontal bars illustrate mean forearm blood flow in both study groups.



**Figure 6.** Nitrite is not subject to tolerance formation in nonhuman primates. A, Nitrite was infused constantly over a period of 14 days at a rate of  $12.5 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  for 24 hours. Every day, nitrite boluses ( $12\,000 \mu\text{g}/\text{kg}$ ) were administered, and before and after the bolus, blood pressure was determined. Nitrite-induced hypotension was similar during the 14 days of continuous nitrite infusion, which indicates the absence of the tolerance formation typical for exogenous NO donors such as inorganic nitrates. B, Infusion of boluses of nitrite did lower mean arterial blood pressure by 18 mm Hg ( $P < 0.0001$ ). MAP indicates mean arterial pressure.

inhalation.<sup>29–33</sup> Human and animal studies have shown that breathing trace quantities of NO can have detectable systemic effects, and therefore, bioactivity is transported in some manner from the lungs to the periphery. These systemic actions include a decrease in systemic vascular resistance,<sup>34</sup> increases in kidney filtration rates,<sup>35</sup> an increase in aortic cGMP levels,<sup>36</sup> restoration of blood flow to the feline intestine<sup>29</sup> and human forearm after infusion of an NO synthase inhibitor,<sup>30</sup> and protection against ischemia reperfusion injury in cats<sup>37</sup> and mice.<sup>31</sup> Furthermore, the infusion of an NO-saturated buffer into the human brachial artery produced an increase in systemic and regional blood flow; this effect was associated with a parallel increase in systemic levels of NO-modified proteins and nitrite.<sup>26,38</sup> These observations are all consistent with intravascular biostabilization, transport, and delivery of NO bioactivity, but the mediator of this phenomenon has remained uncertain.

Authentic NO cannot be responsible for these effects, because it should be scavenged immediately in the lung vasculature and should be incapable of distal transport in blood. This is because NO reacts in a nearly diffusion-limited reaction with both oxyhemoglobin ( $6$  to  $8 \times 10^7 \text{ mol} \cdot \text{L}^{-1} \cdot \text{s}^{-1}$ ) and deoxyhemoglobin ( $2$  to  $6 \times 10^7 \text{ mol} \cdot \text{L}^{-1} \cdot \text{s}^{-1}$ ) to form methemoglobin/nitrate and iron nitrosyl hemoglobin, respectively.<sup>39,40</sup> On the basis of these reaction rates and the high concentration of hemoglobin in blood ( $\approx 10 \text{ mmol/L}$  heme), kinetic calculations suggest that NO could only have a half-life of  $\approx 1 \mu\text{s}$  and diffuse  $< 0.1 \mu\text{m}$  (assuming a diffusion rate of  $3300 \mu\text{m}^2/\text{s}$ ).<sup>41</sup> Diffusion barriers in the unstirred layer around the red blood cell and a cell-free zone along the endothelium in laminar flowing blood reduce the reaction rate of NO and intraerythrocytic hemoglobin by  $\approx 1000$ -fold, which increases the half-life of NO to  $\approx 2 \text{ ms}$ .<sup>41,42</sup> This increases the NO diffusional distances suffi-

ciently enough to allow paracrine signaling from endothelium to smooth muscle but not sufficiently enough to mediate the endocrine or systemic effects of NO inhalation.<sup>30</sup>

In many previous studies, a role for S-nitrosated albumin or hemoglobin, iron nitrosyl complexes, or N-nitrosamines had been considered. However, in studies from our laboratory, normal volunteers were given 80 ppm NO in air to breathe while forearm blood flow was measured with and without endothelial NO synthase inhibition with  $N^G$ -monomethyl-L-arginine. We found that NO inhalation restored forearm blood flow that had been reduced after NO synthase inhibition, a finding consistent with NO transport in blood from the lungs to the tissue. However, no significant changes were observed in the levels of S-nitrosated albumin or hemoglobin; only the nitrite concentration in plasma and iron nitrosyl hemoglobin increased.<sup>30</sup> In the same study, differences in nitrite concentrations across the human peripheral circulation were measured, consistent with the uptake of nitrite in the microvasculature after inhalation of NO.<sup>16</sup> At that time, nitrite was not considered a vasodilator because of the high concentrations required for vasodilation in vitro in aortic ring experiments<sup>43</sup> and the belief within the scientific community that nitrite was an inert metabolite of NO in the human circulation.<sup>1,44,45</sup> To test for the bioactivity of nitrite itself, our group then infused sodium nitrite into the brachial artery in the forearm of healthy volunteers at a rate of  $40 \mu\text{mol}/\text{min}$  ( $39.4 \mu\text{g} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ ). Local vasodilation was observed, and blood pressure decreased 3 mm Hg after 5 minutes of infusion and 7 mm Hg after 20 minutes of nitrite infusion.<sup>4</sup> Although it remains unclear why previous studies by Lauer and colleagues<sup>1</sup> did not observe vasodilation in humans during nitrite infusions, numerous recent studies have now confirmed potent vasodilating effects of nitrite in mice, rats, sheep, dogs, primates, and humans.<sup>5–8,10,46–48</sup>

