Kidney

NADPH Oxidase in the Renal Microvasculature Is a Primary Target for Blood Pressure–Lowering Effects by Inorganic Nitrate and Nitrite

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Abstract—Renal oxidative stress and nitric oxide (NO) deficiency are key events in hypertension. Stimulation of a nitrate–nitrite–NO pathway with dietary nitrate reduces blood pressure, but the mechanisms or target organ are not clear. We investigated the hypothesis that inorganic nitrate and nitrite attenuate reactivity of renal microcirculation and blood pressure responses to angiotensin II (ANG II) by modulating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and NO bioavailability. Nitrite in the physiological range (10^-7–10^-5 mol/L) dilated isolated perfused renal afferent arterioles, which were associated with increased NO. Contractions to ANG II (34%) and simultaneous NO synthase inhibition (56%) were attenuated by nitrite (18% and 26%). In a model of oxidative stress (superoxide dismutase-1 knockouts), abnormal ANG II–mediated arteriolar contractions (90%) were normalized by nitrite (44%). Mechanistically, effects of nitrite were abolished by NO scavenger and xanthine oxidase inhibitor, but only partially attenuated by inhibiting soluble guanylyl cyclase. Inhibition of NADPH oxidase with apocynin attenuated ANG II–induced contractility (35%) similar to that of nitrite. In the presence of nitrite, no further effect of apocynin was observed, suggesting NADPH oxidase as a possible target. In preglomerular vascular smooth muscle cells and kidney cortex, nitrite reduced both basal and ANG II–induced NADPH oxidase activity. These effects of nitrite were also abolished by xanthine oxidase inhibition. Moreover, supplementation with dietary nitrate (10^-2 mol/L) reduced renal NADPH oxidase activity and attenuated ANG II–mediated arteriolar contractile responses and hypertension (99±2–146±2 mmHg) compared with placebo (100±3–168±3 mmHg). In conclusion, these novel findings position NADPH oxidase in the renal microvasculature as a prime target for blood pressure–lowering effects of inorganic nitrate and nitrite. (Hypertension. 2015;65:161-170. DOI: 10.1161/HYPERTENSIONAHA.114.04222.) • Online Data Supplement

Key Words: angiotensin II • hypertension, renovascular • kidney • microcirculation • nitrates • nitric oxide • oxidative stress

Hypertension is a major risk factor for stroke, coronary heart disease, and premature death. The prevalence is continuously increasing, and it is estimated that elevated blood pressure affects >1 of every 3 adults or =1 billion people worldwide.1 The economic burden to the society is enormous, and intensified efforts to prevent and control hypertension are crucial. Nitric oxide (NO) is a key regulator of renal and cardiovascular function, and emerging evidence shows that renal oxidative stress and subsequent NO deficiency are critically associated with development of hypertension and cardiovascular disease.2,3 Therefore, treatment modalities that reduce oxidative stress and increase NO production in the kidney may have important implications for renal and cardiovascular homeostasis.

The kidneys control long-term blood pressure level by adjusting renal peripheral vascular resistance and hence body fluid volume. The afferent arterioles are the major resistance vessels in the kidney, and increased arteriolar reactivity may reduce glomerular perfusion and filtration, increase extracellular volume, and thus contribute to development of hypertension.4 There is a clear relationship between afferent arteriolar resistance and blood pressure, and a reduction in arteriolar diameter may even predict later development of hypertension.5 Preglomerular resistance is determined by the balance between constrictor agents such as angiotensin II (ANG II) and vasodilator pathways, notably NO.6,5 Elevated level of ANG II is a strong stimulus for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase–induced superoxide formation in the vasculature, and such oxidative stress significantly contributes to enhanced arteriolar reactivity and susceptibility to hypertension.10–13

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Research during the past decade has revealed that the endogenous anions nitrate and nitrite are used to generate NO and that stimulation of this nitrate–nitrite–NO pathway can compensate for disturbances in NO generation from NO synthases (NOS). This has interesting nutritional implications because nitrate is also a normal constituent of our daily diet, with vegetables being the main source. Oral commensal bacteria seem obligatory for the reduction of nitrate to nitrite, whereas several mammalian enzymes are capable of subsequent reduction of nitrite to NO and other bioactive nitrogen oxides.

Recent clinical and experimental studies have independently shown that supplementation with inorganic nitrate, by dietary means or with nitrate salt, reduces blood pressure in healthy normotensive individuals as well as in hypertensives. In addition, these anions can partly compensate for metabolic disturbances in aged endothelial NOS knockout mice, reduce hypertension and markers of oxidative stress, and ameliorate organ injuries in models of renal and cardiovascular disease. The mechanisms for nitrate- and nitrite-mediated antihypertensive effects along with renal and cardiac protection are not yet clear. In the present study, we tested the hypothesis that stimulation of a nitrate–nitrite–NO pathway, with inorganic nitrate or nitrite, limits afferent arteriolar responsiveness and hypertension in response to ANG II by modulating the balance between superoxide and NO.

**Methods**

**Animals**

The studies were conducted on male C57BL/6J mice (The Jackson Laboratory, Maine). To specifically investigate the role of bacteria in bioactivation of nitrate, germ-free (n=8) and conventional (n=8) mice were used. To investigate the effects of nitrite in a model with oxidative stress, we used superoxide dismutase 1 (SOD1) knockout (SOD1−/−; n=15) and wild-type (SOD1+/+; n=19). To investigate the effects of dietary nitrate in a model of renal hypertension, we used Sprague-Dawley rats (Charles River Laboratories, Germany) for blood pressure measurements (n=12) (online-only Data Supplement).

**Vascular Studies**

**Isolated and Perfused Afferent Arterioles**

Dissection and perfusion of renal cortical afferent arterioles and contractility studies were performed as described previously (Figure S1 in the online-only Data Supplement). The experiments were digitally recorded and then digitized off-line and analyzed as described before. Changes in luminal diameters were measured to estimate the effect of vasoactive substances.

**Fluorescent Detection of NO Production in Afferent Arterioles**

A highly sensitive, photostable cell-permeable fluorescent probe, 4-amino-5-methylamino-2′,7′-difluorescein diacetate (Invitrogen, Life Technologies Europe BV, Stockholm, Sweden), was used for the detection of NO production with nitrite (10⁻⁵ mol/L) in the isolated and perfused afferent arteriole (Figure S1).

