Dihydrobiopterin (BH$_2$): Key Determinant in Influencing Arginine Mediated Endothelial Tolerance and Dysfunction

Srinidi Mohan, Harsh Patel, Jorge Bolinaga, Nathania Soekamto, Lum Achu and Kefali Teklemariam

Department of Pharmaceutical Sciences, University of New England, Portland, ME 04103, USA

Received 2012-3-24; Revised 2012-5-4; Accepted 2012-5-15

ABSTRACT

The redox-sensitive tetrahydrobiopterin (BH$_4$) is an essential cofactor that is required by endothelial Nitric Oxide Synthase (eNOS) for L-arginine (ARG) mediated Nitric Oxide (NO) generation. Oxidation of BH$_4$ causes cofactor insufficiency and uncoupling of eNOS, resulting in product switching from NO to O$_2^-$ production. Here we tested the hypothesis that eNOS uncoupling is not simply a consequence of BH$_4$ insufficiency, but rather results from a diminished ratio of BH$_4$ versus its catalytically incompetent oxidation product, 7,8-dihydrobiopterin (BH$_2$). Human Umbilical Vein Endothelial Cells (HUVEC) were incubated for 2 h in Locke's buffer with 100 µM ARG with or without other agents for 2 h (acute) or in medium for 7 days and challenged in buffer for 2 h (chronic). eNOS activity was determined by cellular accumulation of nitrite/nitrate and its expression was measured using ELISA method. Dihydroethidium fluorescence technique was used to measure O$_2^-$ accumulation. For binding studies, cell extracts were quantified for levels of BH$_4$, BH$_2$, quinonoid isoform of BH$_2$ (qBH$_2$) and biopterin using a modified HPLC method. [$^3$H]BH$_4$ binding studies revealed BH$_4$ and BH$_2$ bind eNOS with equal affinity and BH$_2$ can efficiently replace BH$_4$ in preformed eNOS-BH$_4$ complexes. While the total pterin pool of HUVEC was unaffected by chronic (7 days) exposure to ARG, BH$_2$ levels increased from undetectable to 40% of total pterin. This BH$_2$ accumulation was associated with diminished NO activity and accelerated O$_2^-$ production. Reciprocally, O$_2^-$ production was found to negatively correlate with intracellular ratio of BH$_4$-to-BH$_2$. Our findings implicate intracellular BH$_4$-to-BH$_2$ ratio, not simply BH$_4$ amount, as a critical in vitro determinant of eNOS product formation during continuous ARG supplementation. Accordingly, diminished ratio of BH$_4$-to-BH$_2$ is likely to be the fundamental molecular link between oxidative stress and endothelial dysfunction during ARG mediated tolerance development.

Keywords: Tetrahydrobiopterin, dihydrobiopterin, eNOS, arginine, super-oxide

1. INTRODUCTION

L-Arginine (ARG) is an essential amino acid that is involved in regulating multiple physiological process (Schulman et al., 2006). It helps in removing ammonia from the body through the urea cycle (Ha and Milner, 1979) and serves as a substrate for the endogenous synthesis of creatine and proline (Wu and Morris, 1998). ARG has also gained popularity in the last decade as a dietary supplement after its role as the endogenous substrate for Nitric Oxide Synthase (eNOS) to produce Nitric Oxide (NO) was identified.

In the National Institute of Health website Medlineplus, the use of ARG in as many as 44 diseases and diagnoses were discussed and categorized according to the strength of scientific evidence supporting its use. Indications graded as “A” (for strong scientific evidence) are growth hormone reserve test/pituitary disorder diagnosis and inborn errors of urea synthesis. Those graded as “B” (for good scientific evidence) are coronary artery disease (angina), critical illness, heart failure, migraine headache and peripheral vascular disease/claudication. Those graded as “C” (for unclear scientific evidence) include diabetes, erectile
dysfunction, myocardial infarction, pre-eclampsia, wound healing, among others (n = 30).

In patients with stable angina pectoris, 6 g ARG/day for 3 days increased their exercise tolerance (Bednarsz et al., 2000) and supplement with 2 food bars enriched with ARG per day for 2 weeks improved vascular function, exercise capacity and quality aspects of life (Maxwell et al., 2002). In patients with congestive heart failure, 9 g ARG/day for 7 days prolonged exercise duration (Bednarsz et al., 2004). In addition, ARG has been found to improve immunity (Popovic et al., 2007), in patients under critical care (Zhou and Martinale, 2007) and in sickle cell disease (Romero et al., 2002; Vichinsky, 2002). The range of diseases that can be potentially benefitted by ARG supplementation is therefore quite wide.

