Effects of Tetrahydrobiopterin on Endothelial Dysfunction in Rats with Ischemic Acute Renal Failure

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Abstract. The role of nitric oxide (NO) in ischemic renal injury is still controversial. NO release was measured in rats kidneys subjected to ischemia and reperfusion to determine whether (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4), a cofactor of NO synthase (NOS), reduces ischemic injury. Twenty-four hours after bilateral renal arterial clamp for 45 min, acetylcholine-induced vasorelaxation and NO release were reduced and renal excretory function was impaired in Wistar rats. Administration of BH4 (20 mg/kg, by mouth) before clamping resulted in a marked improvement of those parameters (10–8 M acetylcholine, ∆renal perfusion pressure: sham-operated control −45 ± 5, ischemia −30 ± 2, ischemia + BH4 −43 ± 4%; ∆NO: control +30 ± 6, ischemia +10 ± 2, ischemia + BH4 +23 ± 4 fmol/min per g kidney; serum creatinine: control 23 ± 2, ischemia 150 ± 27, ischemia + BH4 48 ± 6 μM; mean ± SEM). Most of renal NOS activity was calcium-dependent, and its activity decreased in the ischemic kidney. However, it was restored by BH4 (control 5.0 ± 0.9, ischemia 2.2 ± 0.4, ischemia + BH4 4.3 ± 1.2 pmol/min per mg protein). Immunoblot after low-temperature sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the dimeric form of endothelial NOS decreased in the ischemic kidney and that it was restored by BH4. These results suggest that the decreased activity of endothelium-derived NO may worsen the ischemic tissue injury, in which depletion of BH4 may be involved.

Nitric oxide (NO) participates in the physiologic regulation of cardiovascular and renal functions. In this respect, NO has been suggested to play a pivotal role in ischemic acute renal failure (iARF). However, there have been conflicting reports on the amount of NO itself and its origins. Chintala et al. (1) reported that NO synthase (NOS) inhibition by Nω-monomethyl-L-arginine worsened renal ischemic injury, whereas L-arginine mitigated it, suggesting that a reduction in NO release may be involved in ischemia-induced tissue damage. Conger et al. (2) observed maximal release of endothelium-derived NO in postischemic rat kidneys because endothelium-dependent renal vasodilation was reduced and renal vessels constricted to a greater extent by Nω-nitro-L-arginine methyl ester (L-NAME), but not by aminoguanidine, an antagonist of inducible NOS (iNOS). On the other hand, Noiri et al. (3) showed that iNOS-derived NO increased in ischemic kidneys and that inhibition of iNOS expression decreased the tubular damage.

Endothelium-derived NO, which causes vasodilation, reduces intracellular calcium, and inhibits leukocyte adhesion to vessels (4) and platelet aggregation (5), probably exerts a protective action on ischemia-induced tissue damage. On the other hand, NO derived from iNOS has been considered to enhance the tissue damage observed in ischemia-reperfusion injury, partly via the formation of peroxynitrite anion, an NO-derived oxidant formed from the interaction of NO with superoxide (6–8). However, administration of exogenous NO donors has been shown to be unequivocally beneficial against ischemia-reperfusion insults (9,10).

It has been demonstrated that (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4), a cofactor of all isoforms of NOS, is a rate-limiting factor of NO production by iNOS (11). Several cytokines induce not only iNOS but also guanosine triphosphate cyclohydrolase-1, which is the rate-limiting enzyme for de novo synthesis of BH4 (12), and it may lead to an enhanced production of NO in sepsis (13). It has also been shown that BH4 is essential for NO production by endothelial NOS (eNOS) (14,15) and that in patients with hypercholesterolemia (16), the administration of BH4 improves endothelial dysfunction.