In the present study, forearm blood flow increased within 15 seconds after nitrite infusion began. This rapid response, which occurred before systemic compensations in pressure were observed, is consistent with the local diffusion of NO into the resistance vessel walls from blood. In accordance with the time required for circulatory transport, blood flow increased in the contralateral arm after 40 to 60 seconds and was associated with an increase in circulating nitrite concentrations to 1  $\mu\text{mol/L}$ . Interestingly, these observations suggest that previous contralateral forearm vasodilation observed during infusions of NO solutions could have been nitrite-dependent.<sup>26,38</sup> Additionally, we have now observed vasodilation in the human circulation at nitrite concentrations as low as 350 nmol/L, a level well within the physiological concentration range of nitrite in mammalian species.<sup>27,28</sup> These data in aggregate provide further evidence that nitrite is a systemic vasodilator at pharmacological and physiological concentrations and support the concept that nitrite is a major mediator of the endocrine effects of NO inhalation and NO solution infusions.

Recent studies have compared the potency of nitrite to vasodilate with NO. Compared with NO, nitrite is 1 to 3 log orders less potent depending on whether hypoxic<sup>49</sup> or normoxic<sup>50</sup> conditions are used; however, nitrite is present in blood at 2 orders-of-magnitude-higher concentration.<sup>17</sup>

### Mechanisms of Nitrite-Mediated Vasodilation

On the basis of earlier work showing that nitrite reacts with deoxyhemoglobin to form NO and methemoglobin, we and others have proposed a model in which deoxyhemoglobin functions as an allosterically regulated nitrite reductase that generates NO gas from nitrite under conditions of physiological hypoxia.<sup>4,6,51–53</sup> Although this mechanism is supported in the present study by the observed correlation between vasodilation and formation of NO within the red blood cell, other mechanisms must be considered. It is evident that tissues can directly bioactivate nitrite under hypoxia.<sup>54,55</sup> Additional mechanisms for nitrite reduction to NO that might contribute to an NO-dependent hypoxic vasodilator effect include acidic reduction (disproportionation)<sup>12,20,50,56–58</sup> and reduction by xanthine oxidoreductase.<sup>59</sup> The evaluation of effects of ascorbate and oxypurinol on nitrite vasodilation appears to rule out a major role for these pathways in nitrite-induced vasodilation of the human forearm.

The mechanism of the modest enhancement of vasodilation by ascorbate likely involves an intravascular decrease in redox potential. For example, at supraphysiological doses, ascorbate reduces the concentration of oxidants such as superoxide and hydrogen peroxide<sup>60</sup> and reduces ferric hemoglobin to ferrous hemoglobin, which increases the availability of intravascular nitrite reductants.<sup>61</sup> In addition, ascorbate reacts with protonated nitrite to form NO via direct electron donation and reduction.<sup>62</sup> Interestingly, an ascorbic acid to dehydroascorbic acid shuttle exists in the red blood cell that could potentially modulate red cell redox potential.<sup>63</sup> The enhancement rather than inhibition of nitrite-dependent vasodilation by oxypurinol appears to preclude a mechanism of xanthine oxidoreductase-dependent nitrite reduction dur-

ing physiological conditions in the human circulation; however, a role for this enzyme in nitrite reduction during ischemia cannot be dismissed. The fact that oxypurinol slightly increases blood flow during nitrite infusion is compatible with the thesis that NO intermediates formed by nitrite reduction are partially scavenged by reaction with superoxide (or hydrogen peroxide via peroxidase chemistry) produced by xanthine oxidase. Enzymatic inhibition thus would indirectly increase NO bioavailability after nitrite reduction.