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Nitrite-mediated dilatation of renal microvessels in C57 mice is associated with increased nitric oxide bioavailability. A. Effects of acute administration of nitrite on diameters of afferent arterioles. Nitrite dilated arterioles (n=6) in a concentration-dependent manner, whereas time-controlled placebo treatment (control, n=6) had no significant effects. Each dose was applied for 2 minutes. *P<0.05 compared with control group (2-way ANOVA, Bonferroni multiple comparisons test). B. Effect of nitrite (10⁻⁵ mol/L; n=4) or placebo (n=4) on nitric oxide (NO) production in afferent arterioles, determined as changes in fluorescence during a 20-minute period. Administration of nitrite was associated with increased NO production compared with baseline (paired Student t test). C. Effects of acute administration of nitrite on diameters of afferent arterioles during inhibition of NO synthase with Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME; 10⁻⁴ mol/L). Administration of nitrite (10⁻⁵ mol/L; n=8) was associated with increased arteriolar diameter, whereas L-NAME alone (n=6) reduced diameter. L-NAME-mediated contraction was abolished by simultaneous administration of nitrite (n=6). *P<0.05 compared with baseline; #P<0.05 compared with L-NAME (2-way ANOVA, Bonferroni multiple comparisons test). Values are presented as mean±SEM.
Myograph Studies
Carotid arteries, renal interlobar arteries, and third branch of mesenteric arteries from C57 mice were isolated, and arterial rings were mounted in myograph chambers (online-only Data Supplement). Resting tension was set according to normalization procedure, and vessel viability was assessed by the responses to 0.1 mol/L KCl. After washout, cumulative concentration response for ANG II (10⁻¹²–10⁻⁶ mol/L) was obtained, with or without simultaneous incubation with nitrite (10⁻⁵ mol/L).

Renal NADPH Oxidase Activity

Preglomerular Vascular Smooth Muscle Cells
Isolation and culturing of primary preglomerular vascular smooth muscle cells (PG-VSMCs) were performed as previously described, and the phenotype was confirmed as described by Dubey et al. After 30-minute incubation with different nitrite concentrations (NaNO₂) or placebo (NaCl), the cells were transferred into reaction tubes.

Kidney Cortex
The tissue was homogenized with bullet blender in ice-cold PBS, and the homogenate was centrifuged at 4°C for 20 minutes at 2000g. Fresh tissue homogenates were incubated for 30 minutes with placebo (NaCl), ANG II (10⁻⁶ mol/L), nitrite (10⁻⁷ mol/L), or combinations with ANG II and nitrite before the NADPH oxidase activity measurements.

Chemiluminescence technique was used to determine the NADPH oxidase–mediated superoxide formation. In experiments with both PG-VSMCs and renal cortex, 100 µL of NADPH (10⁻⁴ mol/L) and lucigenin (5×10⁻⁵ mol/L; Sigma-Aldrich) were injected into the reaction tube (final volume of 1 mL), and NADPH oxidase activity was determined by measuring lucigenin chemiluminescence every 3 seconds for 3 minutes with an AutoLumat LB953 Multi-Tube Luminometer (Berthold Technologies, Bad Wildbad, Germany). Results were corrected by cell number (VSMC) or by protein quantification (cortex) using Bradford protein assay (Bio-Rad Laboratories, United Kingdom).

Renal NADPH Oxidase and Xanthine Oxidase Expression
Renal cortex and PG-VSMCs were used for quantitative polymerase chain reaction analysis of NADPH oxidases and xanthine oxidase (XO) expression (online-only Data Supplement).

Plasma Analysis

Cyclic Guanosine Monophosphate
The inhibitor of cAMP/cGMP phosphodiesterases (IBMX [3-isobutyl-1-methylxanthine]) was added to plasma samples (final concentration, 10⁻⁵ mol/L) before freezing and was later analyzed for cGMP content with ELISA method (Biotrak EIA System; Amersham).

Nitrite and Nitrate
Plasma samples were extracted with methanol and analyzed using a highly sensitive high-performance liquid chromatography technique as previously described.

Blood Pressure Response to Prolonged ANG II Infusion
Telemetric devices (PA-C40, DSI, St Paul, MN) were implanted in adult male Sprague-Dawley rats (n=12; body weight=335±4), and was determined by measuring lucigenin chemiluminescence every 3 seconds for 3 minutes with an AutoLumat LB953 Multi-Tube Luminometer (Berthold Technologies, Bad Wildbad, Germany). Results were corrected by cell number (VSMC) or by protein quantification (cortex) using Bradford protein assay (Bio-Rad Laboratories, United Kingdom).