Thus, in the present study we investigated whether NO release increased or decreased, and examined the origin of NO in rats with iARF. Furthermore, we examined whether BH4 exerts beneficial effects on ischemic injury in the rat kidney.
Materials and Methods

Animals

The in vivo model of renal ischemia was prepared in 12-wk-old Wistar rats weighing 313 ± 10 g by clamping both renal arteries for 45 min; then clamps were removed and the rats developed ischemic renal failure 24 h later. The NOS substrate L-arginine (1 g/kg), the NOS cofactor BH4 (5 or 20 mg/kg; Allexis, San Diego, CA), 2,4-diamino-6-hydroxypyrimidine (DAHP; 1 g/kg), which is an inhibitor of guanosine 1,4,5-triphosphate cyclohydrolase-I, and the NOS inhibitor L-NAME (30 mg/kg) was administered by gavage to some of the rats just before anesthesia with pentobarbital, i.e., 5 min before bilateral renal arterial clamping. The effects of BH4 (20 mg/kg) when administered 1 h after reperfusion were also examined. The doses of BH4 and DAHP were determined as described in previous reports (13,17). All animal studies were performed in conformance with the Guide for Animal Experimentation (Faculty of Medicine, The University of Tokyo).

Measurement of Biopterin Levels in Plasma and Renal Tissues in Rats with iARF

To confirm that orally administered BH4 reached the kidneys of rats with iARF, we measured the levels of biopterin in the plasma and kidneys after administration of BH4 or vehicle by gavage to rats subjected to 45 min ischemia and 3 h reperfusion, by differential iodine oxidation in acid and base, using reversed-phase high performance liquid chromatography (18–20).

Isolated Perfused Kidney and Measurement of NO

The kidneys of rats with iARF were isolated and perfused as described elsewhere to evaluate renovascular endothelial function (21,22). The kidneys were perfused with Krebs-Henseleit buffer at 37°C and saturated with 95% O2/5% CO2, which contained 10−6 M phenylephrine and 10−5 M indomethacin, at a constant flow of 5 ml/min. Phenylephrine at 10−6 M was used for the precontraction of renal vasculature to efficiently examine the effects of acetylcholine (ACH) on the change in renal perfusion pressure (RPP). This dose was selected to maintain the RPP at a level similar to the in vivo RPP. Indomethacin was added to exclude the effects of prostanooids on RPP because previous studies have suggested that the effect of ACH on the tone of vasculature is partly mediated by prostanooids (23,24). Renal arterial pressure was continuously monitored with a polygraph recorder and maintained at about 100 mmHg. After a 60-min equilibration period, we investigated the effects of the vehicle, cumulative doses of ACH, and Nω-nitro-L-arginine (L-NNA) on RPP and NO release. The effluent perfusate from the renal vein was collected continuously (2 ml/min), mixed with a chemiluminescence probe (0.5 ml/min; 2 mM H2O2, 18 μM luminol, 2 mM potassium carbonate, and 150 mM desferrioxamine), and then forwarded into a chemiluminescence analyzer (825-CL; Jasco Corp., Tokyo, Japan) to measure the NO output (25).

Histologic Studies

Samples of renal tissue from the control, ischemic, and BH4-treated (20 mg/kg, orally, before ischemia) animals were fixed in 10% formaldehyde, stained with periodic acid-Schiff reagent, and examined under an optical microscope in a blinded manner by a pathologist (A.T.). The kidneys were examined for the presence of tubular dilation, necrosis, casts, interstitial edema, and brush border loss. The degree of renal injury was evaluated using the criteria established by Solecz et al. (26).

Measurement of NOS Activity

The activity of NOS in vitro was determined by the conversion of L-[14C] arginine to L-[14C] citrulline, according to the method described by Breit and Snyder (27). The renal medulla was dissected after sagittal section of the left kidney in ice-cold phosphate-buffered saline and then frozen in liquid nitrogen. Samples were kept at −70°C until assayed. Frozen samples were homogenized using a Polytron homogenizer in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM mercaptoethanol, 0.1 mM ethylenediaminetetra-acetic acid, 0.1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N′-tetra-acetic acid [EGTA], 2 μM leupetin, 1 μM pepstatin A, 1 mM phenylmethysulfonyl fluoride). Forty microliters of the sample was incubated in duplicate in 100 μl of assay buffer containing 0.5 μCi/ml L-[14C] arginine and incubated for 20 min at 37°C. The reaction was stopped with the addition of 500 μl of ice-cold buffer (20 mM Hepes, 2 mM ethylenediaminetetra-acetic acid, 2 mM EGTA, pH 5.5). To separate L-[14C] arginine from L-[14C] citrulline, samples were loaded onto 1-ml columns of Dowex resin (AG50WX-8 Na+ form) and eluted with 500 μl of distilled water. Aliquots were used for liquid scintillation counting. Calcium-dependent activity was determined as the difference between the L-[14C] citrulline produced in control samples and samples containing 3 mM EGTA to bind calcium. Calcium-independent activity was determined as the difference between the L-[14C] citrulline produced from samples containing 3 mM EGTA and samples containing both 3 mM EGTA and 1 mM L-NNa. Activity of the enzyme was normalized by protein content determined by the Bradford method (Bio-Rad, Richmond, CA), using bovine serum albumin as the standard.

