Tetrahydrobiopterin protects the kidney from ischemia–reperfusion injury

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Tetrahydrobiopterin (BH4) is an essential cofactor for the nitric oxide (NO) synthases and represents a critical determinant of NO production. BH4 depletion during ischemia leads to the uncoupling of the synthases, thus contributing to reperfusion injury due to increased superoxide formation. To examine whether BH4 supplementation attenuates ischemia–reperfusion injury, we clamped the left renal arteries of male Lewis rats immediately following right-side nephrectomy. BH4 tissue levels significantly decreased after 45 min of warm ischemia compared with levels in non-ischemic controls. Histopathology demonstrated significant tubular damage and increased peroxynitrite formation. Intravital fluorescent microscopy found perfusion deficits in the microvasculature and leakage of the capillary mesh. Supplemental BH4 treatment before ischemia significantly reduced ischemia-induced renal dysfunction, and decreased tubular histologic injury scores and peroxynitrite generation. BH4 also significantly improved microcirculatory parameters such as functional capillary density and diameter. These protective effects of BH4 on microvasculature were significantly correlated with its ability to abolish peroxynitrite formation. We suggest that BH4 significantly protects against acute renal failure following ischemia reperfusion. Whether BH4 has a therapeutic potential will require more direct testing in humans.

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Renal ischemia–reperfusion injury (IRI) still represents a major clinical complication after both transplantation and renal surgery.[1] Although advanced surgical techniques, novel immunosuppressive regimens, and more effective prophylaxis of infections resulted in markedly improved outcomes, 2–50% of all kidney transplants still manifest some degree of early dysfunction, leading to the clinical syndrome of delayed graft function.[2] This form of acute renal failure[3] (ARF) following ischemia and reperfusion results in post-transplantation oliguria, increased allograft immunogenicity, and risk of acute rejection episodes as well as decreased long-term survival.[4] In addition, it has been shown that IRI is also the main cause of ARF after major renal surgery and trauma.[5–7] IRI entails various pathophysiological events including microcirculatory disorders, endothelial cell activation, and expression of proinflammatory cytokines and adhesion molecules as well as loss of endothelial integrity.[8]

Ischemia and, paradoxically, the reinstitution of blood supply during reperfusion thereby cause not only the resumption of metabolic functions but also the generation of deleterious oxygen-free radicals such as superoxide (O^{2-}), peroxynitrite ($ONOO^{-}$), and nitric oxide (NO). NO is produced by a family of enzymes called the nitric oxide synthases (NOS) that convert the amino acid L-arginine and O_2 to L-citrulline and NO. This NADPH-consuming enzymatic reaction requires Ca_2+/calmodulin, flavin adenine dinucleotide, flavin mononucleotide, and heme and tetrahydrobiopterin (BH4) as cofactors.

Under physiological conditions, NO has been shown to be an important mediator of vascular homeostasis, inflammation, and neurotransmission.[9,10] NO generation can attenuate renal damage due to inhibition of platelet aggregation and regulation of neutrophil recruitment by inhibiting the expression of adhesion molecules. Moreover, exogenous NO was shown to diminish IRI after solid organ transplantation.[11,12]

However, it has also been stated that excessive production of NO predominantly by the inducible NOS isoform can lead

to the disruption of active cytoskeleton, thus resulting in proximal tubular cell detachment and tubular obstruction.[13] Furthermore, an increase in NO during inflammatory responses has also been linked to neutrophil recruitment, increased production of inflammatory cytokines, and increased $ONOO^-$ and O_2^- formation, both potent oxidants that modify proteins by forming 3-nitrotyrosine.[14] Thus, the precise role of NO generated during renal ischemia and reperfusion is discussed controversially and is still under debate.

BH4 as an essential NOS cofactor has profound effects on the structure of all NOS isoforms by stabilizing the active dimeric form of the enzyme and increasing substrate affinity. Furthermore, BH4 is highly redox-sensitive and readily oxidized. Oxidative stress induced by IRI has been shown to deplete intracellular BH4 stores below a critical threshold, resulting in an uncoupling of the NOS enzyme after which the heme group can directly reduce oxygen and release superoxide (O_2^-) instead of NO.[13,15,16] Superoxide in concert with other highly reactive free radicals then form a cytotoxic cocktail capable of initiating inflammatory pathways contributing to IRI.[17]

The aim of this study was to determine whether BH4 supplementation would exert beneficial effects on IRI in terms of improvement of microvascular perfusion, histological tissue damage, and decrease in the generation of oxygen-free radicals in the kidneys undergoing renal artery clamping. On the basis of our results, we hypothesize that BH4 might be a novel promising therapeutic agent to reduce delayed graft function in kidney transplants as well as ARF after major renal surgery.