Of concern, recent studies revealed that the therapeutic benefits of ARG, often clearly observed in short-term studies, are not evident after long-term use. When patients with peripheral arterial insufficiency were dosed with ARG at 3 g/day for 6 months, NO synthesis was not improved (Wilson et al., 2007) in spite of short-term benefits observed at 1 month (Oka et al., 2005). In patients with myocardial infarction, ARG dose at 3 g tid for 6 months led to no improvement in vascular stiffness measurements or ejection fraction (Schulman et al., 2006), again in spite of positive effects observed after 1 month (Bednarsz et al., 2005). These findings have led to the conclusion that clinical tolerance toward ARG supplementation develops after chronic (or continuous) therapy.

Using human endothelial cells in culture, we have recently shown that tolerance to the NO-generating effects of ARG can be reproduced after 7 days of in vitro exposure to 100 µM ARG. Concomitantly, enhanced intracellular superoxide (O$_2^•$) and glucose accumulation, as well as suppressed eNOS expression, were observed (Mohan et al., 2011). Several studies in whole animals have also indicate ARG supplementation to be associated with oxidative stress (Chen et al., 2003; Huang et al., 2008; Simonet et al., 2004) and the specific formation of O$_2^•$-- from ARG was implicated (Simonet et al., 2004). Depletion in redox-sensitive tetrahydrobiopterin (BH$_4$), an essential cofactor for eNOS, has caused uncoupling of eNOS from ARG, oxidation of NADPH and ferrous dioxygen species, thereby resulting in endothelial dysfunction via product switching from NO to O$_2^•$ (Veres et al., 2008).

Restoring BH$_4$ level through subsequent dosing has reduced the symptoms of endothelial dysfunction in chronic smokers and patients with diabetes (Pieper et al., 1995; Shinozaki et al., 2000), hypercholesterolemia (Stroes et al., 1997) or ischemia-reperfusion injury (Tiefenbacher et al., 1996). Treatment of deoxycorticosterone acetate salt induced mice with oral BH$_4$ attenuated vascular reactive oxygen species production, increased NO levels and blunted hypertension compared with non-hypertensive control mice (Landmesser et al., 2003). Besides supplementing BH$_4$, cells exposed to antioxidants such as Glutathione, Vitamin C or E (which are capable of providing chemical stabilization to BH$_4$), preventing BH$_4$ oxidation and increasing cellular eNOS activity (Wolff et al., 1993; Yoshida et al., 1995). These studies provide the initial evidence to suggest oxidation of BH$_4$ during ARG induced O$_2^•$ generation to be the basis for eNOS uncoupling in vascular dysfunctions.

However, the functionally incompetent oxidized species of BH$_4$ (7,8-dihydrobiopterin, BH$_2$) is also known to bind to eNOS (Gross et al., 1991; Kwon et al., 1989; Tayeh and Marletta, 1989). We test here the hypothesis that eNOS dysfunction during continuous ARG exposure is simply not a consequence of BH$_4$ oxidation alone, but rather the ability of the accumulated BH$_2$ to bind eNOS, thereby causing displacement of BH$_4$ and suppression in eNOS activity. The present study will implicate the binding avidity of BH$_2$ as well as the ratio of BH$_2$-to-BH$_4$, rather than BH$_4$ depletion, to be the fundamental molecular link between oxidative stress and endothelial dysfunction.

2. MATERIALS AND METHODS

2.1. Supplies and Reagents

Human Umbilical Vein Endothelial Cells (HUVEC) was purchased from American Type Culture Collection (Manassas, VA), whereas Ea.hy926, an immortalized human endothelial cell line, was obtained as a gift from the University of North Carolina. Culture reagents were obtained from Invitrogen (Carlsbad, CA) and other supplies and chemicals were from Laboratory Product Sales (Rochester, NY), VWR (Franklin, MA) and Sigma-Aldrich (St. Louis, MO). Human eNOS immunoassay kit was purchased from R and D Systems (Minneapolis, MN). QuantiChrom D-glucose assay kit utilizing the o-toluidine method was purchased from Bioassay Systems (Hayward, CA). To characterize BH$_4$ binding 6R-[H(6)]BH$_4$ (abbreviated as [H] BH$_4$) and 7R [H]BH$_2$ (abbreviated as [H] BH$_2$) was custom synthesized and obtained from New England Nuclear/Perkin Elmer (Waltham, MA), Diphenyleneiodonium (DPI); L-nitroarginine methyl ester (L-NAME) an inhibitor of eNOS; rotenone and Thenoyl Tri Fluoro Acetone (TTFA), which are inhibitors of mitochondrial electron transport complexes I and II respectively, were obtained from EMD Chemicals, Inc. (Gibbstown, NJ).
2.2. Cell Culture

