Imaging of redox-imbalance and oxidative stress in kidney in vivo, induced by dietary cholesterol

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ABSTRACT

We used a mitochondria-penetrating nitroxide, mito-TEMPO, as a contrast probe for imaging of kidney dysfunction in mice, based on the redox-imbalance and oxidative stress in the renal tissues. Kidney dysfunction was triggered by hypercholesterolemia. The mice were divided in three groups: (i) on normal diet (ND; control); (ii) on cholesterol diet (CD); (iii) on cholesterol plus cholestyramine diet (CC). CD mice showed increased plasma levels of total cholesterol and non-HDL-cholesterol, as well as increased serum levels of blood urea nitrogen, uric acid and creatinine, compared to ND mice. CC mice showed slightly increased plasma levels of total cholesterol and HDL-cholesterol, but not non-HDL-cholesterol, compared to ND mice. The serum levels of blood urea nitrogen, uric acid and creatinine in CC mice were equal to those in ND mice. The MRI signal of mito-TEMPO in the kidneys was characterized by: high intensity and long life-time in CD mice, indicating a high oxidative capacity of renal tissues; poor intensity and short life-time in ND mice, indicating a high reducing capacity of renal tissues; moderate intensity and relatively short life-time in CC mice, which shows the protective effect of lipid-lowering agents against oxidative damage. The data suggest that hypercholesterolemia induces redox-imbalance and oxidative stress in kidneys and this process could be visualized using MRI and mito-TEMPO as a redox-sensitive contrast substance.

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Introduction

Renal dysfunction is mainly caused by inflammatory and/or atherogenic factors (e.g. environmental pollutants, drugs, insecticides, unhealthy foods, etc.), inducing increased serum levels of cholesterol, lactate dehydrogenase, alkaline phosphatase, aminotransferases, urea, creatinine, creatine kinases, and plasma pro-inflammatory cytokines (interleukin-1-beta, interleukin-6, and tumour necrosis factor-alpha), as well as decreased levels of glutathione and ascorbate and suppression of antioxidant enzymes [1–5]. All these events are accompanied by a redox-imbalance, resulting in decreased antioxidant capacity and oxidative stress [6,7]. The prolonged effect of inflammatory and atherogenic factors leads to irreversible structural damage of kidney and development of renal failure – a severe pathology, in which the life of patient is maintained through haemodialysis to find a suitable donor for transplantation. In this context, early diagnosis of renal dysfunction and prevention of renal failure has a significant social impact. Extensive research has found that many antioxidants and redox-modulators of natural or synthetic origin have a protective effect against renal dysfunction [1–7]. They are suitable candidates for the prevention of renal dysfunction.

The redox-status of cells, tissues and body fluids is one of the basic parameters, monitored in clinical trials of chronic kidney disease and renal transplantation [8,9]. The tissue redox-status is determined by the balance between endogenous redox-active compounds: (i) oxidizers [e.g. reactive oxygen and nitrogen species (ROS/RNS)]; and (ii) reducers (e.g. antioxidant systems, thiol-containing proteins, endogenous redox-pairs) [10]. Significant progress has been made in the
selective localized detection of many redox-active compounds in vitro and in vivo due to the development of new synthetic or genetically encoded redox-sensitive contrast substances and improvement of visualization techniques: fluorescence, chemiluminescence, magnetic resonance, nuclear, ultrasonic imaging [11–13]. At present, the efforts are focused on mapping the redox-status of tissues and organs in intact organisms. The perfect methodology should provide direct and non-invasive detection of the redox-status of the target organ in vivo.

Some of the most attractive redox-sensitive contrast substances are cyclic nitroxide radicals, which can be registered and analyzed in vitro and in vivo by various magnetic resonance techniques, such as electron-paramagnetic resonance imaging (EPRI), magnetic resonance imaging (MRI) and Overhauser-enhanced MRI (OMRI) [13,14]. The paramagnetic nitroxide radical is involved in electron-transfer reactions with oxidizers and reducers, leading to the formation of diamagnetic intermediate products (hydroxylamine and oxoammonium) (Figure 1). The rate constants of these reactions determine the dynamics of nitroxide-enhanced MRI/EPRI signal in living biological objects. Thus, the nitroxide probes allow an assessment of the total (overall) redox-status of cells, tissues and body fluids. Some studies have shown that cyclic nitroxides exist mainly in two forms in vivo, radical and hydroxylamine [13,15]. Various endogenous reducers and oxidizers could be involved (directly or indirectly via oxoammonium) in the formation of diamagnetic hydroxylamine, but only the interaction of hydroxylamine with superoxide can restore the radical form of nitroxide and its contrast properties at physiological pH (7.4) [11,14,16–18]. Thus, the intensity of the nitroxide-enhanced MRI/EPRI signal in a particular organ indicates the tissue redox-status and can serve as a marker for non-invasive assessment of redox-imbalance and oxidative stress in vivo.

