(6R)-5,6,7,8-Tetrahydro-L-Biopterin and Its Stereoisomer Prevent Ischemia Reperfusion Injury in Human Forearm

Lila Mayahi, Simon Heales, David Owen, Juan P. Casas, Joanne Harris, Raymond J. MacAllister, Aroon D. Hingorani

Objective—6R-5,6,7,8-tetrahydro-L-biopterin (6R-BH4) is a cofactor for endothelial nitric oxide synthase but also has antioxidant properties. Its stereo-isomer 6S-5,6,7,8-tetrahydro-L-biopterin (6S-BH4) and structurally similar pterin 6R,S-5,6,7,8-tetrahydro-D-neopterin (NH4) are also antioxidants but have no cofactor function. When endothelial nitric oxide synthase is 6R-BH4–deplete, it synthesizes superoxide rather than nitric oxide. Reduced nitric oxide bioavailability by interaction with reactive oxygen species is implicated in endothelial dysfunction (ED). 6R-BH4 corrects ED in animal models of ischemia reperfusion injury (IRI) and in patients with cardiovascular risks. It is uncertain whether the effect of exogenous 6R-BH4 on ED is through its cofactor or antioxidant action.

Methods and Results—In healthy volunteers, forearm blood flow was measured by venous occlusion plethysmography during intra-arterial infusion of the endothelium-dependent vasodilator acetylcholine, or the endothelium-independent vasodilator glyceryl trinitrate, before and after IRI. IRI reduced plasma total antioxidant status (P<0.03) and impaired vasodilatation to acetylcholine (P<0.01), but not to glyceryl trinitrate (P=0.3). Intra-arterial infusion of 6R-BH4, 6S-BH4 and NH4 at approximately equimolar concentrations prevented IRI.

Conclusion—IRI causes ED associated with increased oxidative stress that is prevented by 6R-BH4, 6S-BH4, and NH4, an effect mediated perhaps by an antioxidant rather than cofactor function. Regardless of mechanism, 6R-BH4, 6S-BH4, or NH4 may reduce tissue injury during clinical IRI syndromes. (Arterioscler Thromb Vasc Biol. 2007;27:1334-1339.)

Key Words: 6R-5,6,7,8-tetrahydro-L-biopterin ■ antioxidant ■ endothelial dysfunction ■ ischemia ■ reperfusion

6R-5,6,7,8-tetrahydro-L-biopterin (6R-BH4) is an essential cofactor for endothelial nitric oxide synthase (NOS) that catalyzes production of the vasodilator and atheroprotective mediator NO.1,2 Biosynthesis of 6R-BH4 occurs in the endothelium, promoting NO synthesis by facilitating electron transfer from the reductase domain of NOS to arginine. 6R-BH4 also stabilizes the NOS dimer and exerts an allosteric effect, enhancing substrate binding.3 Changes in 6R-BH4 availability influence NO production. A reduction in 6R-BH4 leads not only to a diminished NO synthesis but also to the generation of NOS-derived superoxide (O2−) that has been implicated in the development of endothelial dysfunction.4

Ischemia reperfusion injury (IRI), such as occurs after thrombolysis or balloon angioplasty, is associated with endothelial dysfunction, which may contribute to cellular damage. The mechanism underlying endothelial dysfunction in IRI is incompletely understood but adhesion of activated neutrophils to endothelial cells,5 an increase in oxygen radical generation,6,7 the elaboration of inflammatory cytokines, and a reduction in NO production8,9 are all believed to play a role.