RESULTS

Renal BH4 tissue levels after ischemia and reperfusion

Total renal biopterin and BH4 tissue levels were assessed by high-performance liquid chromatography. Values are expressed as mean ± s.e.m. ($n = 6$ animals per group) in pmol/mg protein. Forty-five minutes of ischemia (I/R) led to a significant decrease in total biopterin levels (45 minI: 12.68 ± 3.77) compared with non-ischemic controls (control: 29.53 ± 3.20; $P < 0.01$). This decrease was maintained throughout the entire observation/reperfusion period (15 minR: 12.47 ± 2.77; 2hR: 12.77 ± 2.11; 7dR: 10.17 ± 1.21; Figure 1a). Treatment with BH4 (I/R + BH4) was associated with a massive increase in kidney biopterin concentrations following 45 min of ischemia (45 min + BH4: 2279 ± 672), as well as after 15 min (15 minR: 2211 ± 441.2) and 2 h (2 hR: 2289 ± 298.7) of reperfusion ($P < 0.001$) (Figure 1b). This confirmed that the administered BH4 reached the kidney during the reperfusion period. The high variability in the I/R + BH4 group was caused by animal-to-animal variation in response to BH4 treatment rather than by variability in the measurement method, which showed a coefficient of variation of 3.1 ± 0.8% (mean ± s.d. of triplicate variation of kidney samples of six BH4-treated animals).

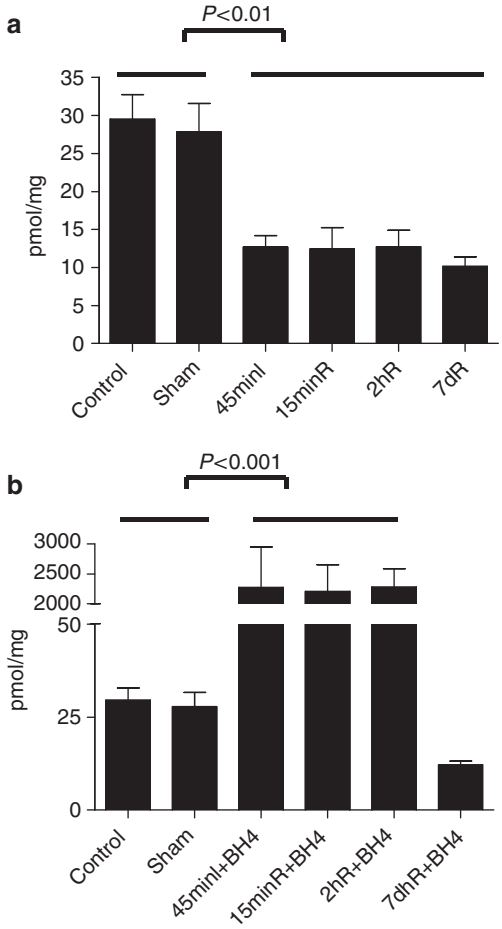

Figure 1 | BH4 tissue levels. (**a**) Total BH4 tissue levels in kidneys subjected to 45 min of ischemia (I) and up to 7 days of reperfusion (R). Ischemia significantly decreased BH4 levels. During reperfusion, BH4 levels remained low throughout the entire observation period. Data are expressed as pmol biopterin per mg protein. (**b**) Total biopterin tissue levels in kidneys treated with 20 mg/kg tetrahydrobiopterin before ischemia and reperfusion. Peak BH4 levels were determined after 45 min of ischemia and 15 min and 2 h after reperfusion. $P < 0.001$ as compared with controls and after BH4 supplementation. Data are means ± s.e.m.; $n = 6$.

Effects of BH4 on kidney microcirculation

Following 45 min of ischemia and 120 min of reperfusion, kidneys were analyzed by means of intravital fluorescence microscopy (IVM). To assess and quantify ischemia-induced microvascular injury, functional defects and the potential protective effects of BH4 microcirculatory parameters such as functional capillary density (FCD) and capillary diameter (CD) were determined. FCD is defined as the length of all blood cell-perfused nutritive capillaries per observation area, and CD as the largest distance of two opposite capillary walls per observation area. Forty-five minutes of ischemia (I/R) caused significant perfusion deficits with leakage of the capillary mesh (Figure 2b and d; supplementary online video 1), compared with the homogeneous perfusion patterns of baseline controls (Figure 2a and d; supplementary online video 2). Mean FCD in controls was 429.9 ± 3.33 cm^{-1}, which was significantly decreased after 45 min of warm

