Dietary nitrate attenuates oxidative stress, prevents cardiac and renal injuries, and reduces blood pressure in salt-induced hypertension

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Aims
Reduced bioavailability of endogenous nitric oxide (NO) is a central pathophysiological event in hypertension and other cardiovascular diseases. Recently, it was demonstrated that inorganic nitrate from dietary sources is converted in vivo to form nitrite, NO, and other bioactive nitrogen oxides. We tested the hypothesis that dietary inorganic nitrate supplementation may have therapeutic effects in a model of renal and cardiovascular disease.

Methods and results
Sprague-Dawley rats subjected to unilateral nephrectomy and chronic high-salt diet from 3 weeks of age developed hypertension, cardiac hypertrophy and fibrosis, proteinuria, and histological as well as biochemical signs of renal damage and oxidative stress. Simultaneous nitrate treatment (0.1 or 1 mmol nitrate kg⁻¹ day⁻¹), with the lower dose resembling the nitrate content of a diet rich in vegetables, attenuated hypertension dose-dependently with no signs of tolerance. Nitrate treatment almost completely prevented proteinuria and histological signs of renal injury, and the cardiac hypertrophy and fibrosis were attenuated. Mechanistically, dietary nitrate restored the tissue levels of bioactive nitrogen oxides and reduced the levels of oxidative stress markers in plasma (malondialdehyde) and urine (Class VI F₂-isoprostanes and 8-hydroxy-2-deoxyguanosine). In addition, the increased circulating and urinary levels of dimethylarginines (ADMA and SDMA) in the hypertensive rats were normalized by nitrate supplementation.

Conclusion
Dietary inorganic nitrate is strongly protective in this model of renal and cardiovascular disease. Future studies will reveal if nitrate contributes to the well-known cardioprotective effects of a diet rich in vegetables.

Keywords
ADMA • DASH • Nitric oxide • Nitrite • S-Nitrosothiol • Uninephrectomy

1. Introduction
The kidneys play a key role in the homeostatic regulation of body fluid volume and electrolyte balance and consequently possess a dominant role in blood pressure control. A high-salt intake is associated with elevated blood pressure and greater risk of cardiovascular morbidity. Intervention studies have shown that blood pressure decreases when sodium intake is restricted and it was recently estimated that a modest overall salt intake reduction in the adult US population would lead to dramatic health benefits with considerable decreases in myocardial infarction, stroke, and overall mortality. Furthermore, compromised renal function plays a central role in linking salt intake to hypertension. Nitric oxide (NO) is a key regulator of cardiovascular homeostasis with reduced NO bioavailability being a central underlying pathological event in many cardiovascular disorders including hypertension and atherosclerosis. An increase in renal and vascular oxidative stress is thought to be involved in salt-induced hypertension and can be effectively counteracted by NO via its rapid inactivation of superoxide (O₂⁻). By the same mechanism, increased production of reactive...
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2. Methods

2.1 Animals and study protocol

Studies were performed on male Sprague–Dawley rats (Møllegaard, Copenhagen, Denmark). The animals were uninephrectomized (UNX) or sham operated at 3 weeks of age. Sham-operated controls were given a normal-salt diet (0.7% NaCl, SD389-R36, Lactamin, Södertälje) and the uninephrectomized animals were subjected to high-salt diet (4% NaCl, SD312-R36, Lactamin) alone, or a high-salt diet supplemented with two different doses of nitrate (0.14 or 1.4 g NaNO₃ kg⁻¹ day⁻¹) to achieve a daily intake of 0.1 and 1 mmol nitrate kg⁻¹ day⁻¹, respectively. In this way, four different experimental groups were created: sham-operated animals raised on a normal-salt diet (controls); UNX animals raised on a high-salt diet (UNX + HS); UNX animals raised on a high-salt diet supplemented with a nutritional dose of nitrate, UNX + HS + nitrate (low); and UNX animals raised on a high-salt diet supplemented with a pharmacological dose of nitrate, UNX + HS + nitrate (high). The total NaCl content was 4% in all high-salt diets. The experimental protocols were carried out in adult animals after 8- to 11-week treatment with nitrate (see Supplementary material online, Figure S1).

2.2 Uninephrectomy

A unilateral nephrectomy was performed as described previously.36 Anaesthesia with spontaneous inhalation of 2–2.5% isoflurane (Foren® Abbot Scandinavia AB, Kista, Sweden) was used; the abdomen was opened sterile through a midline incision, and the left kidney was exposed. The renal vessels and the ureter were carefully isolated and tied, and the distal portions were cut. Sham operations in control animals were performed in the same way, but without removal of the kidney and the ureter. After surgery, all animals were left to grow with free access to the different diets.