In this study, we used a cell-penetrating and mitochon-dria-penetrating nitroxide radical, mito-TEMPO, as a contrast probe for non-invasive imaging of kidney dysfunction in mice, based on the induction of redox-imbalance and oxidative stress in the renal tissues. The renal dysfunction was triggered by hypercholesterolemia. Experimental and clinical studies have shown that hypercholesterolemia is a risk factor for development of chronic kidney disease accompanied by structural and functional changes in this organ [19–21]. High level of cholesterol in the bloodstream and tissues causes inflammation and induces oxidative stress, which leads to renal fibrosis, cell apoptosis and lesions, respectively to severely impaired and reduced filtration [22,23].

We also evaluated the effect of a lipid-lowering drug (cholestyramine) on the tissue redox-status of kidney. Cholestyramine is an ion-exchange polymer and bile acid sequestrant (Figure 2). It can exchange its chloride anions with anionic bile acids in the gastrointestinal tract and bind them in the polymer matrix.

**Materials and methods**

**Ethics of experimentation statement**

The care, maintenance, and experiments with animals were in accordance to the 'Principles of Laboratory
Animal Care’ (NIH publication number 85-23, revised 1985) and the Guidelines of the Animal Investigation Committee of the National Institute of Radiological Sciences (QST-NIRS, Chiba, Japan).

Animals

Male C57Bl/6 mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Mice were subjected to a normal diet (ND mice) (MF; Oriental Yeast Co., Tokyo, Japan) or a cholesterol diet (ATT6492210; 1.25% [wt/wt] cholesterol, Oriental Yeast Co.) without (CD mice) or with 3% cholestyramine (CC mice), starting at 5 weeks of age. Throughout the experiments, the mice were kept in stainless steel cages with food and water available ad libitum and maintained on a 12-h light-dark cycle.

MRI measurements

The MRI measurements were performed on 7.0 Tesla horizontal magnet (Kobelco and Jastec, Kobe, Japan) interfaced to a Bruker Avance-I console (Bruker BioSpin, Rheinstetten, Germany) and controlled with ParaVision 4.0.1 (Bruker BioSpin).

Mice were anesthetized by isoflurane (2.0%, Abbott Japan, Tokyo, Japan) and placed in a body holder (Rapid Biomedical, Rimpar, Germany), stomach side down and fixed head. A polyethylene catheter (PE-10, Becton-Dickinson, NJ, USA) was placed in the tail vein for probe administration. The mouse’s body was then placed in the 1H-volume coil 35 mm in diameter. Rectal temperature of the mouse was maintained at 36.5 ± 0.5°C using a circulating hot water pad and monitored using an optical temperature probe (FOT-L and FTI-10, FISO Technologies Inc., Germany). A respiration sensor (TDS160A, BIOPAC Systems Inc., CA, USA) was placed on the chest of the mouse for monitoring.

Before nitroxide administration, five control images of the mouse body were acquired with the following parameters: T1-weighted incoherent gradient-echo sequence (fast low-angle shot); repetition time = 75 ms; echo time = 3.2 ms; flip angle = 45°; number of averages = 4; scan time = 19.2 s; matrix = 64 × 64; slice thickness = 1.5 mm; number of slices = 5. We selected the coronal slice orientations with a 500 × 625 × 1500 μm³ nominal voxel resolution. Mito-TEMPO was dissolved in phosphate-buffered saline (10 mmol/L PBS, pH 7.4) to 100 mmol/L stock solution. Ninety-six seconds after starting the MRI scan (five scans as pre-administration data), 100 μL of mito-TEMPO per 25 g mouse were injected via the tail vein during scanning. T1-weighted images were acquired continuously within ~15 min.