Immunoblot Analysis for NOS Protein

Each kidney was excised and homogenized on ice in 3 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 10% glycerol, 2 μg/ml leupetin, 2 μg/ml aprotinin, 1 mM phenylmethysulfonyl fluoride), using a Polytron homogenizer. The homogenates were centrifuged at 12,000 × g at 4°C for 20 min. After determining the protein content, appropriate volumes of the supernatant (200 μg/lane) were mixed with an equal volume of 2× sample buffer (100 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate [SDS], 20% glycerol, 10% 2-mercaptoethanol, 0.02% bromphenol blue) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using 6% acrylamide gels. For immunoblot analysis of monomer of eNOS and iNOS, samples were heated at 98°C for 5 min before electrophoresis (28). For immunoblot analysis of the dimeric form of eNOS, samples were not heated and the temperature of the gel was maintained below 15°C during electrophoresis (low temperature SDS-PAGE [LT-PAGE]) (29). The proteins were transferred by semidyed electroblotting to polyvinylidene difluoride membranes for 120 min. The blots were then blocked and incubated with either anti-eNOS antibody (IgG1, monoclonal antibody; 1:100 dilution; Transduction Laboratories, Lexington, KY) or anti-iNOS antibody (IgG2a, monoclonal antibody, 1:500 dilution; Transduction Laboratories) for 60 min at room temperature. To detect bound antibodies, the blots were incubated with horseradish peroxidase-linked rabbit anti-mouse second antibody (IgG1 polyclonal antibody; 1:7000 dilution; Transduction Laboratories, Little Chalfont, United Kingdom) for 60 min at room temperature, and the antibody was visualized using an enhanced chemiluminescence method (ECL; Amersham). The integrated density (density × area) of the bands was quantified using NIH Image 1.61 software. For the immunoblot analysis of iNOS, we prepared two kinds of positive controls; i.e., mouse macrophages and the kidney of rats treated with lipopolysaccharide (20 mg/kg, intraperitoneally) for 6 h.
Statistical Analyses

Data are expressed as the mean ± SEM. Statistical comparisons were made with ANOVA, followed by the Student-Neumann-Keuls test. To compare tissue damage scores, the Mann–Whitney U test was used. Differences with a P value < 0.05 were considered statistically significant.

Results

Biopterin Levels in Plasma and Renal Tissues in Rats with iARF

As shown in Figure 1, the levels of biopterin in the plasma and kidneys of rats with iARF increased in a dose-dependent manner after oral administration of BH4, which confirmed that orally administered BH4 reached the kidneys of rats with iARF during the reperfusion period.

Effects of Ischemia on RPP and NO in Wistar Rats

Figures 2 and 3 illustrate the changes in RPP and NO in response to ACh and L-NNA in the isolated perfused kidney after 45 min ischemia and 24 h reperfusion. As shown in Table 1, the RPP in the ischemic group was slightly lower. However, the differences in the achieved RPP values among groups were not significant when assessed by one-way ANOVA. In the control, sham-operated rat kidney, ACh increased NO release and reduced RPP in a dose-dependent manner; the effects of ACh were antagonized by L-NNA. On the other hand, the changes in RPP and NO induced by ACh or L-NNA were significantly attenuated in ischemic kidneys. Pretreatment with BH4 potentiated the effects of ACh on RPP and NO release in a dose-dependent manner. In particular, 20 mg/kg BH4 restored the parameters of endothelial function to levels similar to those of the control kidney. Administration of BH4 1 h after reoxygenation also significantly restored renal responses to ACh, although the extent of the improvement was smaller. However, pretreatment with BH4 did not influence the responses to ACh in sham-operated controls. Pretreatment with L-arginine did not affect the responses to ACh in ischemic kidneys.