In humans, local delivery of 6R-BH4 by intra-arterial infusion improves endothelial dependent vasodilation in patients with coronary artery disease,10 type II diabetes mellitus,11 elevated cholesterol,12 raised blood pressure,13 and in smokers,14 leading to the proposal that an acquired deficiency in 6R-BH4 and defective NOS catalysis underlies endothelial dysfunction in these states. Supplementation with 6R-BH4 also reduces IRI in the rat heart15 and kidney16 and in the pig heart.17 In animals, the administration of antioxidants such as ascorbic acid or N-acetylcysteine reduces endothelial dysfunction induced by IRI.18–20

Therefore, the effect of 6R-BH4 on endothelial function in IRI could also be mediated by an antioxidant action,21 a property shared with its stereoisomer 6S-5,6,7,8-tetrahydro-L-biopterin (6S-BH4),22 as well as with 6R,S-5,6,7,8-tetrahydro-D-neopterin (NH4),14 rather than by correction of defective NOS catalysis. Although approximately equipotent as antioxidants,14,22 only 6R-BH4 has substantial capacity to support NOS catalysis.23,24

We therefore examined, for the first time, the effect of 6R-BH4 on endothelial dysfunction during IRI in humans and tested whether this was mediated through correction of 6R-BH4 deficiency and defective NOS catalysis or through an antioxidant action.
Materials and Methods
Forty-eight nonsmoking healthy volunteers, 34 males, 14 females, aged 19 to 42, with mean BMI (SD) of 23.4(3.1), blood pressure 127/77 (15/10) mm Hg, heart rate 71 bpm, random glucose 4.8(0.6) mmol/L, and random cholesterol 3.8(1.1) mmol/L, were recruited after written informed consent was obtained. All females were studied during their menstrual period to take into account the variation in endothelial function during the menstrual cycle.\textsuperscript{25} The studies were approved by the UCLH research ethics committee.

Assessment of Flow in Forearm Resistance Arteries
Studies were performed in a temperature-controlled laboratory. Baseline blood pressure and heart rate was recorded. We used venous occlusion plethysmography using mercury-in-silastic strain gauges to measure forearm blood flow in the resistance vessels of both arms as described previously.\textsuperscript{26} Drugs were administered in saline (0.9% sodium chloride) and infused at a rate of 0.5 mL/min through a 27-gauge needle (Cooper’s Needle Works Ltd, Birmingham UK) inserted into the nondominant brachial artery under local anesthesia using 1 to 2 mL of 2% lidocaine. Pterin compounds were prepared immediately before infusion in deoxygenated 0.9% sodium chloride solution. During the recording of forearm blood flow, the hands were excluded from circulation by inflation of the wrist cuffs to 200 mm Hg.

To induce IRI, the nondominant forearm was made ischemic by inflation of the upper arm cuff to 200 mm Hg for 20 minutes followed by reperfusion by deflating the cuff for 15 minutes, as described previously.\textsuperscript{26}

Venous Blood Sampling
Venous plasma was obtained in EDTA tubes from an antecubital vein for measurement of total biopterin, total antioxidant status (TAOS), nitrate and nitrite concentration (NOx), and stored at −80°C until analysis.

TAOS
The TAOS of plasma was determined by its capacity to inhibit the peroxidase-mediated formation of the 2,2-Azino-bis-3-ethylbenothiazoline-6-sulfonic acid (ABTS\textsuperscript{+}) radical.\textsuperscript{27,28} In this assay, the relative inhibition of ABTS\textsuperscript{+} formation in the presence of plasma is proportional to the antioxidant capacity of the sample. Briefly, 2.5 \( \mu \text{L} \) of plasma was incubated for 3 minutes at 37°C in a 96-well plate with a reaction mixture comprising (final concentrations): 20 \( \mu \text{L} \) ABTS (20 mmol/L), 20 \( \mu \text{L} \) horseradish peroxidase (30 mU/mL), and 37.5 \( \mu \text{L} \) phosphate-buffered saline, pH 7.4. The reaction was initiated by the addition of 20 \( \mu \text{L} \) hydrogen peroxide (0.1 mmol/L), and the increase in absorbance over 6 minutes was monitored at 405 nm, using a microplate reader. At 6 minutes, sample absorbance was read together with a control (containing 2.5 \( \mu \text{L} \) phosphate-buffered saline instead of plasma). The proportional difference in absorbance between the control and test samples was used to represent percentage inhibition of the reaction.

Plasma Total Biopterin
Plasma total biopterin concentration was measured using the method of Fukushima and Nixon.\textsuperscript{29} Briefly, reduced pterins were oxidized using 3% iodide solution (2% iodine in 6% KI) after removal of proteins with 60% perchloric acid. Total biopterin was detected by fluorescence set at 360 nm excitation and 440 nm emission after using a high-performance liquid chromatography Spherisorb ODS-1 column with a 5% methanol mobile phase run at a flow rate of 1 mL/min.