2.3 Metabolism studies

After 8 weeks of treatment, rats were placed in metabolic cages for 24 h, with food and water given ad libitum, to study intake and renal excretion. Samples of fresh urine were stored at −80°C until analysed. Water/food consumption and urine production were measured gravimetrically.

2.4 Telemetric measurements

Anaesthesia was used as described above, and the telemetric device (PA-C40) (DSITM, Transoma Medical, St Paul, MN, USA) was implanted as described previously. After surgery, all animals were allowed to recover for at least 10 days before any measurements commenced. Telemetric measurements of blood pressure and heart rate were conducted continuously during a control period (72 h), followed by an additional 72 h period with the NOS inhibitor L-NAME (0.5 g/L) in drinking water.

2.5 Plasma collection

At the end of the study period, animals were anaesthetized by intraperitoneal injection of thiobutabarbital sodium (Inactin® 120 mg/kg body weight) and a catheter was placed in the left carotid artery for blood sampling. Blood samples were collected in tubes containing EDTA (final concentration 2 mM) and N-ethylemaleimide (final concentration 5 mM), immediately centrifuged at +4°C (730g, 5 min) and stored at −80°C for later analysis.

2.6 Tissue collection and histology

The kidneys, heart, and liver were rapidly excised, weighed, rinsed, snap frozen in liquid nitrogen, and stored at −80°C for later analysis, or prepared for histology. Sagittal slices of the heart and renal tissue were fixed in formalin (4% in PBS), embedded in paraffin, cut into 5 µm-thick sections and stained (haematoxylin and eosin, periodic acid-Schiff, and Picro-Sirius) for histopathology evaluation by a blinded pathologist. In the renal tissues, the cortex, medulla, and papilla were investigated for hypertrophy, fibrosis, and inflammatory changes. The hearts were investigated for hypertrophy, fibrosis, and inflammatory changes.

The evaluated tissues, except from arterio-arteriolar sclerosis, were given a score of 0–3 depending on the severity of change (0, no observable changes; 1, mild; 2, moderate; and 3, severe changes). The lowest score was given if the renal/cardiac histoarchitecture was normal, with no changes in any of the investigated parameters. The highest score...
Table 1 Twenty-four-hour metabolism parameters

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>UNX + HS</th>
<th>UNX + HS nitrate (low)</th>
<th>UNX + HS nitrate (high)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>336 ± 2</td>
<td>307 ± 4</td>
<td>301 ± 5</td>
<td>291 ± 9</td>
</tr>
<tr>
<td>Water intake (mL/24 h/bw)</td>
<td>0.107 ± 0.004</td>
<td>0.271 ± 0.019a</td>
<td>0.266 ± 0.014a</td>
<td>0.258 ± 0.009a</td>
</tr>
<tr>
<td>Food intake (g/24 h/bw)</td>
<td>0.087 ± 0.005</td>
<td>0.083 ± 0.004</td>
<td>0.087 ± 0.003</td>
<td>0.089 ± 0.002</td>
</tr>
<tr>
<td>Faece (g/24 h/bw)</td>
<td>0.029 ± 0.002</td>
<td>0.029 ± 0.003</td>
<td>0.027 ± 0.002</td>
<td>0.032 ± 0.003</td>
</tr>
<tr>
<td>Urine (mL/24 h/bw)</td>
<td>0.064 ± 0.004</td>
<td>0.223 ± 0.0131</td>
<td>0.207 ± 0.0111</td>
<td>0.188 ± 0.0104</td>
</tr>
<tr>
<td>Na⁺ (mM)</td>
<td>106 ± 5</td>
<td>304 ± 17</td>
<td>321 ± 16</td>
<td>306 ± 15</td>
</tr>
<tr>
<td>Na⁺ (µmol/24 h/bw)</td>
<td>6.7 ± 0.2</td>
<td>62.7 ± 5.0</td>
<td>65.6 ± 2.1</td>
<td>59.2 ± 2.4</td>
</tr>
<tr>
<td>K⁺ (mM)</td>
<td>168 ± 13</td>
<td>40 ± 2</td>
<td>42 ± 2</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>K⁺ (µmol/24 h/bw)</td>
<td>10.0 ± 0.5</td>
<td>7.8 ± 0.6</td>
<td>8.7 ± 0.6</td>
<td>7.8 ± 0.4</td>
</tr>
<tr>
<td>Osmolarity (mOsm)</td>
<td>1063 ± 82</td>
<td>781 ± 33</td>
<td>804 ± 42</td>
<td>806 ± 42</td>
</tr>
<tr>
<td>Osmolarity (mOsm/24 h/bw)</td>
<td>66.8 ± 3</td>
<td>153 ± 11</td>
<td>163 ± 6</td>
<td>156 ± 7</td>
</tr>
</tbody>
</table>