These effects of BH4 were confirmed by changes in serum urea nitrogen and creatinine levels. As shown in Figure 4, the level of urea nitrogen in rats with iARF was much higher than in the control rats (35 ± 2 versus 8 ± 1 mM, P < 0.01).
However, administration of BH4 prevented the increase in urea nitrogen substantially (16 ± 2 mM). Similar results were obtained in the case of serum creatinine levels. In contrast, L-arginine did not affect urea nitrogen or creatinine in iARF.

As shown in Figure 5, DAHP, an inhibitor for de novo synthesis of BH4, attenuated the effects of ACh on RPP and NO release in the sham-operated kidney. However, DAHP did not affect the responses to ACh in iARF. The NOS inhibitor L-NAME diminished the responses to ACh in both the sham-operated kidney and in iARF. This effect of L-NAME was associated with further increases in urea nitrogen and creatinine in iARF (Figures 4 and 5).

Histopathologic Analysis of Kidneys with Ischemia-Reperfusion Injury

Ischemia-induced morphologic changes, including tubular necrosis, tubular casts, interstitial edema, tubular dilatation, and brush border loss, were clearly observed in the ischemic kidney of Wistar rats. BH4 significantly reduced the scores for the renal tissue damage (Figure 6).

Effects of Ischemia on NOS Activity and Expression of eNOS and iNOS

In Wistar rats, calcium-dependent NOS activity, which was measured based on the conversion of L-arginine to L-citrulline, significantly decreased in the medulla of the kidney in iARF, and it was partially reversed by treatment with BH4 (Figure 7). On the other hand, calcium-independent NOS activity was not detected in the kidney of any group of animals.

The expression of eNOS and iNOS protein in iARF was evaluated in the whole kidney of Wistar rats. In conventional immunoblot analysis (Figure 8A), the immunoreactivity of total eNOS protein significantly increased in iARF, and it was not significantly affected by BH4 (n = 8 each; iARF 196 ± 26; iARF + BH4 250 ± 48% of optical densities in the control; both P < 0.05 versus control). However, in immunoblot analysis after LT-PAGE (Figure 8B), the immunoreactivity of the eNOS dimer significantly decreased in iARF, which was restored by BH4 (n = 6 each; iARF 64 ± 9; P < 0.01 versus sham-operated control; iARF + BH4 124 ± 20% of optical densities in the control; P < 0.05 versus iARF). iNOS was not

*P < 0.05, ‡P < 0.01 versus sham-operated control; §P < 0.05, iP, 0.01 versus ischemia. Each group consists of five or six rats.

Figure 3. Effects of ACh and L-NNa on RPP and NO in Wistar rats with iARF. pre 5 and pre 20, rats administered 5 and 20 mg/kg BH4 orally before ischemia, respectively; post 20, rats administered 20 mg/kg BH4 by gavage 1 h after reoxygenation; *P < 0.05, ‡P < 0.01 versus sham-operated control; §P < 0.05, iP, 0.01 versus ischemia. Each group consists of five or six rats.
detected in any group of rats, whereas it was clearly detected in mouse macrophages and in the kidneys from lipopolysaccharide-treated rats (Figure 8C).