Figure 2. Nitrite attenuates renal microvascular responses to angiotensin II (ANG II) via xanthine oxidase (XO)–dependent nitric oxide (NO) formation. A, ANG II concentration response curves (control) in isolated and perfused afferent arterioles of C57 mice (n=6). Simultaneous administration of nitrite (10⁻⁵ mol/L) attenuated ANG II–mediated contraction (n=8). B, Effect of nitrite on ANG II–induced contractions (control) during inhibition of NO synthase (NOS) with l-NAME alone (10⁻⁴ mol/L; n=8) or in combination with nitrite (10⁻⁵ mol/L; n=8) for 15 minutes, followed by cumulative doses of ANG II (2 minutes each dose). During NOS inhibition, both sensitivity and contractility to ANG II were strongly enhanced. At the same time, the attenuating effect of nitrite on ANG II–mediated contraction was more pronounced. C, The attenuated contractile response to ANG II+l-NAME in the presence of nitrite was blocked by simultaneous administration of the XO inhibitor oxyquinol (0.5×10⁻³ mol/L; n=7) or by scavenging of NO with 2-(4-carboxyphosphoryl)-4,5-dihydro-4,4,5,4-tetramethyl-1H-imidazolyl-1-oxy-3-oxide (cPTIO; n=7). However, the effect of nitrite was only partially blocked by inhibition of soluble guanylyl cyclase (sGC) with 1H-[1,2,4]oxadiazolo[4,3-a]quinolin-1-one (ODQ; n=6). Oxyquinol, cPTIO, and ODQ were administered 15 minutes before the ANG II dose response. D, Maximal arteriolar contraction for each group. *P<0.05 compared with ANG II (control; A) or ANG II+l-NAME (control; B); †P<0.05 compared with nitrite in combination with ODQ, cPTIO, and oxyquinol; ‡P<0.05 compared with nitrite, nitrite+cPTIO, and nitrite+oxyquinol (C; 2-way ANOVA, Bonferroni multiple comparisons test). Values are presented as mean±SEM.
measurements were conducted as previously described. After surgery, the animals were allowed to recover for 10 days before measurements were started. Blood pressure and heart rate were measured continuously during (1) baseline conditions (72 hours), followed by giving (2) Nω-nitro-l-arginine methyl ester hydrochloride (l-NAME) in drinking water (500 mg/L) supplemented with placebo (NaCl) or sodium nitrate (NaNO₂; 10⁻³ mol/L) for 72 hours. The rats were then anesthetized by spontaneous inhalation of isoflurane (Forene, Abbott Scandinavia AB, Sweden) in air (+2.2%), and an osmotic minipump (Alzet, Durect, CA) was implanted subcutaneously, delivering ANG II (Sigma-Aldrich) at 120 ng/kg per 24 hours for 20 days. Two days after implantation, telemetric measurements were performed continuously for 15 days with ANG II infusion. During the past 4 days, measurements were conducted without placebo or nitrate supplementation.

Drugs and Reagents
Drugs and chemicals used for this study were obtained from Sigma, unless otherwise stated. In the arteriolar contraction experiments, all compounds were applied to the bath solution.

Statistical Analysis
Values were presented as mean±SEM. ANOVA, followed by post hoc comparison, was used to test time- or concentration-dependent changes in the arteriolar diameter and to allow for >1 comparison with the same variable among groups. Single comparisons among normally distributed parameters were tested for significance with Student paired or unpaired t test as appropriate. Experiments were evaluated in a blinded fashion. Statistical significance was defined as P<0.05.

Ethics
The experiments were approved by the Uppsala or Stockholm Ethical Committee for Animal Experiments and were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Results
Effect of Nitrite on Renal Afferent Arteriolar Diameter and NO Production
Nitrite dilated arterioles in a concentration-dependent manner, whereas no change in diameter was observed for the time control (Figure 1A). Significant vasodilatation was observed already at 10⁻⁷ mol/L nitrite, which is well within the physiological range. The nitrite-evoked dilatation (10⁻⁵ mol/L) was paralleled by increased NO formation as measured with 4-amino-5-methylamino-2′,7′-difluorescein fluorescence (Figure 1B). Inhibition of NOS with l-NAME reduced arteriolar diameter, and simultaneous administration of nitrite completely prevented this l-NAME-mediated contraction (Figure 1C).

Nitrite Attenuates ANG II–Induced Constrictions in the Renal Microvasculature
ANG II is a strong modulator of preglomerular resistance. Afferent arteriolar contraction to ANG II involves NADPH oxidase–derived superoxide, but the response to this is dampened by a concomitant increase in NO formation by NOS. ANG II (10⁻¹²–10⁻⁸ mol/L; 2 minutes each dose) constricted arterioles in a concentration-dependent manner (threshold response, 10⁻⁹ mol/L), with a maximum response of 34% (Figure 2A). Simultaneous treatment with nitrite (10⁻⁵ mol/L) attenuated both sensitivity (threshold response, 10⁻⁴ mol/L) and contractility to ANG II (maximum response, 18%).

To investigate whether the renal micrcirculation is especially sensitive to nitrite, we performed similar experimental protocols in conductance vessels, larger renal vessels, and small extrarenal resistance arteries. The demonstrated attenuating effect of nitrite (10⁻⁵ mol/L) on ANG II–induced constrictions in pregglomerular arterioles was not observed in carotid, renal interlobar, or mesenteric arteries (Figure S2). These results suggest the renal microcirculation as a primary target of nitrite.

To investigate the role of NOS-derived NO in offsetting ANG II–induced arteriolar contraction, vessels were treated with l-NAME (10⁻⁴ mol/L) for 15 minutes before and during treatment with ANG II. As expected, l-NAME enhanced both sensitivity (threshold response, 10⁻¹² mol/L) and contractility to ANG II (maximum response, 56%; Figure 2B). In arterioles exposed to nitrite, these effects were markedly attenuated (threshold response, 10⁻¹¹ mol/L and maximum response, 26%).

Nitrite Effects Are Mediated by XO-Catalyzed NO Formation
Next, we aimed to investigate the mechanism(s) for nitrite-mediated inhibition on ANG II contractility. To further
investigate whether the nitrite-mediated attenuation of arteriolar contractility was dependent on generation of NO, a NO scavenger (2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide [cPTIO]) was administered together with l-NAME and nitrite. In the presence of cPTIO, the effect of nitrite was abolished (Figure 2C), and the contractile response was indistinguishable from that of ANG II+NAME (Figure 2D). A variety of proteins and enzymes including hemoglobin, myoglobin, mitochondrial complexes, aldehyde oxidase, and XO can catalyze the reduction of nitrite to NO in blood and tissues.17,32 Although oxypurinol alone did not significantly alter the contractile response to ANG+NAME (data not shown), it prevented the effects of nitrite on arteriolar responses (Figure 2C and 2D). Interestingly, the effect of nitrite was only partially attenuated during simultaneous inhibition of soluble guanylyl cyclase with ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one). Taken together, these findings demonstrate that nitrite can attenuate ANG II–induced arteriolar contraction via XO-dependent NO generation. Moreover, the subsequent events are partially independent of the NO–soluble guanylyl cyclase–cGMP pathway.