Figure 1. Effect of 6R-BH\textsubscript{4} on venous biopterin concentrations (a) and the baseline forearm blood flow (b). Dotted line indicates 6R-BH\textsubscript{4} \( K_m \) for endothelial nitric oxide synthase in vitro.
Measurement of Nitrite and Nitrate Concentration
Plasma NO$_2^-$ and NO$_3^-$ (NOx) were measured using the Griess assay. Nitrate was converted to nitrite with nitrate reductase before the addition of the Griess reagent as described previously.30

Drugs and Infusates
Acetylcholine chloride (ACh) was obtained from Merck Biosciences, and glyceryl trinitrate (GTN), lidocaine hydrochloride, and normal saline (0.9% sodium chloride) solution were from East Anglia Pharmaceutical, Phoenix, and Baxter, respectively. 6R-BH4, 6S-BH4, and NH4 were purchased from Schircks Laboratories.

Reagents for Assays
For measurements of total plasma antioxidant capacity, Dulbecco phosphate-buffered saline was purchased from Invitrogen, horseradish peroxidase was from Merck Biosciences, and hydrogen peroxide and 2,2-Azino-bis-3-ethylbensthiazoline-6-sulfonic acid (ABTS) were from Sigma-Aldrich. For plasma total biopterin and measurement of nitrate (NO$_3^-$) and nitrite (NO$_2^-$), perchloric acid 60%, sodium ascorbate, iodine and potassium iodide solution, glucose-6-phosphate dehydrogenase, nitrate reductase, glucose-6-phosphate, and reduced form of nitrate reductase were purchased from Sigma-Aldrich.

Experimental Protocols
Endothelial and smooth muscle function were assessed by measuring the forearm blood flow response to intra-arterial infusion of ACh (25, 50, and 100 nmol/min) and GTN (8, 16, and 32 nmol/min), respectively. Each dose was infused for 3 minutes; 0.9% sodium chloride was infused between study drugs. The effect of infusion of pterin compounds on endothelial and smooth muscle dilator function to IRI was assessed in separate studies as follows.

Protocol 1: Effect of 6R-BH4 on Baseline Forearm Blood Flow
Forearm blood flow was measured during intra-arterial infusion of 50 or 500 μg/min 6R-BH4, each dose for 30 minutes. Venous blood was drawn from the same arm at 10-minute intervals during and after the infusions.

Protocol 2: Effect of Pterins on Vasodilation to ACh
Forearm blood flow was measured in response to ACh before and after infusion of 500 μg/min 6R-BH4 or NH4.

Protocol 3: Effect of IRI on Plasma TAOS, Biopterin, and NOx
In 8 subjects an antecubital vein was cannulated in both arms for blood sampling. The nondominant arm was then subjected to 20 minutes of ischemia and 15 minutes of reperfusion as described previously. Venous samples were obtained simultaneously from both arms before initiation of ischemia, at the end of ischemia, 15 minutes after reperfusion, and then in recovery 4 hours later.

Protocol 4: Effect of IRI on Endothelial Function
Forearm blood flow responses to ACh or GTN were assessed before and immediately after IRI.

Protocol 5: Effect of Pterins on IRI Induced Endothelial Dysfunction
In separate experiments, to assess the effect of pterin compounds on endothelial function after IRI, vasodilation to ACh and GTN was measured before and immediately after IRI. 6R-BH4, 6S-BH4, or NH4 (500 μg/min) were infused intra-arterially in the study arm during both ischemia and reperfusion.

For all the protocols, please see supplemental Figure I (available online at http://atvb.ahajournals.org).

Statistical Analysis
Forearm blood flow was measured as mL/100 mL forearm volume/min. The average ratio of flow in the infused (active)/noninfused (control) arm was calculated over the last minute of infusion for each dose of drug and comparisons were made by 2-way ANOVA. A value of P<0.05 was considered statistically significant. Where an alteration in baseline flow was observed between study periods, responses were evaluated after adjustment for these differences using logistic regression analysis. Within-group comparisons for other parameters were analyzed by paired Student t test. All analyses were performed using Graph Pad Prism or Stata 8.2.