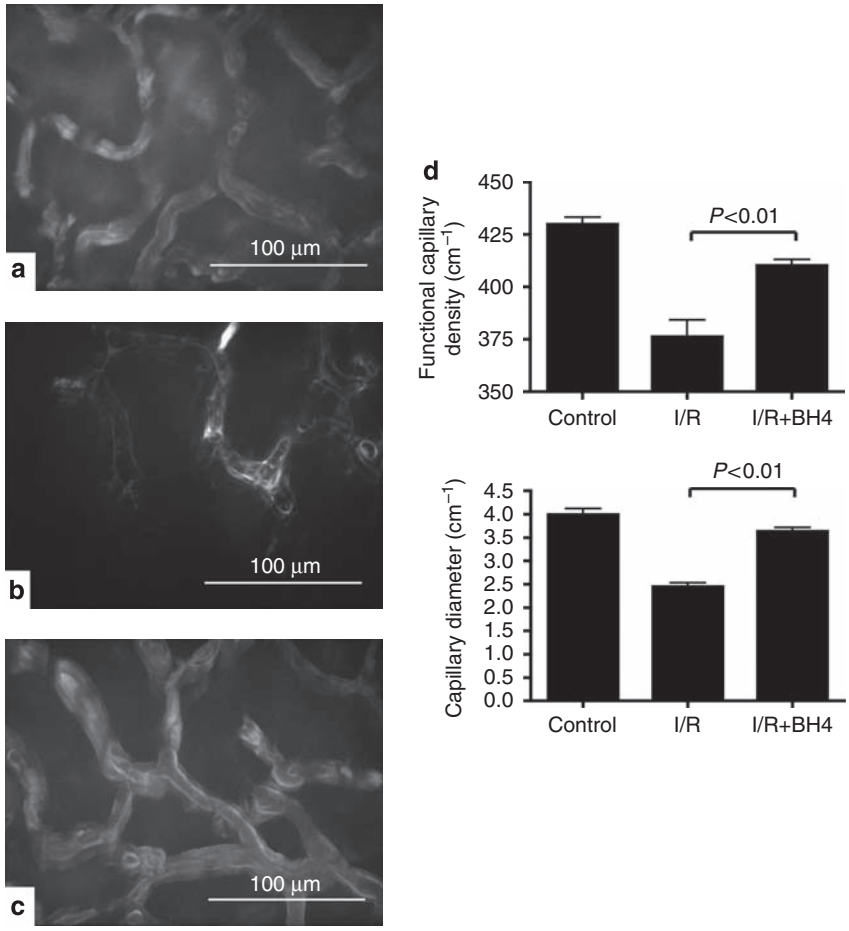

Figure 2 | Intravital fluorescence microscopy. (**a**) Kidney microcirculation in non-ischemic controls lacks signs of microvascular injury. (**b**) In contrast, significant microvascular impairment was evident following 45 min of ischemia. (**c**) BH4 supplementation shows reversal of microcirculatory changes. Original magnification $\times 350$. (**d**) Mean functional capillary density and capillary diameter. BH4 significantly improved microcirculatory parameters compared with no treatment (I/R). Data presented as means ± s.e.m. ($P<0.01$) $n=6$.

ischemia (I/R) $376.2 \pm 8.10\,cm^{-1}$ ($P<0.01$). In parallel, ischemia significantly decreased intercapillary distance (I/R) $2.50 \pm 0.08\,cm^{1}$ compared with controls $4.0 \pm 0.13\,cm^{-1}$ ($P<0.01$). BH4 administration before ischemia, however, significantly improved all microcirculatory parameters resulting in increased FCD (I/R + BH4) $410.3 \pm 2.72\,cm^{-1}$ ($P<0.01$) and increased CD (I/R + BH4) $3.63 \pm 0.08\,cm^{-1}$ ($P<0.01$), comparable with non-ischemic controls (Figure 2c and d; supplementary online video 3).

Histopathology

Histological findings determined by hematoxylin and eosin staining were classified using the Solez score.[18] Kidneys undergoing 45 min of renal artery clamping and 7 days of reperfusion (I/R) thereby showed significant ischemia-induced damage particularly within the renal tubuli, compared with non-ischemic controls, including detachment of the tubulus epithelial cells (I/R: 1.40 ± 0.10 versus control: 0.39 ± 0.08; $P<0.01$), interstitial edema (I/R: 1.25 ± 0.13 versus control: 0.37 ± 0.07; $P<0.01$), and tubular cell casts (I/R: 2.25 ± 0.24 versus control: 0.42 ± 0.07; $P<0.01$) (Figure 3b and d) (Figure 3a and d). In contrast, renal histology obtained after BH4 supplementation revealed significantly diminished changes and reduced quantitative renal tissue damage scores (epithelial detachment: I/R 1.40 ± 0.10 versus I/R + BH4: 0.45 ± 0.06; $P<0.01$; interstitial edema: I/R: 1.25 ± 0.13 versus I/R + BH4: 0.51 ± 0.08; $P<0.01$; tubular cell cast: I/R: 2.25 ± 0.24 versus I/R + BH4: 1.33 ± 0.11; $P<0.01$) (Figure 3c and d).

Peroxynitrite formation

The nitrosylation of tyrosine is paradigmatic for the reaction of peroxynitrite ($ONOO^-$) with aromatic amino acids and is therefore an indirect marker for this strong oxidative agent. Immunostaining for nitrotyrosine was thus performed to indirectly estimate peroxynitrite generation (Figure 4). Compared with non-ischemic controls (Figure 4a), peroxynitrite formation and hence nitrotyrosine staining were significantly increased following 45 min of warm ischemia (Figure 4b), which could be abrogated almost entirely by BH4 treatment (Figure 4c).