The MRI data were analyzed using the ImageJ software (National Institute of Health, Bethesda, MD, USA). The intensity of the nitroxide-enhanced MRI signal in the kidney area (region-of-interest, ROI) was normalized to the average intensity of the MRI signal in the same area before injection of mito-TEMPO (first five scans).
**Analysis of plasma cholesterol levels**

Blood samples were taken from the tail vein in a heparinized microhematocrit tubes. The samples were centrifuged at 12,000×g for 5 min at room temperature, to obtain plasma. Plasma was stored at −80°C until cholesterol analysis. The total cholesterol level was determined by a modification of the cholesterol oxidase method with the use of kit reagents (Wako Pure Chemical Industries, Osaka, Japan). The high-density lipoprotein (HDL) cholesterol levels were measured by the cholesterol oxidase assay of the supernatant from the precipitate of non-HDL lipoproteins with phosphotungstic acid and magnesium chloride using the kit reagents (Wako Pure Chemical Industries). The non-HDL cholesterol levels were calculated as HDL cholesterol levels subtracted from total cholesterol levels.

**Histochemical staining**

Isolated kidney was fixed with 4% formaldehyde in PBS overnight. The tissue was embedded in paraffin. Tissue sections were prepared and stained with haematoxylin and eosin. The analysis of stained tissue sections was performed with a microscope (Olympus FV1000, Olympus, Japan).

**Statistical analysis**

The results are expressed as means with standard error (±SE) or means with standard deviation (±SD). Comparisons between the groups were performed using Student's t-test. A value of \( P < 0.05 \) was considered significant.

**Results and discussion**

The mice were divided in three groups: (i) group 1 – on normal diet (control) (ND mice); (ii) group 2 – on cholesterol diet (CD mice); (iii) group 3 – on cholesterol plus cholestyramine diet (CC mice). After 15 weeks of feeding, the mice were subjected to the following analyses: (i) plasma cholesterol levels; (ii) serum test, representative for renal functionality; (iii) evaluation of redox-status of the kidneys in vivo, using nitroxide-enhanced MRI and mito-TEMPO as a redox-sensor.

The CD mice were characterized by significantly elevated levels of total plasma cholesterol and non-HDL cholesterol, and decreased levels of HDL cholesterol, compared to the ND mice (Figure 3(A)). CC mice were characterized by slightly elevated total plasma cholesterol, control level of non-HDL cholesterol and slightly increased HDL cholesterol, compared to the ND mice. These data prove the development of hypercholesterolemia in CD mice and lipid-lowering effect of cholestyramine.

The hypercholesterolemia compromised the renal function of CD mice: blood urea nitrogen, creatinine and uric acid increased significantly, compared to the control group (ND mice) (Figure 3(B)). In CC mice, all these parameters were almost equal to the reference levels.
values, measured in the control group. Histochemical analysis, performed at the end of the study, showed glomerular and tubular lesions in CD mice (Figure 4). Similar results, showing the development of renal dysfunction in experimental models of hypercholesterolemia, have been also reported by other authors [22,24,25].

Representative nitroxide-enhanced magnetic resonance images of kidney are shown in Figure 5. The nitroxide-enhanced signal was extracted from each image after injection of mito-TEMPO and normalized to the average baseline signal, obtained before the injection. Thus, it is possible to assess the redox-status of the nitroxide probe (oxidized/reduced), respectively to assess the redox-capacity of the tissues in vivo.

In ND mice, a weak MRI signal was detected in the kidney immediately after injection of mito-TEMPO, but it disappeared completely within 3 minutes (Figure 5(A,D)). This indicates a conversion of nitroxide radical to its diamagnetic form (hydroxylamine), which can be explained by the high reducing capacity of normal (healthy) renal tissues. In CD mice, a strong MRI signal was detected in the kidney immediately after injection of mito-TEMPO (Figure 5(C)). The intensity decreases within 15 min but is still above the baseline (Figure 5(D)). This indicates the presence of nitroxide in paramagnetic form, which can be explained by the high oxidative capacity of the renal tissues. In CC mice, the nitroxide-enhancement was very well expressed immediately after injection of mito-TEMPO, but the signal disappeared completely within 10 min (Figure 5(B,D)). Obviously, the renal tissues of cholestyramine-treated mice were characterized by lower oxidative and higher reducing capacity than those of untreated CD mice. The kinetic curves of nitroxide-enhanced MRI signal in Figure 5(D) support this assumption. In CD mice, the signal was long-lived and had a significantly higher intensity than that of ND mice. The differences were statistically significant even in the ‘washout period’ (P < 0.05). No statistically significant difference was found between the integrated MRI signals (area under the curve) of CC mice and ND mice.