HUVEC were cultured in physiological F-12K medium containing 100 µM ARG and 5 mM glucose, supplemented with 20% horse serum. Ea.hy926 cells were cultured in Duboecco’s Modified Eagle Medium (DMEM) containing 100 µM ARG, 5 mM D-glucose, supplemented with 10% fetal bovine serum. All culture media contained 100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin. Cells were maintained in a humidified chamber at 37°C with 5% CO₂ and passages between 6 and 16 (mean passage number = 9-13) were used in all experiments.

2.3. Cell Culture Studies

For acute studies, HUVEC and Ea.hy926 cells grown to confluence in 6 well dishes (well area of 9.6 cm²) were incubated in Locke’s buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 2.3 mM CaCl₂, 5.6 mM D-glucose, 5 mM HEPES, pH 7.4) for 2 h containing 100 µM ARG with or without 30 µM of eNOS inhibitor (L-NAME), 20 µM DPI, or inhibitors of electron transport chain complex I (2 µM rotenone) or complex II (5 µM TTFA). Continuous effect was assessed by pre-incubating with 50 µM [³H] BH₃ for 1 h. Control cells pre-exposed to 50 µM [³H] BH₃ in 2 ml of Locke’s buffer for 1 h was washed twice in 1 X PBS, before incubating for 1 h in 2 ml Locke’s buffer alone, without any added agents. The dissociating avidity between BH₃ and BH₄ after forming a complex with eNOS was tested by initially pre-exposing both cell lines to either 100 µM [³H] BH₃ or [³H] BH₄ for 1 h in Locke’s buffer, followed by subsequent incubation in Locke’s buffer for 1 h with 10⁻⁹-10⁻⁴ molar concentrations of BH₄ and BH₃ respectively. All cellular exposures were washed twice with 1 mL of 1X PBS and the extracted cell lysate samples were quantified by HPLC. All binding and dissociation studies were conducted at 22 °C and 0.1 mM dithiothreitol (DTT) was used to minimize [³H] BH₄ oxidation.

2.5. Pterin Quantification by HPLC

Cellular levels of BH₄, BH₃ and the quinonoid isomer of BH₂ (qBH₂) and Bipterin (B) levels were quantified aspreviously described (Heales and Hyland, 1989). The mobile phase consisted of sodium acetate (50 mmol l⁻¹), citric acid (5 mmol l⁻¹) pH 5.2, containing EDTA (48 mmol l⁻¹) and DTT (0.1 mmol l⁻¹). The samples were passed through a 5 µm Agilent ZORBAX Eclipse plus (25×4.5 cm I.D) column (Santa Clara, CA) at a flow rate of 1 mL min⁻¹. The column temperature was maintained at 40°C. Samples were detected by an ESA Coulochem 5100 A electrochemical detector using a model 5011 high sensitivity electrode (Bedford, MA) with upstream and downstream electrodes set at +0.06 and -0.46 V, respectively. Quantification of BH₄ and BH₃ was done by comparison with external standards after normalization for total protein content.

2.6. Inorganic Nitrate and Total Nitrite/Nitrate Determination

Cell lysate samples or freshly prepared nitrate standard were first brought to volume of 100 µL with double-deionized water. Samples were protected from light and 10 µL of freshly prepared diaminonaphthalene (DAN, 0.05 mg mL⁻¹ in 1 M HCl) was added and mixed immediately. After 10 min incubation at room temperature, the reaction was terminated with 5 µL of 2.8 N NaOH. The intensity of the fluorescent signal produced was measured using a plate reader with excitation at 360 nm and emission read at 420 nm, with a gain setting at 100%. In order to measure total nitrate/nitrate, nitrate was converted to nitrite by the action of nitrate reductase from Aspergillus niger. Briefly, the samples were incubated with 40 µM NADH and 14 µM of enzyme in
a final volume of 50 µL of 20 mM Tris, pH 7.6, followed by 30 min incubation with 10 µL of DAN at room temperature. The reaction was terminated after 30 min with 20 µL of NaOH. Nitrite contents in the samples were then calculated by first subtracting the value of the enzyme blank containing NADH. The values were further normalized using total protein concentration, which were measured according to the method (Lowry et al., 1951).