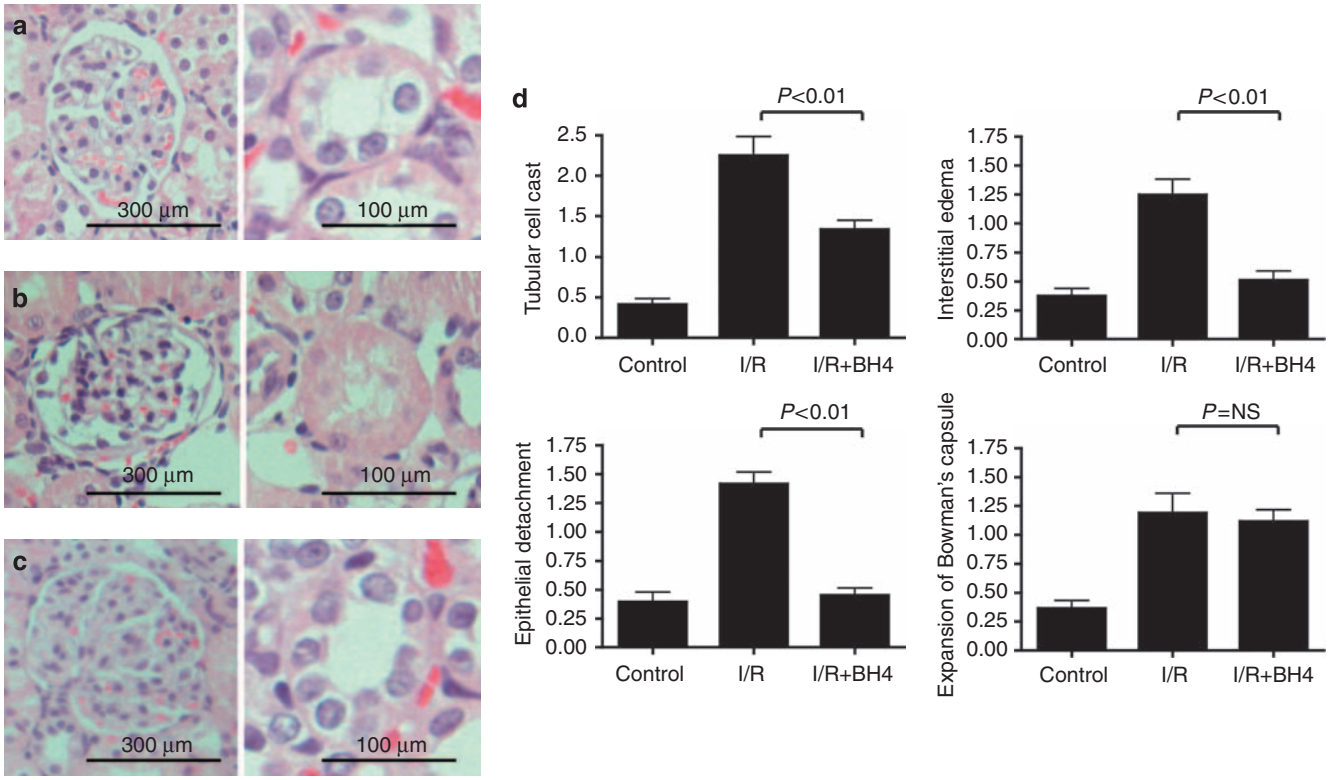

Figure 3 | Histopathology (hematoxylin and eosin staining). (**a**) Non-ischemic controls. (**b**) Tubular cell cast, interstitial edema, epithelial detachment, and expansion of Bowman's capsule were observed in kidneys undergoing ischemia and 7 days of reperfusion. (**c**, **d**) BH4 supplementation significantly improved semiquantitative histological scores. Data presented as means ± s.e.m. ($P < 0.01$).

For quantification purposes, the product of the proportion of positive cells and the staining intensity was calculated, yielding a total immunostaining score ranging from 0 to 12 (Figure 4d). Immunostaining was significantly higher in untreated kidneys (I/R: 7.56 ± 0.45) compared with BH4-treated kidneys (I/R + BH4: 2.22 ± 0.45) or non-ischemic controls (control: 0.85 ± 0.54). In addition, intragraft peroxynitrite generation correlated significantly with the impairment of renal microcirculation and FCD scores. (Spearman: $r = -0.58$; $P < 0.01$; data from Figure 4d). A similar staining pattern was also observed at 2 h following reperfusion (I/R: 5.53 ± 0.88; I/R + BH4: 1.65 ± 0.58; control: 1.08 ± 0.49), whereas histomorphology only revealed moderate tissue edema but no other morphologic changes such as tubular cell cast or detachment of tubulus epithelial cells at such an early time point following ischemic injury (data not shown).

Effect of BH4 treatment on renal function

Renal function was assessed using serum creatinine and urea as indirect parameters of glomerular function. Both parameters were significantly increased after renal ischemia in untreated animals (I/R) after 24 h (Cr: 2.14 ± 0.81 mg/dl; U: 286.67 ± 55.28 mg/dl) and 48 h (Cr: 1.34 ± 0.24 mg/dl; U: 196.02 ± 40.00 mg/dl) following reperfusion compared with non-ischemic sham-operated controls (sham) Cr: 0.65 ± 0.18 mg/dl; U: 82.58 ± 14.25 mg/dl at 24 h and Cr: 0.44 ± 0.24 mg/dl; U: 68.27 ± 21.94 mg/dl at 48 h. The administration of a single dose of BH4 (I/R + BH4) before ischemia resulted in significantly improved renal function and reduced serum creatinine and urea levels Cr: 0.58 ± 0.19 mg/dl; U: 84.10 ± 20.70 mg/dl at 24 h and Cr: 0.48 ± 0.13 mg/dl; U: 81.77 ± 22.48 mg/dl at 48 h after reperfusion (Figure 5a and b).