Figure 2. Effect of IRI on venous plasma TAOS (a), biopterin (b), and NOx (c) (probability values refer to the difference between control and active arms at IRI.)
Results

A total of 109 forearm studies were conducted among 48 healthy volunteers.

Effect of 6R-BH4 and NH4 on Baseline Forearm Blood Flow and Venous Biopterin Concentrations

6R-BH4 (50 μg/min intra-arterial for 30 minutes) increased forearm venous total biopterin concentrations to approximately 6±1 μmol/L. At a rate of 500 μg/min for 30 minutes the forearm venous concentration of total biopterin increased to 108±40 μmol/L (Figure 1a). There was no alteration in forearm blood flow at either dose of 6R-BH4 (Figure 1b). Forearm blood flow was also unchanged during infusion of NH4 (500 μg/min for 30 minutes; data not shown).

Effect of Pterins on Vasodilation to ACh

Neither 6R-BH4 nor NH4 altered the dilator response to ACh in the absence of IRI (supplemental Figure II).

Effect of IRI on TAOS, Biopterin, and NOx

There was a significant reduction of forearm venous plasma TAOS (Figure 2a) immediately after IRI with no detectable change in plasma total biopterin or NOx (Figure 2b, 2c).

Effect of IRI on Endothelial Function and the Effect of Pterins on Endothelial Function Following IRI

As reported previously,26 IRI attenuated endothelium-dependent vasodilation to ACh (P=0.01) but did not affect the response to endothelium-independent dilator GTN (P=0.3; Figure 3a , 3b). Continuous infusion of 6R-BH4, 6S-BH4, or NH4 (all at 500 μg/min) during ischemia and reperfusion preserved the response to ACh dilation, in keeping with protection from reperfusion injury (Figure 4a, 4b, 4c). The response to GTN was unaltered (data not shown).

Discussion

In this study 6R-BH4, an essential endogenous NOS cofactor, prevented endothelial dysfunction caused by IRI, consistent with observations in animal models. The protection from endothelial dysfunction observed here with 6R-BH4 is similar to that seen in humans after other acute endothelial insults such as cigarette smoking or an oral glucose load, and after chronic exposure to cardiovascular risk factors. However, in our studies, a similar protective effect on endothelial IRI was also observed with 6S-BH4, and with NH4, at doses that would have resulted in approximately equimolar intra-arterial concentrations. 6S-BH4 is 60-times less potent as a NOS cofactor than 6R-BH4,23,31–33 and NH4 has no cofactor activity.24 Therefore, it is unlikely that this protective action is the result of increased NOS catalysis. However, all 3 pterins are equally efficacious as scavengers of the superoxide radical.21,22,34 Because superoxide may quench the effect of NO by a rapid chemical interaction altering its vasodilator and other actions,35–37 this might provide a mechanism for the observed protection from endothelial IRI produced by all 3 pterins.
IRI is associated with increased generation of $O_2^-$ and related radicals during the early phase of reperfusion, although evidence for this is indirect and based on experiments where there has been reduction in injury with use of antioxidants such as ascorbic acid or N-acetylcysteine or superoxide dismutase. Potential sources of $O_2^-$ include cyclo-oxygenases, xanthine oxidase, NADPH oxidase, uncoupled NOS, and the mitochondrial electron transport system, all of which have been implicated in IRI. In this study, consistent with increased $O_2^-$ production, there was a marked reduction in TAOS of venous plasma after IRI. Therefore, the simplest explanation of our findings is that all 3 pterins exert their protective effects in this model through free-radical scavenging.