**Discussion**

In the present study, we found that in the isolated rat kidney exposed to ischemia-reperfusion, vasorelaxation and NO release in response to ACh were markedly decreased, and vasoconstriction in response to L-NNA was attenuated. Calcium-dependent NOS activity was also attenuated. Furthermore, the activity and expression of iNOS were very little in the kidney with iARF. These results indicate that basal and stimulated NO release from eNOS is reduced in the ischemic kidneys, although there have been conflicting reports about the amount of NO and the isoform of NOS responsible for NO in ischemia. It is striking that preischemic administration of BH4, a

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**Table 1.** Values of body weight (BW), kidney weight (KW), baseline renal perfusion pressure (RPP), and renal perfusion rate (RPR) in all groups of rats

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BW (g)</th>
<th>KW (g)</th>
<th>RPP (mmHg)</th>
<th>RPR (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>5</td>
<td>313 ± 10</td>
<td>1.36 ± 0.07</td>
<td>107 ± 5</td>
<td>5.0</td>
</tr>
<tr>
<td>Sham + BH₄</td>
<td>5</td>
<td>339 ± 14</td>
<td>1.45 ± 0.01</td>
<td>97 ± 2</td>
<td>5.0</td>
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<tr>
<td>Sham + DAHP</td>
<td>4</td>
<td>333 ± 10</td>
<td>1.45 ± 0.09</td>
<td>97 ± 4</td>
<td>5.0</td>
</tr>
<tr>
<td>Sham + L-NAME</td>
<td>6</td>
<td>302 ± 11</td>
<td>1.37 ± 0.06</td>
<td>95 ± 5</td>
<td>5.0</td>
</tr>
<tr>
<td>Ischemia</td>
<td>5</td>
<td>309 ± 9</td>
<td>1.36 ± 0.08</td>
<td>87 ± 2</td>
<td>5.0</td>
</tr>
<tr>
<td>Ischemia + BH₄ (pre; 5)</td>
<td>5</td>
<td>332 ± 10</td>
<td>1.36 ± 0.04</td>
<td>88 ± 2</td>
<td>5.0</td>
</tr>
<tr>
<td>Ischemia + BH₄ (pre; 20)</td>
<td>5</td>
<td>302 ± 7</td>
<td>1.20 ± 0.03</td>
<td>99 ± 3</td>
<td>5.0</td>
</tr>
<tr>
<td>Ischemia + BH₄ (post; 20)</td>
<td>6</td>
<td>322 ± 7</td>
<td>1.43 ± 0.03</td>
<td>95 ± 5</td>
<td>5.0</td>
</tr>
<tr>
<td>Ischemia + DAHP</td>
<td>5</td>
<td>313 ± 5</td>
<td>1.36 ± 0.03</td>
<td>85 ± 6</td>
<td>5.0</td>
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<tr>
<td>Ischemia + L-NAME</td>
<td>5</td>
<td>288 ± 12</td>
<td>1.57 ± 0.08</td>
<td>90 ± 6</td>
<td>5.0</td>
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<tr>
<td>Ischemia + L-arginine</td>
<td>5</td>
<td>319 ± 16</td>
<td>1.53 ± 0.07</td>
<td>97 ± 2</td>
<td>5.0</td>
</tr>
</tbody>
</table>

*Values are expressed as mean ± SEM. Sham, sham-operated rats; BH₄, tetrahydrobiopterin; DAHP, 2,4-diamino-6-hydroxypterimidine; L-NAME, N⁷-nitro-L-arginine methyl ester; (pre; 5 or 20), oral administration of 5 or 20 mg/kg before ischemia; (post; 20), oral administration of 20 mg/kg after ischemia.*

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**Figure 4.** Values of serum urea nitrogen and creatinine in rats with iARF. L-NAME, N⁷-nitro-L-arginine methyl ester. *P < 0.05, †P < 0.01 versus sham-operated control; ‡P < 0.05, §P < 0.01 versus ischemia. Each group consists of five or six rats.
cofactor of NOS, dose-dependently restored not only endothelium-dependent vasorelaxation, but also renal excretory function and morphologic changes in iARF. In contrast, administration of L-NAME resulted in a deterioration of the endothelial function and renal excretory function under ischemic conditions. These findings suggest that the reduction in endothelium-derived NO is an important factor that accelerates renal damage in iARF.