In line with animals subjected to ischemic injury, only rats undergoing life-supporting kidney transplantation following bilateral nephrectomy (TX) also showed significant increases in serum creatinine and urea after 24 h (Cr: 2.41 ± 0.43 mg/dl; U: 292.0 ± 51.40 mg/dl), 48 h (Cr: 3.75 ± 1.20 mg/dl; U: 452.40 ± 202.5 mg/dl), and 7 days (Cr: 1.40 ± 0.13 mg/dl; U: 210.50 ± 226.3 mg/dl) after transplantation without treatment. The administration of a single dose of BH4 (20 mg/kg per body weight) to the donor and recipient before organ recovery and revascularization (TX + BH4), respectively, resulted in significantly improved renal function at 24 h (Cr: 1.53 ± 0.92 mg/dl; U: 266.50 ± 71.55 mg/dl), 48 h (Cr: 1.65 ± 0.73 mg/dl; U: 341.50 ± 230.20 mg/dl), and 7 days (Cr: 0.56 ± 0.20; U: 119.60 ± 44.89 mg/dl) after transplantation. In addition to improved graft function, BH4 therapy also increased animal survival rates from 33.2% in the untreated control group (TX) to 66.7% in the BH4-treated group (TX + BH4) ($P < 0.01$).

DISCUSSION

In this study, we were able to show that BH4 supplementation significantly protects kidneys from IRI. Moreover,

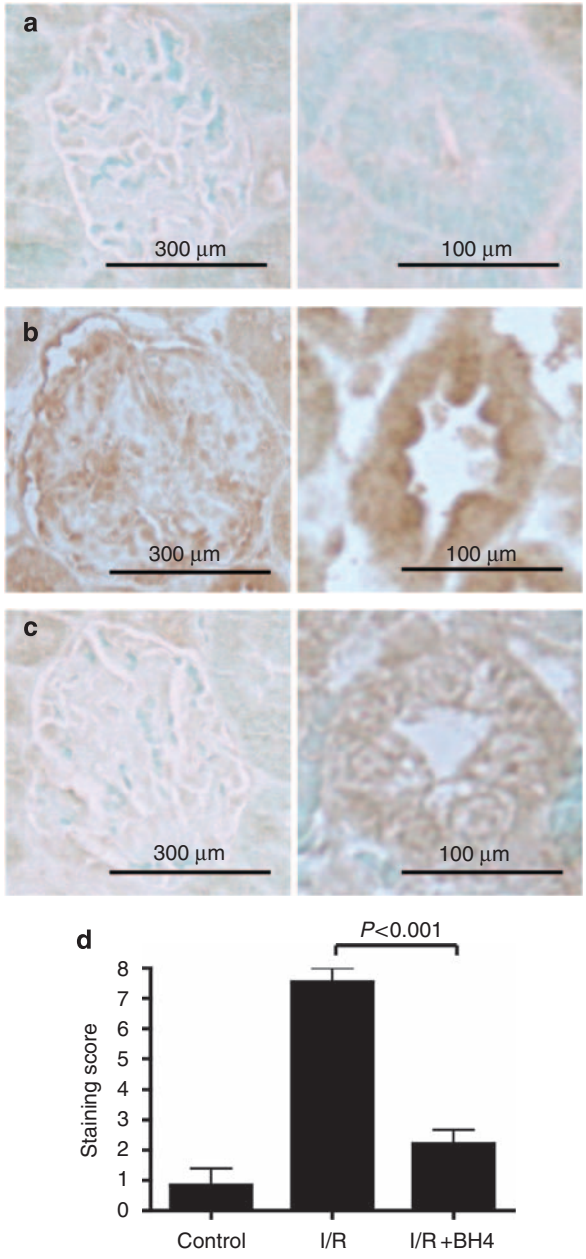

Figure 4 | Immunostaining for nitrotyrosine of kidneys with ischemia–reperfusion injury. (**a**) Non-ischemic controls. (**b**) Ischemia resulted in prominent kidney nitrotyrosine staining as observed on day 7 after reperfusion. (**c**) This staining was abolished by BH4 treatment. Original magnification × 200. (**d**) Quantitative immunostaining score as the product of the proportion of positive cells and the staining intensity. Data presented as means ± s.e.m. ($P<0.001$).

BH4 restores ischemia-induced microcirculatory disorders, diminishes histological damage such as epithelial detachment, tubular cell cast, and interstitial edema, and abrogates peroxynitrite formation consequently resulting in improved renal function.

It has been previously shown that BH4, a cofactor of all NOS isoforms, is one of the most powerful naturally occurring reducing substances having direct antioxidant effects contributing to its cytoprotective action.[19] Several studies have suggested that the availability of BH4 is essential for the formation and stabilization of NOS. The absence of BH4 results in an uncoupling of the NOS enzyme and subsequently causes superoxide formation instead of NO, thereby contributing substantially to oxidative injury following ischemia reperfusion.[20]

The loss of BH4 is likely the result of oxidative degradation secondary to the formation of oxidants and oxygen-free radicals that are enhanced during kidney ischemia and reperfusion. In particular, the endothelium is considered to generate free radicals during IRI. Furthermore, oxyradical injury alters the redox state of endothelial cells and thus impairs BH4 availability, as the biosynthesis of BH4 depends on a normal cellular redox state.[21] In this study, BH4 concentrations were found to be significantly decreased following 45 min of warm ischemia compared with non-ischemic controls; however, this could be reversed sufficiently by a single dose of exogenous BH4 delivery.