2.8. Statistical Analysis

The resulting mixtures were harvested in acetonitrile (0.2 ml/well), sonicated (10 s) and centrifuged (13,000 rpm, 15 min at 4°C). The supernatant fraction was air-dried, reconstituted in PBS and the fluorescence intensity (in arbitrary units, AU) was determined, in duplicate, using a micro-plate reader at excitation and emission wavelengths of 405 and 570 nm, respectively.

2.7. Superoxide (O$_2^-$) Measurement

O$_2^-$ production was assessed by Dihydroethidium (DHE) fluorescence (Zhao et al., 2003). At the end of the incubations, cells were washed and incubated in Locke’s buffer at a final DHE concentration of 10 nM for 20 min. The resulting mixtures were harvested in acetonitrile (0.2 ml/well), sonicated (10 s) and centrifuged (13,000 × g for 5 min at 4°C). The supernatant fraction was air-dried, reconstituted in PBS and the fluorescence intensity (in arbitrary units, AU) was determined, in duplicate, using a micro-plate reader at excitation and emission wavelengths of 405 and 570 nm, respectively.

2.8. Statistical Analysis

Data are presented as mean ± standard deviation (n = 6 replicates) unless otherwise stated. Statistical comparisons among groups were performed using one-way analysis of variance (ANOVA), followed by Fisher’s and Tukey’s post-hoc test procedure (version 15.x; Minitab). Statistical significance was concluded when p < 0.05.

3. RESULTS

3.1. Effects of Short Term and Continuous ARG Exposure in Endothelial Cells

Short-term exposure of endothelial cells to 100 µM ARG showed an increase in eNOS activity (quantified as total nitrite-nitrate) glucose and O$_2^-$ accumulation, while the levels of eNOS protein expression, BH$_4$ and BH$_2$ remained unchanged from control, as displayed in Table 1. When cells were continuously exposed to ARG, a 30% reduction in eNOS expression and about 50% decrease in BH$_4$ content (P < 0.01) was observed, as shown in Fig. 1 and Table 1. The accumulation of BH$_4$ was found to be almost exclusive, as the quinonoid tautomer qBH$_2$ and bipterin, were not detected (data not shown). This increase in BH$_4$ accumulation in endothelial cells occurred progressively with subsequent increases seen in glucose accumulation and O$_2^-$ generation during the 7 days of continuous ARG treatment, as given in Table 1.

Cells subjected to continuous ARG exposure in the presence of a NOS specific inhibitor, L-NAME retained 76.5±8.1 pmol of BH$_4$ per milligram of cellular protein in their functionally reduced form, thereby avoiding oxidation of BH$_4$ by 67% and also suppressed O$_2^-$ accumulation. However neither DPI nor L-NAME involvement during continuous ARG exposure was successful in suppressing ARG mediated cellular accumulation of glucose, as shown in Fig. 2 and 3. The ARG elicited oxidation of BH$_4$ was totally abolished by DPI, an agent that inhibits O$_2^-$ production by NOS and other flavoproteins, including NADPH oxidase (data not shown). The significant suppression of O$_2^-$ observed in cells treated with L-NAME, that was identical to those achievable with DPI treatment, as shown in Fig. 3 identifies uncoupled eNOS to have a major impact in causing BH$_4$ oxidation and subsequent O$_2^-$ production, thereby initiating a vicious tolerance generating cycle during continuous ARG supplementation in endothelial cells.