Nitrite Modulates NADPH Oxidase Activity

Recent reports have suggested that nitrate and nitrite may reduce oxidative stress in models of renal and cardiovascular disease.24–26 Here, inhibition of NADPH oxidases with apocynin markedly attenuated the contractile response to ANG II during NOS inhibition (Figure 3A). This supports the notion that ANG II–induced contraction is linked to increased NADPH oxidase activity. Simultaneous administration of nitrite and apocynin had no further attenuating effect compared with that of nitrite alone (Figure 3B and 3C), suggesting that nitrite reduces ANG II–induced vasoconstriction by modulating NADPH oxidase function. Next, we investigated whether nitrite modulates NADPH oxidase activity in renal cortex and primary PG-VSMCs. Nitrite attenuated basal NADPH oxidase–derived superoxide production in cortical tissue compared with control (Figure 3A). Incubation with ANG II significantly increased NADPH oxidase activity, and in the presence of nitrite, this effect was abolished (Figure 4A). In PG-VSMCs, incubation with nitrite reduced basal NADPH oxidase activity in a dose-dependent fashion (Figure 4B). Similar to renal cortex, ANG II increased NADPH oxidase–derived superoxide production in PG-VSMC that was abolished by nitrite (Figure 4C). As

![Figure 4](https://hyper.ahajournals.org/)

**Figure 4.** Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity is attenuated with nitrite. **A**, In the renal cortex from C57 mice, incubation with angiotensin II (ANG II; 10−6 mol/L; n=5) for 30 minutes increased NADPH oxidase activity compared with control group. Incubation with nitrite (10−5 mol/L) for 30 minutes attenuated NADPH oxidase–derived superoxide production compared with control and abolished the effect of ANG II. n=6 (kidneys) for each group. **B**, In pregglomerular vascular smooth muscle cells (PG-VSMCs), incubation with nitrite (30 minutes) attenuated NADPH oxidase–derived superoxide production compared with control. The attenuating effect with nitrite was dose dependent. Control: n=14; nitrite: n=11 (10−3), n=13 (10−4), n=14 (10−5), n=10 (10−6 mol/L). *P<0.05 compared with control. **C**, Similar to that in the renal cortex, ANG II increased NADPH oxidase activity in PG-VSMCs, which was abolished by nitrite. **D**, Inhibition of xanthine oxidase (XO) with febuxostat (3×10−8 mol/L) abolished nitrite-mediated effects on NADPH oxidase activity under both basal and ANG II–stimulated conditions. Control, n=10; ANG II, n=10; nitrite, n=10; febuxostat, n=10; ANG II+febuxostat, n=10; nitrite+febuxostat, n=10; nitrite+ANG II+febuxostat, n=10 (1-way ANOVA, Bonferroni multiple comparisons test). Values are presented as mean±SEM.
Nitrate Bioactivation Is Dependent on Bacteria

Oral commensal bacteria have been suggested to be important in the bioactivation of nitrate by first reducing it to the more reactive nitrite anion.\textsuperscript{18,33,34} To investigate this, we used germ-free mice that are born and raised under completely sterile conditions. The ability of nitrite in reducing NADPH oxidase activity (Figure 2C) was dependent on functional XO. In PG-VSMCs, inhibition of XO with febuxostat abolished the ability of nitrite in reducing NADPH oxidase activity (Figure 4D). Expression of XO was confirmed in both renal cortex and PG-VSMCs (online-only Data Supplement). In aggregate, these results suggest that nitrite modulates NADPH oxidase function in the kidney, primarily in preglomerular vessels, and this effect requires functional XO.

Nitrite Restores Contractile Responses in a Mouse Model of Oxidative Stress

Next, we investigated the effects of nitrite in SOD1-knockout mice, a transgenic mouse model characterized by increased oxidative stress and NO deficiency. In SOD1 wild-type mice, the contractile response to ANG II (maximal response, 41%) was similar to that observed in C57 mice (compare Figure 2A), and simultaneous incubation with nitrite attenuated arteriolar contraction (maximum response, 24%; Figure 5A). Arterioles from SOD1 knockouts displayed greatly enhanced contractile responses to ANG II (threshold response, 10\(^{-11}\) mol/L; maximum response, 90%; Figure 5B). Treatment with nitrite markedly attenuated the arteriolar responses (threshold response, 10\(^{-9}\) mol/L; maximum contraction, 44%), which were similar to that observed in placebo-treated C57 (Figure 2) and SOD1 wild-type mice (Figure 5A). Maximal arteriolar responses to ANG II are summarized in Figure 5C. Again, this supports that nitrite interferes with reactive oxygen species generating systems to elicit vasodilation.

Dietary Nitrate Attenuates NADPH Oxidase Activity and Microvascular Responses to ANG II

Supplementation with dietary nitrate can fuel a nitrate–nitrite–NO pathway and increase levels of bioactive nitrogen oxides,\textsuperscript{14,15,32} and we have reported therapeutic effects with inorganic nitrate in renal and cardiovascular disease models associated with NO deficiency and oxidative stress.\textsuperscript{33,24} Next, we investigated whether chronic dietary supplementation with nitrate could modulate renal arteriolar function and NADPH oxidase activity. One week of dietary nitrate treatment was associated with increased plasma nitrate but did not significantly (\(P=0.10\)) alter circulating nitrite or cGMP levels (Figure S3). However, arterioles from mice treated with dietary nitrate displayed reduced ANG II–induced contractions (maximum response, 29%) compared with placebo-treated mice (maximum response, 58%; Figure 6A and 6B). Analysis of renal NADPH oxidase activity from the same groups showed that nitrate supplementation was associated with an \(\approx50\%\) reduction in NADPH oxidase–derived superoxide production (Figure 6C). The effect of dietary nitrate on NADPH oxidase activity was not associated with any significant changes in mRNA expression of NADPH oxidase isoforms or its subunits (Figure S4).

Dietary Nitrate Attenuates Blood Pressure Elevation in a Model of Renal Hypertension

Following the demonstration of pronounced effects of nitrate supplementation on arteriolar contractile responses ex vivo, together with inhibition of ANG II–induced NADPH oxidase activity, we investigated whether dietary nitrate would also affect cardiovascular function in a model of hypertension. Here, arteriolar responses, the inhibitory effect of nitrate supplementation on NADPH oxidase activity was not observed in germ-free mice (Figure 6D). Overall, this demonstrates a crucial role of commensal bacteria in bioactivation of dietary nitrate.
we find that the blood pressure elevation in response to chronic ANG II infusion was markedly attenuated in animals with dietary nitrate supplementation (Figure 7A). The placebo group displayed a characteristic slow pressure response to ANG II with progressive hypertension throughout the study period. No differences in heart rate were observed between the placebo and nitrate groups (Figure S5). Finally, when the nitrate supplementation was stopped, the blood pressure increased in the nitrate group, whereas no change was observed in the placebo group. Maximal changes in blood pressure for the different treatment periods are shown in Figure 7B for the placebo and nitrate groups.