### Differences Between Nitrite and the Organic Nitrates (Nitroglycerin)

The pharmacological actions of nitrite differ from those of organic nitrates despite their involvement in similar mechanisms of NO-dependent vascular relaxation. Conversion of nitrite ( $\text{NO}_2^-$ ) to NO occurs specifically under acidic and hypoxic conditions present during physiological hypoxemia and tissue ischemia and is facilitated by deoxyhemoglobin and deoxymyoglobin.<sup>4,52,64,65</sup> On the other hand, the organic nitrates (nitroglycerin) must be metabolized enzymatically to form both nitrite and NO via the actions of the mitochondrial isoform of aldehyde dehydrogenase, glutathione-S-transferase, the cytochrome p450 superfamily, or xanthine oxidoreductase.<sup>66,67</sup> The organic nitrates, nitroglycerin in particular, are susceptible to tolerance; nitroglycerin tolerance is well known to limit its therapeutic efficacy. In 1908 and 1930, 2 studies compared the effects of nitroglycerin and nitrite on development of tolerance.<sup>68,69</sup> They demonstrated in dogs, rabbits, and humans that oral doses of nitrite ranging from 150 to 300 mg lowered systolic blood pressure up to 24 mm Hg and increased heart rate by 20 bpm. Nitrite was given repeatedly over 6 days, 2 to 3 times per day, and the same lowering of blood pressure was observed.<sup>68,69</sup> In a third study, however, repeated doses of nitrite in rabbits did cause tolerance formation and decreased the responsiveness of the vasculature to nitrite.<sup>70</sup> The results of the present study suggest that one of the major metabolites of the organic nitrates, namely, nitrite, does not induce enzymatic tolerance and may contribute substantially to the bioactivity and therapeutic action of this class of medicines. These data suggest that direct therapy with nitrite may thus bypass enzymatic nitroglycerin metabolism and tolerance.

### Nitrite Safety and Therapeutics

High doses of ingested nitrite have been associated with toxic methemoglobinemia.<sup>71</sup> Nitrite has been used for decades as an antidote to cyanide poisoning by inducing methemoglobinemia.<sup>72</sup> The present results demonstrate that vasodilating concentrations of nitrite do not induce clinically significant methemoglobinemia. The methemoglobin levels did not exceed a value of 3.2%, even at the highest doses of nitrite administered in the present human studies. Thus, the major safety assessment in clinical trials will be blood pressure rather than methemoglobinemia.

Although concerns about nitrite-dependent N-nitrosamine formation have been raised in the context of gastric cancer for the last 40 years, modern epidemiological studies have not confirmed this association.<sup>73</sup> Indeed, a revisionist evaluation



of this biology suggests that ingested nitrate is concentrated in saliva to form nitrite, which after ingestion is converted to NO in the acidic stomach. This NO increases mucous production and regional blood flow and protects against ulcer formation.<sup>12</sup>

Nitrite has been shown to exert cytoprotective and antiapoptotic effects after tissue ischemia and reperfusion.<sup>18,31,74</sup> In a primate model of cerebral vasospasm after subarachnoid aneurysmal hemorrhage, nitrite has been observed to prevent delayed cerebral vasospasm,<sup>5</sup> a complication that causes permanent neurological deficits or death in at least 15% of patients after otherwise successful surgical treatment of ruptured intracranial aneurysms. Inhaled nitrite has been shown to be a selective, long-lasting, hypoxia-sensitive pulmonary vasodilator and is currently being evaluated in pulmonary hypertension of the newborn.<sup>6</sup> The present evaluation of the pharmacokinetics of nitrite in humans now allows us to safely target blood nitrite levels that were therapeutically effective in the animal models.

## Conclusions

These studies reveal that nitrite is a potent and rapid regional and systemic vasodilator with evident bioactivity observed at physiological concentrations, an effect slightly potentiated by systemic reductants and inhibition of xanthine oxidase. This vasodilation is temporally associated with intraerythrocytic reduction of nitrite to NO by deoxyhemoglobin. The vasodilatory effects of nitrite do not appear to be susceptible to tolerance, which sets this anion apart from the organic nitrates. These data in aggregate continue to support the hypothesis that nitrite is a circulating endocrine NO-generating molecule that regulates basal vascular function. Nitrite therapy offers the potential to bypass nitrate tolerance and modulate vascular tone and cellular function during pathological tissue ischemia and infarction.

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## Disclosures

Drs Gladwin, Cannon, Schechter, Pluta, Oldfield, and Hunter are all listed as coinventors on a National Institutes of Health government patent application on the use of nitrite salts for cardiovascular conditions. The remaining authors report no conflicts.

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### CLINICAL PERSPECTIVE

Nitrite is present in the diet and is naturally formed in human blood and tissues. In the present studies, nitrite was infused into the human circulation and found to be a fast endocrine vasodilator that was converted to nitric oxide in the bloodstream via reactions with deoxygenated hemoglobin. Other enzyme pathways for activation, such as xanthine oxidoreductase, were evaluated and found not to contribute to this effect. Nitrite differed from the organic nitrates (nitroglycerin) in that it did not induce tolerance. Nitrite may represent a circulating molecule that regulates blood flow, hypoxic vasodilation, and blood pressure in humans.