Sham-operated animals (controls) and uninephrectomized animals treated chronically with high-salt diet (UNX + HS), or high-salt diet supplemented with nitrate in a nutritional dose; UNX + HS + nitrate (low), or pharmacological dose; UNX + HS + nitrate (high). Values are mean ± SEM.

*P < 0.05 vs. controls.

**P < 0.05 vs. UNX + HS.

represented major distortion of the normal histoarchitecture. Arterio-arteriolar sclerosis was scored in 20–30 arteries and arterioles in each section as described by Kobayashi et al.37 (i.e. 0, normal; 1, thickening of media; 2, focal hyalinosis with thickening of media; 3, medial thickening with global hyalinosis; 4, fibrinoid necrosis and/or cellular hyperplasia with narrowing of arteriolar lumen and/or thrombus formation).

2.7 Measurements of tissue nitrate, nitrite, and nitros(y)ation products

A highly sensitive chemiluminescence system32 was used for quantification of nitrate, nitrite, and nitros(y)ation products in kidney, liver, and heart tissue (see Supplementary material online).

2.8 Urine and plasma analysis

Urine sodium and potassium concentrations were determined by flame photometry (model FLM3; Radiometer, Copenhagen, Denmark) and osmolality was determined by using an osmometer (model 210 MicroSample Osmometer; Fiske, Norwood, MA, USA).

2.8.1 Creatinine

Plasma content of creatinine was determined using an enzymatic method with the Abbott Diagnostics ARCHITECT analyzer (see Supplementary material online).

2.8.2 Nitrate and nitrite

Urinary and plasma content of nitrate and nitrite were measured with a dedicated high-performance liquid chromatography (HPLC) system (ENO-20; EiCom, Kyoto, Japan) as described12 (see Supplementary material online).

2.8.3 Arginine, ADMA, and SDMA

Concentrations of arginine, ADMA, and SDMA in plasma and urine were measured by HPLC with fluorescence detection (see Supplementary material online).

2.8.4 Malondialdehyde

Total, i.e. free and protein-bound, plasma malondialdehyde (MDA) was measured by HPLC and fluorescence detection after alkaline hydrolysis and reaction with thiobarbituric acid, as described previously.38 The intra- and inter-run variations were 3.5 and 8.7%, respectively.

2.8.5 Class VI F2-isoprostanes

The concentration of Class VI F2-isoprostanes (iPF2α-VI) in urine was determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) (see Supplementary material online).

2.8.6 8-hydroxy-2-deoxyguanosine

The concentration of 8-hydroxy-2-deoxyguanosine (8-OHdG) in urine was determined by LC-MS/MS (see Supplementary material online).

2.8.7 Protein

Urinary protein content was determined by the colorimetric method of Detergent Compatible Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Plates were read from the bottom using a microplate reader (model Safire II; Tecan Austria, Grödig, Austria) (absorbance at 750 nm).

2.9 Statistical analysis

Values are presented as means ± SEM. Single comparisons between normally distributed parameters were tested for significance with Student’s paired or unpaired t-test. For multiple comparisons, ANOVA followed by the Mann–Whitney U test. Statistical significance was defined as P < 0.05.

2.10 Ethics

The investigation was approved by the Uppsala Ethical Committee for Animal Experiments and conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

3. Results

At the beginning of the experiments (i.e. 8-week post-initial surgery), the body weights were slightly lower in uninephrectomized animals than in the control group (Table 1). However, no differences in body weights were found among the groups at the end of the experiments [controls: 554 ± 26 g; UNX + HS: 513 ± 17 g; UNX + HS + nitrate (low): 495 ± 11 g; and UNX + HS + nitrate (high): 527 ± 27 g].
Twenty-four-hour food and water intake, faeces production, and renal excretion data for the different groups are summarized in Table 1. As expected, both water intake and urine production increased in all groups with high-salt intake. Despite differences in body weights, food intake and faeces production were similar in all groups. Interestingly, in the high-dose dietary nitrate group, urinary output was lower compared with that of untreated rats, possibly as a consequence of lower blood pressure. Excretion of sodium was increased and potassium reduced in the UNX+HS group compared with the controls. Nitrate supplementation had no significant effects on electrolyte excretion. Moreover, plasma creatinine was similar in all experimental groups [controls: 27 ± 2 μmol/L; UNX + HS: 29 ± 1 μmol/L; UNX + HS + nitrate (low): 27 ± 1 μmol/L; and UNX + HS + nitrate (high): 27 ± 1 μmol/L] (see Supplementary material online).