**Discussion**

The present study provides novel evidence for a role of dietary nitrate and nitrite in long-term blood pressure regulation via effects on the renal microvasculature. Together, our data show that nitrate and nitrite dilate renal afferent arteriole and counteract ANG II–induced vasoconstriction by generating NO-like bioactivity and reducing NADPH oxidase activity.

Blood pressure–lowering effect of dietary nitrate has been demonstrated in healthy humans and rodents, but the mechanisms remain uncertain. Systemic vasodilatation by activation of the NO–soluble guanylyl cyclase–cGMP pathway has been suggested and is likely of importance in the acute effects of dietary nitrate and nitrite. However, we show that the renal microcirculation is exquisitely responsive to nitrite. Indeed, in larger vessels such as the aorta, the threshold for dilatation by nitrite under normoxia is >10⁻⁵ mol/L,³⁵,³⁶ whereas in renal arterioles, significant vasodilatation was observed already at 10⁻⁷ mol/L, that is, well within the physiological range and actually lower than the measured basal plasma levels of nitrite. Moreover, the other vascular beds (carotid, renal interlobar, and mesenteric arteries) examined in the present study were unresponsive to nitrite under similar conditions. These data suggest that basal circulating levels of nitrite are sufficient to participate in physiological regulation of preglomerular arteriolar tone. The reason for variable responses to nitrite among different vascular beds may be related to the abundance of nitrite reductases in the vascular wall as well as the degree of superoxide generation by NADPH oxidases.

Many of the biological effects of nitrite are thought to proceed via its reduction to NO. Several enzymes and proteins have been implicated in nitrite reduction in the vasculature, including heme proteins such as hemoglobin and myoglobin, enzymes of the respiratory chain, aldehyde oxidase, and XO.¹⁵,³⁷ Our findings emphasize XO as a major nitrite reducer in the renal microvasculature, because XO was highly expressed and inhibitors of the enzyme completely inhibited the nitrite response. The generated NO operates partly by stimulating a NO–soluble guanylyl cyclase–cGMP pathway, but more importantly by inhibiting vascular NADPH oxidase–dependent superoxide signaling. Evidence for the involvement of NO was supported by the visualization of NO formation using 4-amino-5-methylamino-2′,7′-difluorescein probe and more importantly by the functional inhibition of the nitrite response with the NO scavenger cPTIO. In all cases, the nitrite response was evident also in the presence of l-NAME, thereby excluding any involvement of NOS.

![Figure 6](http://hyper.ahajournals.org/)

**Figure 6.** Commensal bacteria bioactivate dietary nitrate resulting in attenuated renal microvascular responses to angiotensin II (ANG II). Concentration responses to ANG II in afferent arterioles from mice with nitrate (NaNO₃, 10⁻² mol/L in drinking water) or placebo (NaCl; control) supplementation for 7 to 10 days. Isolated and perfused arterioles were pretreated with Nω-nitro-L-arginine methyl ester hydrochloride (l-NAME) alone (10⁻⁴ mol/L). A, In conventional mice, dietary nitrate treatment (n=8) was associated with reduced ANG II–induced contractions compared with placebo-treated mice (n=6). This effect with nitrate was abolished in the germ-free mice (curves not shown). *P<0.05 compared with placebo-treated conventional mice (2-way ANOVA, Bonferroni multiple comparisons test). B, Maximal arteriolar contraction to ANG II+l-NAME in conventional and germ-free mice with placebo (conventional, n=6; germ free, n=6) or nitrate treatment (conventional, n=8; germ free, n=8). P<0.05 between indicated groups, 1-way ANOVA, Bonferroni multiple comparisons test). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity in the renal cortex from conventional (C) and germ-free (D) mice with placebo (conventional, n=10; germ free, n=10) or nitrate treatment (conventional, n=5; germ free, n=5) for 7 to 10 days. P<0.05 between indicated groups (unpaired t test equal SD). Values are presented as mean±SEM.
The renal afferent arterioles are the major resistance vessels of the kidneys and play an important role in blood pressure regulation. Early studies show a relationship between afferent arteriolar diameter and blood pressure and indicate that development of hypertension can be predicted by a reduced renal afferent arteriolar diameter in the prehypertensive state. The afferent arteriolar tone and resistance are determined by the balance between constrictor agents such as ANG II and vasodilators, particularly NO. The interactions between the nitrate–nitrite–NO pathway and reactive oxygen species signaling were thoroughly examined in the present study. In the renal cortex and PG-VSMCs, ANG II–stimulated NADPH oxidase–dependent superoxide generation was abolished by nitrite. Interestingly, generation of superoxide in nonstimulated PG-VSMCs was dose-dependently reduced by nitrite. The involvement of reactive oxygen species was further investigated in mice with compromised antioxidant capacity (ie, SOD1−/−). In these mice, ANG II–mediated arteriolar contractions were greatly enhanced and pretreatment with nitrite effectively attenuated the vascular response. Kidneys from mice with dietary nitrate treatment had reduced NADPH oxidase activity, and the arterioles displayed similar attenuation of ANG II–induced contractions as observed with nitrite in vitro. These results demonstrate the functional significance of the entire nitrate–nitrite–NO pathway and strongly support the notion that nitrate and nitrite modulate reactive oxygen species signaling in the kidney, thereby inhibiting vasoconstriction. The fact that the vasodilatory effect of nitrite in nonstimulated vessels was modest in comparison to the degree of inhibition after ANG II stimulation further supports this notion. The functional importance of these findings was further investigated in vivo in animals chronically infused with ANG II. In this model of renal hypertension, ANG II induces a slow pressor response and gradual increase in blood pressure that is preceded by a selective increase in renal vascular resistance. These responses are accompanied by activation of NADPH oxidase and increased generation of superoxide. The essential role of the kidneys in ANG II–induced hypertension has been elegantly demonstrated by Crowley et al. Wild-type mice became resistant to ANG II–induced hypertension after receiving kidneys from ANG II type 1 receptor–deficient mice, whereas hypertension developed in ANG II type 1 receptor knockout mice receiving wild-type kidneys. In the present study, control animals gradually developed hypertension during ANG II infusion, whereas this blood pressure response was markedly attenuated in nitrate-treated animals. By the end of the study period, blood pressure was 25 mm Hg lower in the nitrate group.