It is known that hypercholesterolemia provokes a significant decrease of reduced glutathione and ascorbate in renal lesions, which indicates a redox-imbalance [25–27]. Ascorbate is the main endogenous reducer of nitroxide radical and ‘quencher’ of its MRI contrast [17,28]. Ascorbate and glutathione are major

![Figure 4. Haematoxylin and eosin staining of tissue sections of kidneys isolated from mice on normal diet (A) and cholesterol diet (B). The mice were 20 weeks of age. The images indicate glomerular and tubular damage.](image-url)
factors responsible for the rapid decay of nitroxide-enhanced MRI signal in normal (healthy) kidney. On the other hand, hypercholesterolemia-induced oxidative stress is accompanied by up-regulation of NADPH-dependent oxidase complex (NOX) and mitochondrial dysfunction in kidney [29,30], which results in overproduction of superoxide. Superoxide is the main oxidizer that can restore the nitroxide-enhanced MRI contrast [11,14,18].

Another important factor can also influence the dynamics of MRI contrast in this organ. This is the penetration of nitroxide into the tissues and the rate of excretion from the organism.

Recently, we demonstrated that the relationship between the physicochemical properties of nitroxide probes and their rate of penetration and distribution in cells and tissues is crucial for the proper interpretation of the data from nitroxide-enhanced MRI studies in vivo [31,32]. The rate of metabolism and excretion of nitroxide radical from the organism compete with its penetration in the target tissue and its resistance to reduction. The dynamics of nitroxide-enhanced MRI signal in vivo follow all these processes.

In vitro MRI/EPRI studies have also shown that the nitroxide should penetrate easily and quickly into the cells and interact with intracellular reducers and oxidizers, to serve as a sensor of cellular redox-status [33,34]. Nevertheless, most of the in vivo MRI/EPRI studies of tissue redox-status have been conducted with pyrrolidine-type nitroxides, carboxy-PROXYL (CPx) and carbamoyl-PROXYL (CMPx) [35–38], due to their higher resistance to reduction in biological specimens compared to the piperidine-type nitroxides (as mito-TEMPO). CPx and CMPx are hydrophilic and non-penetrating or poorly penetrating in cells and tissues; they are also excreted very rapidly through the kidney in normal conditions [36].

In our previous study on CD mice, we did not observe any nitroxide-enhanced MRI signal in the kidney, using CMPx as a contrast probe [39]. However, this observation was not a result of a rapid reduction of CMPx to its diamagnetic form. This was a result of strongly decreased renal perfusion, which was proved by a gadolinium-enhanced MRI.

CMPx was recorded by MRI in the bladder of healthy mice at the second minute after tail injection [36], but not in the bladder of mice with hypercholesterolemia [39]. Moreover, we found that the serum albumin and total protein decreased significantly in CD mice (Figure 3(B)). This should accelerate the renal filtration of nitroxide and its excretion from the organism, compared to ND mice. However, the nitroxide-enhanced MRI signal had higher intensity and longer life-time than in ND mice. All these data provide indirect evidence that the higher intensity of MRI signal of mito-TEMPO in the kidneys of CD mice was mainly due to the higher oxidative capacity of renal tissues, compared to ND mice. The comparative analysis with our previous study [39] shows that hydrophilic nitroxide radicals (such as CMPx), that poorly penetrate or
do not penetrate in the cells, are not suitable for ‘redox-imaging’ of kidney dysfunction, accompanied by oxidative stress and severely decreased filtration due to glomerulosclerosis.

Cyclic nitroxides are relatively low toxic (much better than gadolinium complexes) and are not mutagenic [40]. They are characterized by beneficial biomedical effects, such as: anticancer effect, regulation of body weight, protection against ischemia-reperfusion injury, protective effect against cataract, sensitizing cancer cells and tissues to ionizing radiation and protecting normal cells and tissues, etc. [15,41,42]. This increases the interest in nitroxides as contrast substances for MRI/EPRI in vivo. Nitroxide radicals appear to be appropriate candidates as new contrast probes for redox-imaging in translational studies. Our study showed that the higher sensitivity of cell-penetrating piperidine-type nitroxides to reduction should not be considered as a disadvantage. This provides a new opportunity for MRI/EPRI analysis of metabolic pathways, accompanied by minor changes in the reducing capacity of biological objects and induction of oxidative stress.

Conclusions

The present study showed that mito-TEMPO is an appropriate contrast probe for magnetic resonance imaging of hypercholesterolemia-induced kidney dysfunction based on impaired redox-capacity of renal tissues. The probe is also suitable for assessing the effect of anti-lipidemic drugs. The experimental data suggest that hypercholesterolemia induces oxidative stress in kidney and this process could be visualized using MRI and cell-penetrating nitroxide radicals as a redox-sensitive contrast probe (especially mito-TEMPO).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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