Intrabrachial infusion of 6R-BH4 at a dose of 500 $\mu$g/min also markedly improves endothelium-dependent vasodilatation in patients with cardiovascular risk factors. This observation has been taken as evidence that these risk factors lead to a depletion of endogenous 6R-BH4. Because all of these exposures have also been associated with the increased generation of oxygen radicals by the vascular wall, and because 6R-BH4 is itself susceptible to oxidative inactivation, this model has good biological plausibility. However, as we demonstrate in this study, the dose of 6R-BH4 commonly used (500 $\mu$g/min) raises its intravascular concentration to $\approx 100$ $\mu$mol/L, $\approx 1000$-times the Michaelis constant ($K_m$; 100 nmol/L) of the isolated NOS enzyme for 6R-BH4. Though it is uncertain whether intracellular concentrations would be quite as high, the free radical scavenging action of 6R-BH4 at these concentrations could outweigh its specific "physiological" role in NOS catalysis at this dose. Few of the prior studies in humans have compared the effects of 6R-BH4 on endothelial function with those of 6S-BH4 or NH4, which are equipotent as antioxidants but have little or no NOS cofactor actions. The beneficial effect of 6R-BH4 on endothelial function in smokers was not shared by NH4, nor by 6S-BH4, after an oral glucose load in healthy subjects. These observations suggest that, in some situations, the effects of 6R-BH4 could be mediated by direct improvement of NOS catalysis, possibly by restoration of 6R-BH4 deficiency, while in others by an antioxidant effect. Further work will be required to understand which of the 2 actions of 6R-BH4 is important in the endothelial dysfunction induced by diabetes, elevated levels of cholesterol, or high blood pressure.

Although the evidence suggests that most of the injury in IRI occurs early after reperfusion, we chose to infuse 6R-BH4 throughout the IRI period to maximize protection against injury. To further understand the mechanism of action of BH4 in IRI, it will be important to establish the critical period during which pterins exert their protective actions, particularly if these agents are to be considered as potential therapies for IRI in clinical situations.

Understanding the role of 6R-BH4 in endothelial dysfunction has been limited by the lack of a simple specific assay in plasma. In our experiments we measured total plasma biopeterin after acid oxidation of plasma. The total biopeterin thus measured derives from both 6R-BH4 and its oxidized product 7,8-dihydrobiopterin that are thought to circulate in plasma in the approximate ratio of 3 to 2. Measuring each species separately in human plasma has proved challenging, although other workers have developed assays that are reliable in animals. However if a simple method was developed for human plasma, this might enhance understanding of whether 6R-BH4 concentrations are reduced in situations in which endothelial dysfunction occurs.

In summary we have shown, for the first time in humans, that reduced pterins 6R-BH4, 6S-BH4, and NH4 prevent endothelial dysfunction induced by IRI in the human forearm. Regardless of mechanism, this may represent a new therapeutic approach in clinical settings where ischemia-reperfusion injury occurs.

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Disclosures
None.

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Figure I

a. Protocol 1. Effect of 6R-BH4 on forearm blood flow. Forearm blood flow (FBF) was measured at 10 minute intervals during i.a infusion of 50 and 500mcg/min 6R-BH4 and 0.9% saline. Venous blood samples (BS) were also taken at these time points.

b. Protocol 2. Effect of pterins on vasodilation to ACh. Cumulative dose responses to ACh were measured at baseline and after 30 minutes of i.a 6R-BH4 or NH4.

c. Protocol 3. Effect of IRI on plasma TAOS, biopterin and NOx. BS were taken from active (IRI) and control arms. The active arm was subjected to the IRI and the control arm remained at rest with no intervention (the time line is not to scale).

d. Protocol 4. Effect of pterins on endothelial dysfunction following IRI. FBF responses to cumulative doses of ACh and GTN were measured before and after IRI. This was repeated with i.a infusion of 6R-BH4, 6S-BH4 or NH4 (500mcg/min) during IRI.

Figure II. Effect of pterins on vasodilation to ACh. Forearm blood flow responses to cumulative doses of ACh before and after 500mcg/min i.a 6R-BH4 (a) and NH4 (b) show no significant differences
Figure II

(a) Forearm flow ratio in response to ACh nmol/min before and after BH4 with n=10 and p=0.12.

(b) Forearm flow ratio in response to ACh nmol/min before and after NH4 with n=8 and p=0.6.