A remaining question that warrants further investigations is the exact mechanism for nitrate/nitrite-mediated reduction in NADPH oxidase–dependent superoxide formation and signaling. Direct scavenging of superoxide by the NO is plausible, but inhibition of NADPH oxidase activity by nitrite-derived reactive nitrogen oxides is also possible, for example, through nitration of critical thiols in the catalytic site of the enzyme or via nitration reactions. Several NADPH oxidase homologs have been described in renal and cardiovascular systems. We have previously used transgenic mice to illustrate the importance of gp91phox (NADPH oxidase 2) and p47phox in mediating renal afferent arteriolar contraction and blood pressure responses to ANG II. Mechanistically, reduction of NADPH oxidase expression by nitrite would represent a third alternative, although our mRNA expression data of NADPH oxidase and its subunits did not support this.

Recent studies together with the current in vitro findings suggest that the biological effects of dietary nitrate proceed via intermediate generation of the more reactive nitrite anion. Oral bacteria seem central in this process because they most efficiently reduce the nitrate that accumulates in saliva to nitrite. Nitrite is then swallowed and further reduced to NO and other reactive nitrogen oxides in blood and tissues. To test the involvement of bacteria in this model, we used germ-free mice that are unable to use this prokaryotic pathway for nitrate reduction. Interestingly, dietary nitrate treatment had no effect on contractility in renal arterioles from germ-free mice, whereas vessels from conventionally housed mice displayed reduced contractility to ANG II. Moreover, NADPH oxidase activity in renal tissue was reduced by nitrate in conventional mice, but unaffected in the germ-free animals. This finding confirms...
what has been previously proposed, namely a central role of oral commensal bacteria for the bioactivation of dietary nitrate.

The increased incidence of hypertension, cardiovascular disease, and diabetes mellitus in developing countries follows the trend of urbanization and lifestyle changes, perhaps most importantly a Western-style diet. The present findings may have intriguing dietary implications building on a series of recent studies showing beneficial effects of dietary nitrate on cardiovascular function in health and disease. Inorganic nitrate is predominantly found in green leafy vegetables, which figure prominently in cuisines renowned for their cardioprotective properties. Future studies will reveal whether the high content of nitrate in vegetables contributes to the blood pressure–lowering effect of this food group.

We conclude that the renal microvasculature is a primary target for blood pressure regulation by inorganic nitrate and nitrite (Figure 8). Dietary nitrate is reduced to nitrite in vivo by oral commensal bacteria, and nitrite further regulates superoxide signaling in the renal vasculature through XO-mediated NO formation. Preglomerular arterioles seem particularly sensitive to nitrite, where vasodilatation occurred at concentrations below basal circulating plasma levels. This indicates a physiological role for nitrate and nitrite in regulation of renal microvascular tone and in extension of blood pressure.

**Perspectives**

Stimulation of a nitrate–nitrite–NO pathway is associated with reduction in blood pressure, but the mechanisms or the target organs have remained obscure. Preglomerular vascular resistance is important for long-term blood pressure control, and emerging evidence suggests renal oxidative stress in hypertension. The profound effects of inorganic nitrate and nitrite on afferent arteriolar regulation, as demonstrated in this study, may explain their cardiovascular effects. Mechanisms are linked to NO generation and inhibition of NADPH oxidase in preglomerular VSMCs. Clinical trials will determine the therapeutic potential of these inorganic anions in preventing development or persistence of hypertension and how they influence glomerular hemodynamic and filtration properties.

**Acknowledgments**

We thank Margareta Stensdotter, Carina Nihlén, and Annika Olsson (Karolinska Institutet, Stockholm) for technical assistance.

**Sources of Funding**

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**Disclosures**

E. Weitzberg and J.O. Lundberg are codirectors of Heartbeat Ltd., a company that owns patent applications relating to therapeutic uses of inorganic nitrate and nitrite. The other authors report no conflicts.

**References**

What Is New?

- The renal microcirculation is exquisitely responsive to nitrate–nitrite–nitric oxide signaling, as demonstrated by vasodilatation and reduced contractility to angiotensin II.
- The observed effects of inorganic nitrates are dependent on functional xanthine oxidase, and nitric oxide deposition of dioxygen in the vasculature is a primary target.

What Is Relevant?

- Dietary supplementation with inorganic nitrate or nitrite has proven beneficial effects in models of renal and cardiovascular disease.
- Our findings demonstrate that boosting this alternative pathway for nitric oxide production modulates oxidative stress and preeclampsia resistance, which may help to explain the antihypertensive effects of inorganic nitrate and nitrite.

Clinical trials are warranted to determine the therapeutic potential of these inorganic anions.

Summary

In contrast to other vascular beds (eg, carotid, renal interlobar, and mesenteric arteries), the microvasculature of the kidney is particularly sensitive to inorganic nitrite, as demonstrated by vasodilatation at physiological levels. Moreover, stimulation of a nitrite–nitrite–nitric oxide pathway attenuated angiotensin II–mediated arteriolar contraction and hypertension by lowering nitrosoamine dioxide nitrite–nicotinamide adenine dinucleotide phosphate oxidase activity.
NADPH Oxidase in the Renal Microvasculature Is a Primary Target for Blood Pressure–Lowering Effects by Inorganic Nitrate and Nitrite

Xiang Gao, Ting Yang, Ming Liu, Maria Peleli, Christa Zollbrecht, Eddie Weitzberg, Jon O. Lundberg, A. Erik G. Persson and Mattias Carlström

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NADPH Oxidase in the Renal Microvasculature is a Primary Target for Blood Pressure Lowering Effects by Inorganic Nitrate and Nitrite