3.1 Dietary nitrate increases tissue levels of nitrosylation products

After ingestion, the nitrate anion is stepwise metabolized to potentially bioactive nitrogen oxides including nitrite, S-nitrosothiols,
and nitros(yl)ated species. The levels of nitrate and other nitrogen oxide species were measured in plasma and tissues after 10 weeks of dietary supplementation with nitrate. As expected, the circulating levels of nitrate were dose-dependently higher in rats receiving dietary nitrate supplementation (Figure 1). However, nitrite levels in plasma were not significantly changed in rats treated with nitrate, even at the higher dose (controls: 0.658 ± 0.102 μM; UNX + HS: 0.786 ± 0.066 μM; UNX + HS + nitrate (low): 0.460 ± 0.084 μM; and UNX + HS + nitrate (high): 0.852 ± 0.095 μM). This differs from studies with acute nitrate administration which have shown marked increases in plasma nitrite in humans and rodents. Tissue evaluation did not reveal any significant differences in nitrite levels among the groups, whereas nitrate supplementation restored or elevated nitrate levels in UNX + HS animals (Table 2).

Although nitrite levels were unaffected by the nitrate supplementation, the levels of nitros(yl)ation products (RXNO) in kidney, liver, and heart tissues were increased (Figure 1). Interestingly, nitrate treatment restored or even elevated RXNO levels in UNX + HS animals compared with controls. These findings are consistent with an initial reduction of nitrate to nitrite and then further metabolism of nitrite to form nitrosylated species.

### 3.2 Chronic dietary nitrate supplementation reduces blood pressure in hypertensive rats

Mean arterial blood pressures and responses to L-NAME are presented in Figure 2. Animals subjected to unilateral nephrectomy and chronic high-salt intake developed hypertension, confirming previous findings. Heart rates were similar in the four groups: controls, 364 ± 4; UNX + HS, 379 ± 7; UNX + HS + nitrate (low), 378 ± 6; and UNX + HS + nitrate (high), 372 ± 4 b.p.m. No differences in mean locomotor activity levels were found among the groups. With the low nitrate dose, blood pressure was not significantly decreased; however, with the higher nitrate dose, blood pressure was 24 mmHg lower compared with the untreated hypertensive rats. The magnitude of this blood pressure reduction was considerably greater than what we recently observed in healthy normotensive rats using the same nitrate dose. The blood pressure reduction by nitrate was sustained after 8 weeks dietary supplementation period. Thus, there seemed to be no development of tolerance, which is classically seen with repeated administration of organic nitrates. The blood pressure increase after L-NAME was augmented in untreated UNX + HS rats compared with controls, which indicates a compensatory up-regulation of NOS in the disease model. In rats receiving the higher nitrate dose, this L-NAME response was similar to in controls which may suggest the existence of a crosstalk between the NOS-independent and NOS-dependent NO generation.

### 3.3 Dietary nitrate prevents the renal damage caused by a high-salt diet

The addition of a high-salt diet to uninephrectomized rats was clearly associated with renal impairment. As expected, total kidney weights increased in untreated UNX + HS rats compared with controls (3.2 ± 0.1 g). Nitrate supplementation dose dependently reduced renal hypertrophy in the UNX + HS group (2.9 ± 0.1 and 2.6 ± 0.1 g, respectively).

Furthermore, UNX + HS rats developed proteinuria compared with controls which was completely prevented by nitrate supplementation (Figure 3). The histological examination revealed focal fibrotic changes, infiltration of inflammatory cells, tubular changes (atrophy, thickening of the tubular basal membrane, dilatation with accumulation of hyaline cast), glomerular changes (i.e. mesangial matrix increase, wrinkling and thickening of glomerular basement membrane, subendothelial widening with reduplication of glomerular basement membrane, shrunk and/or sclerotic glomeruli), and increased arterio-arteriolar sclerosis. As shown in Figure 3, dietary supplementation with nitrate substantially improved the appearance of these pathological events. Remarkably, even the low dose of nitrate, which is equivalent to ingestion of 100–300 g of a nitrate-rich vegetable in a human, was strongly protective. In fact, apart from a slight increase in renal fibrosis, the histological renal injury score in animals receiving a low dose of nitrate was similar to that seen in healthy control animals.