We therefore hypothesized that: (i) BH4 depletion in post-ischemic kidneys may lead to the uncoupling of NOS, increased peroxynitrite ($ONOO^-$) formation, and loss of microcirculatory integrity resulting in impaired renal function and (ii) that BH4 supplementation would be effective in restoring these alterations. To examine precisely the role of BH4 in post-ischemic kidney microcirculation, studies were performed in a rat kidney ischemia–reperfusion model using the renal pedicle clamp approach. Clinical effects in terms of improved renal function were additionally assessed in a rat kidney transplant model.

One of the hallmarks of ARF and delayed graft function are ischemia–reperfusion-related disturbances in microcirculation with subsequent endothelial dysfunction, enhanced leukocyte–endothelial interaction, and hypoxic tissue damage.[22,23] Certainly, endothelial injury and peritubular capillary dysfunction may initiate and extend the pathogenesis of ARF, and also contribute to ischemic forms of ARF by compromising renal vascular responsiveness and tubule function.[24] In addition, localized alterations in kidney blood flow persist after ischemic injury and have a major role in the extension of ischemic injury following reperfusion. Congestion in renal microcirculation, especially in the capillaries of the outer medullary vasa recta, contributes to deficits in oxygen and substrate delivery.[3,25] As microcirculatory dysfunction is a common feature of various forms of renal injury, IVM has become the preferred and unique mode of documenting and directly monitoring changes in peritubular capillary flow following renal ischemia.[26]

Microcirculation as assessed by IVM in our study was significantly impaired in post-ischemic kidneys reflected by persistently reduced FCD and CD. This impairment might occur secondary to a loss of NO production from NOS with a shift of the enzyme toward the generation of O_2^- instead of NO. This shift may be triggered by the observed near-total depletion of BH4 during ischemia.[27] Exogenous BH4 treatment probably restores NO production from NOS and

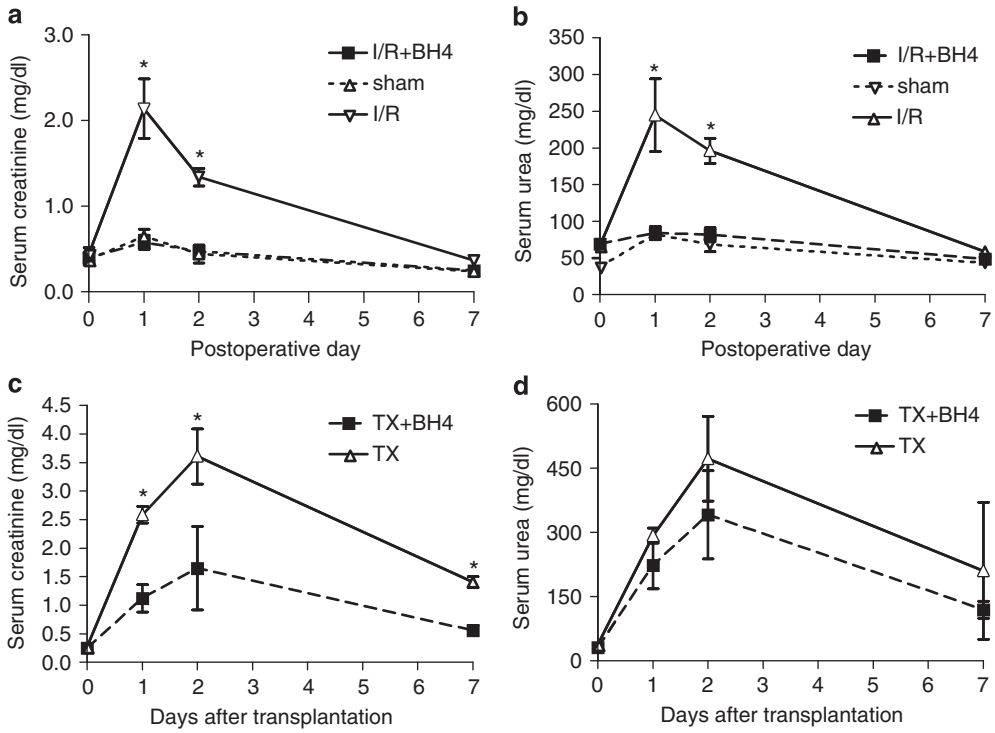

Figure 5 | Serum creatinine and urea. (**a**) Serum creatinine and (**b**) urea levels of sham-operated rats (sham) or rats subjected to renal ischemia either treated with BH4 (I/R + BH4) or untreated (I/R). BH4 treatment significantly improved renal function during the first 2 postoperative days. Data presented as mean ± s.e.m. (*$P < 0.01$; $n = 6$). (**c**) Serum creatinine and (**d**) urea levels of transplanted rats either treated with BH4 (TX + BH4) or untreated (TX). Data presented as means ± s.e.m. (*$P < 0.05$; $n = 6$).

therefore enhanced the post-ischemic recovery of kidneys. Thus, it is reasonable to speculate that BH4 levels are a key factor modulating NOS activity, NO production, peroxynitrite generation, and microcirculation in post-ischemic kidneys.

Indeed, in this study, BH4 supplementation not only attenuated microcirculatory damage but also significantly decreased peroxynitrite formation, as reflected by diminished nitrotyrosine staining scores.