Short title: Gao & Yang – The NO₃-NO₂-NO Pathway in Hypertension

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SUPPLEMENTAL METHODS

Animals

The studies were conducted on male C57BL/6J mice (The Jackson Laboratory, Maine, USA). To specifically investigate the role of bacteria in bioactivation of nitrate, germ free (n=8) and specific pathogen free (conventional) NMRI mice (n=8) were used. The GF status was checked weekly by culturing faecal samples, both aerobically and anaerobically at +20 and +37°C.1 To investigate the effects of nitrite in a model with oxidative stress we used homozygous littermates (SOD1-/-; n=15 and SOD1 +/+; n=19) from heterozygous breeding pairs of SOD1-ko mice (B6;129S7-Sod1tm1Leb/J (stock# 002972) from The Jackson Laboratory, Maine, USA. Genotyping of the offspring was performed as previously described.2 To investigate the effects of dietary nitrate in a model of renal hypertension we used Sprague-Dawley rats (Charles River Laboratories, Germany) for telemetric blood pressure measurements (n=12). All animals, housed in temperature-controlled rooms with 12hr light/dark cycles, were fed standardized rodent chow (R36, Lactamin, Kimstad, Sweden) and tap water ad libitum. Body weights were between 20-27g for mice and 310-350 g for rats.

Vascular Studies

Isolated and Perfused Afferent Arterioles

Dulbecco’s modified Eagle’s medium (DMEM/F12) with 0.01 mol/L HEPES (Invitrogen AB, Lidingö, Sweden) was used for dissection, bath, and perfusion. The pH was adjusted to 7.4 after addition of bovine serum albumin (BSA:SERVA Electrophoresis Heidelberg, Germany). The concentration of BSA was 0.1% in dissection and bath solutions, and 1% in the perfusion solution. Dissection of renal cortical afferent arterioles was performed at 4°C. Single isolated arterioles with their glomeruli were perfused in a thermo-regulated chamber (37°C) using a micromanipulator perfusion system (Vestavia Scientific, Vestavia Hills, AL, USA), which allowed adjustment of outer holding and inner perfusion pipettes. The chamber and the perfusion system were fixed to the stage of an inverted microscope (Nikon, Badhoevedorp, Netherlands). The perfusion pipette, with a diameter of the tip of 5-µm, was connected to a reservoir containing the perfusion solution. The pressure in the pressure head was 100 mmHg, which corresponds to physiological pressure of 60 mmHg and flow of about 50 nl/min in the connected arteriole. The criteria for the use of an arteriole were: an intact myogenic response and a satisfactory, remaining basal tone. Increasing perfusion pressure rapidly and assessing the change in the luminal diameter, which produced a constriction, tested both criteria. A further criterion was a fast and complete constriction in response to KCl (100 mmol/l) solution. The experiments were digitally recorded and then digitized off-line and analyzed as described before.3 Changes in luminal diameters were measured to estimate the effect of vasoactive substances. The equipment used allowed a resolution of 0.2 µm of the vessel structures.4 In all series, the last 10 seconds of a control or treatment period were used for statistical analysis of steady state responses. Each experiment used a separate dissected afferent arteriole.

Fluorescent detection of NO production in afferent arterioles

A highly sensitive, photo-stable cell-permeable fluorescent probe, 4-Amino-5-methylamino-2,7′-difluorescein diacetate (DAF-FM DA) (Invitrogen, Life Technologies Europe BV, Stockholm, Sweden), was used to measure NO production in the isolated and perfused afferent arteriole. DAF-FM DA is diacylated intracellular by esterases to DAF-FM. It reacts
with NO in presence of oxygen to form green-fluorescent triazolofluoresceins. The fluorescent signal provides a temporally integrated estimate of intracellular NO bioavailability. DAF-FM DA was loaded into afferent arterioles by adding the esterified form to the bath (2x10^{-5} mol/L, 45 min, room temperature). DAF-FM was excited with light at 480nm from a Thiel monochromator using a Nikon eclipse, TE300 microscope (Thiel, Munich, Germany, Nikon Stockholm, Sweden), and the emission isolated at 535nm detected with back-illuminated EMCCD camera (DU-887, Andor Technology, Belfast, Northern Ireland) under software control by MetaFluor (Molecular Devices Corporation, Downingtown, PA, USA). Digital pictures were acquired every 30 seconds at a magnification of x1300. Change in fluorescence intensity of DAF-FM with nitrite (10^{-5} mol/L), as an indicator for NO production was quantified as the percent change from the initial value as previously described.5

Myograph Studies in Carotid, Renal Interlobar and Mesenteric Arteries

Carotid arteries, renal interlobar arteries (ILA) and third-order branch of mesenteric resistance vessels (MRV) from C57BL6 mice were isolated and dissected in ice-cold Krebs solution (composition in mmol/L: NaCl 119; KCl 4.7; CaCl 2 1.6; KH 2PO 4 1.2; MgSO 4·7H2O 1.2; NaHCO 3 25.1; glucose 5.5; EDTA 0.026). Arterial rings (2mm) were mounted on 40µm stainless steel wires (carotid artery) or 25µm tungsten wires (ILA and MRV) in a small vessel myograph (Model 620M, Danish Myo Technology, Denmark) for recording isometric force by transducers (PowerLab 4/30, AD Instruments, Australia). The chambers were filled with Krebs solution (37 °C, pH 7.4) aerated with Carbogen (95% O2, 5% CO2). Resting tension of arteries was set according to the normalization procedure described by Mulvany and Halpern,6 and vessel viability was assessed by the responses to 0.1 mol/L KCl. After washout, cumulative concentration response for ANG II (10^{-12}-10^{-6} mol/L) was obtained, with or without simultaneous incubation with nitrite (10μM). Contractile responses were expressed as percentage of constriction to 0.1 mol/L KCl (% of KCl).