### 3.4 Cardiac hypertrophy and fibrosis is attenuated by dietary nitrate

Similar to the kidneys, the hearts were hypertrophic in the uninephrectomized rats on a high-salt diet compared with the controls. This hypertrophy was associated with an increased thickness of the left ventricular wall and increased fibrotic changes in the left ventricular wall.
Figure 2: Mean arterial pressures from 72 h of telemetric measurements in conscious sham-operated animals (control), and uninephrectomized animals treated chronically with high-salt diet (UNX + HS); or high-salt diet supplemented with nitrate in a nutritional dose, UNX + HS + nitrate (low); or pharmacological dose, UNX + HS + nitrate (high) (A and B). L-NAME was administered in drinking water during an additional 72 h, and mean arterial pressure responses are shown in (C). Values are mean ± SEM. *P < 0.05.
Figure 3 (Upper panels) Renal protein excretion (A) and evaluation of fibrosis (B), inflammation (i.e. infiltration of plasma cells and lymphocytes) (C), tubular changes (i.e. atrophy, dilatation, and hyaline material) (D), glomerular changes (i.e. mesangial matrix increase, changes in glomerular basement membrane, sclerosis) (E), and arterio-arteriolar sclerotic changes (F) in sham-operated animals (controls), and uninephrectomized animals treated chronically with high-salt diet (UNX + HS); or high-salt diet supplemented with nitrate in a nutritional dose, UNX + HS + nitrate (low); or pharmacological dose, UNX + HS + nitrate (high). (Lower panels) Representative photomicrographs of kidney sections (cortex), stained with Picro-Sirius (G–I) for fibrosis and PAS (J–R) for inflammation, glomerular, and tubular changes as well abnormalities in arteriole and small artery in the different groups: control animal with a normal renal tissue with no or minimal changes in all investigated parameters (G, J, M, and P). UNX + HS animal with focal interstitial fibrosis (H and K), segmentally sclerotic (K) and shrunken glomeruli (H) (black arrow), scanty inflammatory cells infiltration (K) (red arrow head), and atrophic tubules (H and K) (black arrow head). UNX + HS animal with sclerotic changes in arteriole of the vascular pole (N) (red arrow) and in small artery (Q). Nitrate supplementation attenuated interstitial fibrosis, with no signs of inflammatory and tubular changes (I and L), only minimal glomerular changes (O), preserved arteriole (O) (red arrow), and mild arterial changes (R). Scale bars are 50 μm. Values are mean ± SEM. *P < 0.05 vs. controls. +P < 0.05 vs. UNX + HS. #P < 0.05 vs. UNX + HS + nitrate (low).
ventricular wall (Figure 4). Dietary nitrate supplementation partly prevented the development of cardiac hypertrophy and the higher dose significantly reduced the fibrotic changes observed in the high-salt group. None of the investigated groups demonstrated any significant inflammatory changes in the heart.

3.5 L-Arginine and endogenous dimethylarginine levels are normalized by dietary nitrate

Dimethylarginines including ADMA and SDMA are endogenous inhibitors of NOS-dependent NO generation that are formed during proteolysis of methylated proteins. ADMA inhibits NOS, whereas SDMA competes with L-arginine for cellular uptake via cationic amino acid transporters. Renal injuries may increase the levels of circulating ADMA, thus contributing to the pathogenesis of hypertension and other cardiovascular diseases via the inhibition of NO biosynthesis and concomitant facilitation of O₂⁻ production with increased oxidative stress. The levels of ADMA and SDMA in plasma and urine were increased in the uninephrectomized animals on a high-salt diet compared with control animals (Figure 5). This increase was dose dependently attenuated in rats receiving nitrate supplementation. Plasma levels of L-arginine were increased in UNX + HS animals (188 ± 11 μM) compared with those of controls (155 ± 8 μM; P < 0.05), which could be a result of elevated SDMA levels and impaired cellular arginine uptake. In nitrate-supplemented animals, the L-arginine levels were normalized (UNX + HS + nitrate (low): 163 ± 12 μM and UNX + HS + nitrate (high): 153 ± 11 μM). Lower levels of endogenous NOS inhibitors will likely result in enhanced generation of NO from NOS. This would help to further increase the amounts of bioavailable NO, in addition to that formed directly from nitrate and nitrite.
3.6 Dietary nitrate reduces the level of oxidative stress