Peroxynitrite is generated by the rapid interaction of superoxide anions with NO and has been associated with various deleterious effects on both cellular and tissue function including increased oxidative reactions, lipid peroxidation, and reduction of plasma antioxidants.[28,29] Nitration of protein tyrosine residues leads to the formation of 3-nitrotyrosine, and thus may be considered as an indirect marker of peroxynitrite-dependent oxidative damage.[30] The generation of oxygen-free radicals is increased during reperfusion, depending on the severity and duration of the preceding ischemic period, and subsequently might also increase peroxynitrite formation.[31] This is in accord with recent data showing that reduced peritubular capillary perfusion may contribute to the generation of tubular epithelial oxidant generation. Wu and Mayeux[24] showed that both reactive oxygen species[32] and reactive nitrogen species were significantly increased in the tubular epithelium following a decrease in capillary perfusion resulting in a microenvironment that promotes oxidant generation and tubular injury. Also in line with this suggestion, 45 min of warm ischemia in this study revealed a significant increase in peroxynitrite formation and hence nitrotyrosine staining compared with non-ischemic controls, which significantly correlated with microcirculatory defects. Thus, our results showing decreased nitrotyrosine immunostaining owing to BH4 supplementation indicate that BH4 prevents the tyrosine-nitrating properties of peroxynitrite following IRI, leading to overall reduced injury. Therefore, BH4 may be considered to be protective against manifestations of oxidative and nitrosative stress in this experimental model of renal IRI.

In this regard, apart from the protective effects on microcirculation and peroxynitrite formation, BH4 supplementation also significantly attenuated histopathologic tubular cell damage and improved kidney function as reflected by attenuated creatinine and urea levels during the early period after ischemia and reperfusion. Our data are also in line with previous findings by Kakoki *et al.*[33] who were the first to show, using a similar model of ischemic ARF and treatment regimen, that a single oral dose of BH4 administered before ischemia markedly improved acetylcholine-induced vasorelaxation and NO release in the isolated perfused kidney. In addition, exogenous supplementation of BH4 in their study restored calcium-dependent NOS activity, immunoreactivity of endothelial NOS dimers, renal excretory function, and histomorphologic changes.[33] Recent evidence in the literature further supports the concept that BH4

treatment has favorable actions in animals with renal disease.[34] In addition, significantly improved graft function after renal transplantation in BH4-treated recipients, as shown in our study, further substantiates the clinical potential of BH4 supplementation to minimize IRI.

Several human studies have also assessed the beneficial effects of BH4 on endothelial dysfunction. It has been shown that BH4 supplementation improves and corrects endothelium-dependent vasodilatation in patients with coronary artery disease,[35] type II diabetes mellitus,[36] hypercholesterinemia,[37] hypertension and in smokers.[38] Furthermore, BH4 prevents kidneys from IRI in human form.[39] BH4 can thereby restore the proper redox state in vascular cells by modulating the relative expression of NOS leading to the proposal that a deficiency in BH4 and defective NOS catalysis underlies endothelial dysfunction in these states. However, although the models and protocols are different in some situations, the protective effects of BH4 could be mediated by the direct improvement of NOS catalysis, possibly by the restoration of BH4 deficiency, whereas in others they might be mediated by its direct antioxidant effect. Further work will be required to completely understand which of the two actions of BH4 is more important in ischemia-induced ARF.

In summary, our data suggest that exogenous BH4 treatment is able to protect kidneys from IRI, by significantly decreasing microvascular changes, histological damage, and peroxynitrite formation and improving renal function parameters. Therefore, BH4 might provide a novel and potent therapeutic option to prevent delayed graft function following kidney transplantation as well as ARF after major surgery or trauma. The clinical availability of BH4 might further facilitate rapid clinical translation of the present findings.

MATERIALS AND METHODS

Animal experiments – renal ischemia/reperfusion

Male inbred Lewis rats with a body weight of 200–250 g were obtained from Harlan Winkelmann (Borchen, Germany) and used for ischemia–reperfusion and transplant experiments. All animals received human care in compliance with the 'Principles of Laboratory Animal Care' formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication no. 86-23, revised 1985). All experiments were approved by the Austrian Ministry of Education, Science and Culture (BMBWK-66011/0067-II/10b/2008). As described in detail previously, after right-sided nephrectomy, rats were subjected to left renal occlusion for 45 min using artery clips to clamp the renal artery. Reperfusion commenced once the arterial clips were removed. Occlusion was verified visually by a change in the color of the kidneys to a paler shade and reperfusion by a blush. At the end of all experiments, animals were killed by an overdose of anesthetics.

Renal transplantation

Orthotopic renal transplantation in male inbred Lewis rats was performed as previously described.[40] Briefly, after bilateral nephrectomy, a syngeneic kidney is transplanted in the orthotopic position. The University of Wisconsin solution was used for cold (1–4°C) preservation and storage (120 ± 30 min). Revascularization was performed within 20 min (± 3 min) using a modified non-suture cuff technique.

Experimental groups

To evaluate the effect of BH4 on IRI and ischemic ARF following transplantation, respectively, animals were divided into five groups. Animals in the first group received 20 mg/kg per body weight of BH4 intramuscularly, 15 min before renal artery clamping (I/R + BH4), whereas those in the second group were given an equal volume of 0.9% normal saline (I/R). Sham-operated animals served as controls (sham). In the transplanted group, both donor and recipient received 20 mg/kg per body weight of BH4 intramuscularly, 15 min before organ recovery and revascularization (Tx + BH4). Saline-treated recipients served as controls (Tx).