The beneficial effect of BH4 on endothelial function was also significant when given 1 h after reperfusion, in contrast to a reported finding that preischemic administration of L-arginine, a NOS substrate, had a beneficial effect, whereas its postischemic administration was harmful (30). On the other hand, BH4 had little effect on the endothelial function in nonischemic controls. These results are in accord with the idea that vessels exposed to ischemia-reperfusion are depleted from BH4 (31). It has been suggested that BH4 has a direct antioxidant effect and reduces reactive oxygen species (32), which would also contribute to its cytoprotective action in ischemic injury. However, it has also been shown that BH4 generates superoxide during its autooxidation (33,34) and exerts a suppressive effect on endothelium-dependent vasodilation under normal conditions (35). Therefore, the content of BH4 as a cofactor of NOS is intact in normal vessels, and the supplementation of BH4 does not have additional effects.

However, the fact that administration of DAHP attenuated endothelial function under nonischemic conditions suggested that production of BH4 was important for the maintenance of endothelium-dependent relaxation, as previous studies have already demonstrated (31,36). Furthermore, DAHP did not affect the changes in RPP and NO release in response to ACh under ischemic conditions, indicating that de novo production of BH4 might be impaired in iARF. In contrast, administration of L-NAME resulted in a deterioration of the endothelial function under both nonischemic and ischemic conditions to a similar extent. Thus, the induction of endogenous NOS inhibitors (37,38) is not the main cause for endothelial dysfunction after ischemia.

The regulation of eNOS expression in hypoxia remains controversial. Several studies have shown that hypoxia decreases eNOS expression in human umbilical vein endothelial cells (39), in human pulmonary artery endothelial cells (40), and in bovine pulmonary artery endothelial cells (41). On the other hand, it has been reported that hypoxia increases eNOS protein in bovine aortic endothelial cells (42), hypoxic rat lungs (43,44), and hypoxic rat systemic organs (45). The discrepancies in the regulation of eNOS expression among
these studies may be due to differences in the experimental preparation or the duration and severity of hypoxia.

In the present study, conventional immunoblot analysis revealed that total eNOS expression was increased in iARF, which was unaltered by the supplementation of BH4. In contrast, LT-PAGE revealed that the immunoreactivity for dimeric eNOS was decreased in iARF, which was restored by the supplementation of BH4. BH4 has been shown to stabilize eNOS in its dimeric form, which is an active form (46,47). Therefore, BH4 may determine the effective amount of eNOS regardless of the total quantity of eNOS protein. These results suggest that the impairment of dimerization of eNOS by depletion in BH4 plays an important role in endothelial dysfunction in iARF. It has also been demonstrated that L-arginine facilitates eNOS dimerization (29). However, administration of L-arginine did not improve NO release in ischemic kidneys, suggesting that L-arginine is not depleted in iARF.

In view of the evidence provided by previous studies concerning the role iNOS plays in the pathogenesis of iARF, the present results seem to be inconsistent with them. Because BH4 has been shown to be a rate-limiting factor of the activity of iNOS (11), supplementation of BH4 would aggravate tissue damage. However, at least in the present preparation, BH4 restored endothelium-dependent relaxation, renal excretory function, and renal morphology. Moreover, iNOS protein and calcium-independent NOS activity were very scant in iARF and in BH4-treated iARF rats. It has been suggested that iNOS is induced in hypoxia and that hypoxia-responsive elements exist in the promoter region of mouse iNOS gene (48). However, some authors have shown that hypoxia fails to induce iNOS in rat cultured cardiac myocytes (49); rat cultured mesangial cells (50); rat liver, ventricles, lungs, and kidneys (45); and rat splanchic artery (51). Additional studies are required to elucidate the role of iNOS in iARF.

In conclusion, endothelium-dependent vasorelaxation and NO release were impaired in rat kidneys with iARF. The endothelial dysfunction in iARF was associated with decreased NOS activity and decreased immunoreactivity of eNOS dimers. The expression of iNOS and calcium-independent NOS activity was very little in iARF. Exogenous supplementation of BH4 markedly restored endothelial function, renal morphology, renal excretory function, NOS activity, and immunoreactivity of eNOS dimers. These results suggest that the impairment of dimerization of eNOS by depletion in BH4 plays an important role in endothelial dysfunction and tissue damage in iARF.

Acknowledgments

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