Renal NADPH Oxidase Activity

Chemiluminescence technique was used to determine the NADPH oxidase-mediated superoxide formation in in renal cortex or in preglomerular vascular smooth muscle cells (PG-VSMC). The isolation and culturing of primary PG-VSMC were performed as previously described,7 and the phenotype was confirmed as described by Dubey and colleagues.8 Experiments were conducted between passage 5 and 15 and PG-VSMC were cultured in DMEM/F12 supplemented with fetal bovine serum (10%), penicillin (100 U/mL), streptomycin (100 mg/mL) and glutamine (200 mg/mL) at 37°C (5% CO2 and 95% air) at 98% humidity. Equal numbers of cells were seeded into 6–well plates (2 mL of DMEM/F-12 medium per well), incubated and left overnight to attach. After 24 h, the cells were rinsed twice and incubated with serum free medium for another 48 h. After incubation, and before the experiment, random plates were used for cell count (in quadruplicate) and cellular viability was evaluated with Trypan Blue Solution, 0.4% (Gibco®) exclusion test. After 30 min incubation with different concentrations of nitrite (NaNO 2) or placebo (NaCl) the cells were collected with 300 μL/well dissociation buffer (Invitrogen) and 500 μL/well DPBS and transferred into reaction tubes.

In experiments with kidney cortex, the renal tissue was homogenized with bullet blender in ice cold PBS, and the homogenate was centrifuged at 4°C for 20 min at 2000g. Fresh tissue
homogenates were incubated for 30 min with placebo (NaCl), ANG II (10^{-6} \text{ mol/L}), Nitrite (10^{-5} \text{ mol/L}) or combinations with ANG II and nitrite. In experiments with both PG-VSMC or renal cortex, 100 \mu\text{L} of NADPH (100\mu\text{M}) and lucigenin (50\mu\text{M}) (Sigma-Aldrich) were injected into the reaction tube (final volume of 1 \text{ mL}), and NADPH oxidase activity was determined by measuring lucigenin chemiluminescence every 3 seconds for 3 minutes with an AutoLumat LB953 Multi-Tube Luminometer (Berthold Technologies, Bad Wildbad, Germany). Results were corrected by cell number (VSMC) or by protein quantification (cortex) using Bradford protein assay (Bio-Rad Laboratories, UK).

**Renal NADPH Oxidase and Xanthine Oxidase Expression**

Renal cortex and PG-VSMC were used for expression studies of NADPH oxidases and xanthine oxidase (XO). Infusion of cold PBS (Phosphate Buffered Saline) was started once the vena cava was cut to remove the blood. The kidneys were explanted, blotted and weighed. The renal cortex was separated, homogenized, and quantitative PCR analysis was performed. PG-VSMC were washed with PBS and harvested in RLT buffer for RNA extraction. Total RNA of tissue or cells was isolated using RNeasy Mini Kit (QIAGEN, Valencia, CA), and cDNA was synthesized with High Capacity cDNA Reverse transcription kit (Applied Biosystems). Quantitative PCR analysis was performed according to the Applied Biosystems 7500 protocol. Power SYBR Green Master mix (Applied Biosystems) was used for amplification and detection of DNA. PCR reaction was performed in 96-well plates with 20\mu\text{L} mixer/well (0.25 \mu\text{mol/L of each primer and 5 \mu\text{L of cDNA corresponding to 25 ng (tissue) and 62.5 ng (cells) of RNA, Applied Biosystems}}). The efficiency of PCR was calibrated according to the standard curve and the mRNA level was normalized with \beta-\text{actin by the } \Delta\text{Ct method. Primer sequences and amplification profiles used for Nox2, Nox4, p22phox, p47phox, p67phox, XO and } \beta-\text{actin are described as Table S1.}
SUPPLEMENTAL RESULTS

Renal Xanthine Oxidase Expression

We have previously demonstrated XO activity in the kidney. In the present study we showed that the relative expression of xanthine oxidase to beta-actin is high, with ΔCt value in renal cortex of -0.56±0.13 (Mean Ct(XO) = 24.2±0.1; Mean Ct(beta-actin) = 24.8±0.0) and ΔCt value in PG-VSMC of -1.79±0.02 (Mean Ct(XO) = 24.3±0.0; Mean Ct(beta-actin) = 26.1±0.0).
SUPPLEMENTAL REFERENCES


### Table S1. Primers for PCR Analysis

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Figure S1. Vascular Studies – Effects of nitrite on vascular reactivity and NO formation. Photomicrographs demonstrating the set-up for vascular reactivity (Panel A) and NO measurements (Panel B) in isolated and perfused renal afferent arterioles.
Figure S2. Myograph Vascular Studies – Nitrite do not change vascular response to angiotensin II in carotid, renal interlobar or mesenteric arteries. Angiotensin II concentration response curves (ANG II) in isolated carotid arteries (Panel A), renal interlobar arteries (Panel B) and mesenteric resistance arteries (Panel C) of C57 mice. Simultaneous administration of nitrite (10^{-5} mol/L) did not change ANG II-mediated contraction, as observed in renal afferent arterioles. Values are presented as mean ± SEM of n=8-12 in each experimental group.
Figure S3. Plasma Levels of Nitrate, Nitrite & cGMP
Circulating nitrate (NO₃⁻), nitrite (NO₂⁻) and cGMP levels in conventional mice (n=6 per group) following dietary nitrate supplementation (NaNO₃ 10⁻² mol/L, in drinking water) or placebo treatment (NaCl; Control) for 7-10 days in. Values are presented as mean ± SEM.
Figure S4. Expression of NADPH Oxidase in the Kidney
NADPH oxidase and subunit expressions in renal cortex of conventional mice (n=6 per group) following dietary nitrate supplementation (NaNO3 10^{-2} mol/L, in drinking water) or placebo treatment (NaCl; Control) for 7-10 days in. Values are presented as mean ± SEM.
Figure S5. Supplementation with dietary nitrate does not influence heart rate in a model of renal hypertension. Heart rates measured with telemetry in conscious Sprague-Dawley rats. The telemetric measurements were conducted continuously during control conditions (Baseline, 3 days), without any other treatment. This was followed by a period with L-NAME drinking water (500 mg/L) supplemented with either nitrate (NaNO3, 10⁻² mol/L, n=6) or placebo (NaCl, n=6) for 3 days. Heart rate was then measured with chronic ANG II infusion at a slow-pressor rate (120 ng/kg/min, 15 days). During the last 4 days, heart rate was recorded after stopping the placebo and nitrate supplementation. Values are presented as mean ± SEM.