Intravital fluorescence microscopy

IVM was used to analyze kidney microcirculation by means of FCD and CDs after 2-h of reperfusion. FCD was defined as the cumulative length of all blood cell perfused nutritive capillaries per observation area and CD as the largest distance between two opposite capillary walls per observation area. FCD and CD are expressed as cm/cm^2 (cm^{-1}).[27,41] IVM was performed using an inverted IX-70 microscope (Olympus, Nagano, Japan). To enhance the contrast of the microvessels, 0.3 ml of a 0.4% fluorescein-isothiocyanate-labeled dextran (molecular weight 150,000 (Da); 50 lg/kg per body weight; SIGMA, Deisenhofen, Germany) was injected through the penile vein. Confocal microscopy was performed with a microlens-enhanced Nipkow disk-based system UlraVIEW RS software (Perkin-Elmer GmbH, Vienna, Austria). Selection of observation areas as well as image acquisition was blinded. Images consisted of a z-stack of 20 planes acquired with a × 40 objective at a wavelength of 488 nm. For each rat, five videos of 10 s each were captured at ~12 frames per second to analyze and determine perfusion in the vessels. From each video, one randomly selected picture displaying a minimum of three vessels was analyzed in terms of FCD and CD as described above. Quantitative image analysis was performed with PicEd Cora (JOMESA, Munich, Germany) software.

Histological assessment of renal injury

For histology, tissue samples were fixed in 10% formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin. A semiquantitative histologic analysis was performed. Twenty tubules or glomeruli in each kidney were randomly selected at × 400 magnification, and the degree of renal damage was scored using the renal injury scoring system reported by Solez *et al.*[18] We calculated the mean renal injury score in each rat and then averaged the scores for each group. All sections were examined by a single pathologist (PO) in a blinded manner. Tissue samples were examined for the presence of expansion of Bowman's space, interstitial edema, epithelial detachment, and tubular cells casts. Renal morphologic changes were graded on a scale of 0 to 3+: 0, normal; 1+, slight; 2+, moderate; and 3+, severe.

Immunohistochemistry

Tissue sections (5 μm) were cut from paraffin blocks, mounted on slips, and the paraffin was removed by heating in citrate buffer, pH 6.0. Endogenous peroxidase was blocked with hydrogen

peroxide 0.3%. Immunohistochemistry was then performed in a diaminobenzidine tetrahydrochloride (DAKO) autostainer (DAKO, Copenhagen, Denmark), using an antinitrotyrosine rat polyclonal antibody from Upstate Biotechnology (Lake Placid, NY, USA) at a dilution of 1:100. For staining, secondary antibody peroxidase-labeled polymer and 3,3′ DAKO were used. Hemalaun was used for counterstaining. For quantification, the product of the proportion of positive cells in quartiles (0,1,2,3,4) and the staining intensity (0-no staining; 1-weak; 2-moderate; 3-strong) was calculated, yielding a total immunostaining score ranging from 0 to 12.

BH4 tissue levels

To determine BH4 concentrations, kidney samples were homogenized on ice with a microblender in distilled water containing 5 mM dithioerythrol, centrifuged at 12,000 *g* at 4°C for 10 min, and then subjected to oxidation in acid or base by a method modified from Fukushima and Nixon.[43] To 100 µL supernatant, 20 µL containing 0.5 mol/l HCl and 0.05 mol/l iodine were added for acidic or 20 µL 0.5 mol/l NaOH plus 0.05 mol/l iodine for basic oxidation. After incubation for 1 h in the dark at room temperature, 20 µL HCl was added to the basic oxidation only, and all mixtures received 20 µL 0.1 mol/l ascorbic acid for the reduction of excess iodine. Samples were then centrifuged for 10 min at 12,000 *g* and 4%C. Biopterin concentrations were determined by high-performance liquid chromatography using 10-µL injection volume, a Nucleosil 10SA column (250 mm long, 4 mm i.d., Macherey Nagl, Düren, Germany), eluted with 1.5 ml/min 50 mM potassium phosphate buffer, pH3.0, and fluorescence detection (excitation 350 nm, emission 440 nm). BH4 concentrations were calculated as the difference in results from oxidation in acid and base, respectively.

Serum creatinine and urea levels

Before ischemia as well as at serial time points after reperfusion/transplantation (24, 48 h, and 7 days), blood samples (0.5 ml) were collected from the retrobulbar venous plexus. Samples were centrifuged (390 *g* for 5 min) and plasma concentrations of urea (U) and creatinine[42] were measured using a Hitachi automatic analyzer (Boehringer Mannheim, Mannheim, Germany).

Statistical analyses

Results are expressed as mean ± s.e.m. Statistical analysis was performed with Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). When two groups were compared, a two-tailed Student's *t*-test was used. Spearman's rank coefficient correlation test was used to examine the relationship between FCD and nitrotyrosine immunostaining. A *P*-value of <0.05 was considered to be of statistical significance.

DISCLOSURE

All the authors declared no competing interests.

SUPPLEMENTARY MATERIAL

Movie S1. IVM following 45 min ischemia and 120 min reperfusion.
Movie S2. IVM non-ischemic baseline control.
Movie S3. IVM following 45 min ischemia and 120 min reperfusion + BH4 supplementation.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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