Increased oxidative stress is a proposed central pathophysiological mechanism in salt-induced hypertension and renal dysfunction. ROS can cause damage via direct scavenging of NO, depletion of NOS cofactors, generation of vasoconstrictor lipid peroxidation products, as well as causing structural and functional alterations within the vasculature.9-11 In the current study, we measured the levels of three well-established oxidative stress markers; MDA in plasma, and iPF2α-VI and 8-OHdG in urine. The presence of oxidative stress in this disease model was evident by increases in circulating MDA and urinary excretion of 8-OHdG compared with control animals. The levels of all three markers decreased with both low- and high-dose dietary nitrate (Figure 6). Again, the low dose of nitrate was surprisingly effective, and the levels of oxidative stress markers in these rats did not differ from those seen in healthy control animals.

4. Discussion

In the present study, the effects of dietary nitrate in a model of renal and cardiovascular disease were investigated. We show that chronic dietary supplementation with inorganic nitrate, in amounts resembling a rich intake of vegetables, attenuates hypertension and prevents the development of cardiac and renal damage in uninephrectomized rats subjected to high-salt intake. Nitrate is the stable oxidation product of NO metabolism, and until recently, this anion was universally considered to be biologically inert. However, several lines of research have surprisingly revealed the existence of a reverse pathway in which nitrate is stepwise reduced to form nitrite and then NO and other bioactive nitrogen oxides.15,45 We and others have recently shown acute NO-like effects of dietary nitrate in healthy volunteers23,24,46,47 as well as in normotensive control animals.25 The present study extends those findings by demonstrating long-term effects of nitrate in a clinically relevant model of hypertension and renal disease.

A number of studies have shown that bioactivation of nitrate requires its initial reduction to nitrite.24,25,48 Such bioactivation is mainly carried out by commensal bacteria in the oral cavity18 and to a lesser extent by mammalian enzymes.39 Nitrite, which is much less stable than nitrate, is then further reduced to NO and other bioactive nitrogen oxides by a variety of non-enzymatic as well as enzymatic pathways in blood and tissues.15,16 The nitrate-mediated effects appear to be independent of NOS activity, as a similar elevation in nitrogen oxides was observed in nitrate-supplemented eNOS-knockout mice32 and rats given a non-selective NOS-inhibitor.32 The role of NO as a final mediator of nitrite bioactivity has been established in numerous studies where the effects of nitrite have been abolished by the NO scavenger PTIO or by an inhibitor of guanylyl cyclase (GC).27,49 In addition to the activation of GC and formation of cGMP, NO or its reaction products can nitros(yl)ate proteins and thereby directly modulate their activity. In the present study, we did indeed observe increased formation of nitros(yl)ated products in tissues after chronic dietary supplementation with nitrate.

Remarkably, for the majority of the studied parameters, the low nitrate dose was as protective as the higher one with the exception of blood pressure which was significantly reduced only in the high......
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Figure 6 Plasma levels of MDA (A) and renal excretion rates of iPF2α-VI (B) and 8-OHdG (C) in sham-operated animals (control), and uninephrectomized animals treated chronically with high-salt diet (UNX + HS); or high-salt diet supplemented with nitrate in a nutritional dose, UNX + HS + nitrate (low); or pharmacological dose, UNX + HS + nitrate (high). Values are mean ± SEM. *P < 0.05.

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In conclusion, dietary supplementation with inorganic nitrate in modest amounts prevents kidney and heart injuries and attenuates hypertension in a model of renal and cardiovascular disease. Mechanistically, dietary nitrate restored the tissue levels of bioactive nitrogen oxides and reduced or normalized the levels of oxidative stress markers and dimethylarginines. Future studies will reveal if nitrate contributes to the well-known cardioprotective effects of a diet rich in vegetables.

Supplementary material

Supplementary material is available at Cardiovascular Research online.
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Conflict of interest: J.O.L. and E.W. are named co-inventors on a patent application relating to the therapeutic use of nitrate and nitrite salts.

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References

8. Petersson J, Carlström M, Schreiber O, Phillipson M, Christoffersson G, Jagare A et al. Nitrate salts. J.O.L. and E.W. are named co-inventors (Karolinska Institute, Stockholm), and Sigrid de Jong (VU University Medical Centre, Amsterdam), and Vinnova (CIDaT) and EUs 7th Framework Program (Flavio).