3.2. BH$_2$ Effectively Competes with BH$_4$ for eNOS Occupancy with Greater Avidity

eNOS bound [³H] BH$_4$ dissociated from its complex in the presence of unbound BH$_4$. The dissociation occurred via monophasic kinetics and was 50% completed at (T$_{1/2}$) = 30.1±2.3 min, as shown in Fig. 4. Similar progressive loss of [³H] BH$_4$ bound eNOS complex occurred with increase in BH$_2$ concentration, to a maximum half-maximal displacement of 80% when the concentrations of BH$_2$ and [³H] BH$_4$ approached equivalence, as shown in Fig. 5. In contrast, tetrahydropterin (PH$_4$), an analog of BH$_4$ that differs in the lack of 6-position dihydroxypropyl side chain, was capable of binding with eNOS with >1000 fold less affinity than BH$_4$ or BH$_2$ as shown in Fig. 5; which suggests the importance of retaining the 6-position side chain of bipterin for high-affinity binding of pterin species to eNOS. In addition to testing the binding efficiency of the various pterin in forming complexes with cellular eNOS, the avidity of retaining the formed complex was also tested. Cells pre-exposed to [³H] BH$_4$ (thereby forming the eNOS-[³H] BH$_2$ complex) remained unaltered to subsequent exposures to varying concentrations of BH$_2$> 10$^{-3}$ M, as shown in Fig. 6. However, the eNOS-[³H]BH$_4$ complex formed in cells was disrupted when exposed to > 10$^{-8}$ M concentration of BH$_2$, indicating a greater avidity (> 100,000 fold) for eNOS in retaining its complexation with BH$_2$ than with BH$_4$. Together these studies suggest that BH$_4$ accumulation in endothelial cells to effectively compete with BH$_4$ for eNOS occupancy and since BH$_2$ is known to cause eNOS uncoupling, its increase favors eNOS derived O$_2^-$ over NO production.
Table 1. Analysis of cellular conditions during continuous ARG exposure

| Treatments                  | Acute ARG (2 h challenge dose) | Chronic ARG (7 Days + 2 h challenge dose) |
|-----------------------------|---------------------------------|-------------------------------------------|
| BH₄ pmol/mg protein         | 93±9.2                          | 45.8±7.1 *                               |
| BH₂ pmol/mg protein         | 15.3±1.1                        | 42.4±5.2 #                               |
| NO₂⁻ + NO₃ pmol/µg protein  | 3.0±0.37                        | 1.28±0.3 *                               |
| Glucose mg/dl               | 54.1±6.6                        | 128±11.2 *                               |
| eNOS ng/µg protin           | 0.72±0.15                       | 0.51±0.15 *                               |
| O₂⁺ Arb. Units              | 1.64±0.49                       | 3.49±0.32 *                               |

* p < 0.05 versus control, # p < 0.05 versus ARG acute treatment. (n = 6)

Table 2. Analysis of mitochondrion involvement in endothelial tolerance development during ARG supplementation

| Units                  | Control | 100µM ARG | 7 day Chronic Treatments | 100 µM ARG + 5 µM |
|------------------------|---------|-----------|--------------------------|------------------|
| BH₄ pmol/mg protein    | 93±9.2  | 45.8±7.1 *| 100 µM ARG + 2 µM        | 110 µM ARG       |
| BH₂ pmol/mg protein    | 15.3±1.1| 42.4±5.2 *| Rote none                | 89.6±10.2       |
| NO₂⁻ + NO₃ pmol/µg protein | 3.0±0.37 | 1.28±0.3 *| 17.4±2.2 #               | 13.4±3.2        |
| Glucose mg/dl          | 54.1±6.6| 128±11.2 *| 2.81±0.26 #              | 3.32±0.31       |
| eNOS ng/µg protin      | 0.72±0.08| 0.51±0.11 *| 118.7±8.2 *              | 120.8±6.3       |
| O₂⁺ Arb. Units         | 1.64±0.49| 3.49±0.32 *| 0.69±0.12 #              | 0.71±0.10       |

* p < 0.05 versus control, # p < 0.05 versus ARG chronic treatment. (n = 6)

3.3. Role of Mitochondrial Electron Transport Chain in ARG Induced O₂⁻ Generation and BH₄ Oxidation

The mitochondrion electron transport chain is notably a predominant source of O₂⁻ generation in normal respiring cells. Since eNOS derived O₂⁻ was found to be necessary for oxidation of BH₄ to BH₂ in a concentration dependent manner, BH₄ oxidation was not evident during short term (2 h) exposure to ARG. Points shown in the figure are representing mean ± SE of six determinants.

Fig. 1. Continuous ARG exposure in endothelial cells increases oxidation of BH₄ to BH₂ in a concentration dependent manner. BH₄ oxidation was not evident during short term (2 h) exposure to ARG. Points shown in the figure are representing mean ± SE of six determinants.

Fig. 2. Cellular glucose accumulation during continuous ARG supplementation for 7 days in culture medium followed by 2 h challenge in Locke’s Buffer. L-NAME and DPI exposures did not suppress glucose accumulation during continuous ARG exposure. All indicative values are mean ± SE (n = 6). *, p < 0.05 versus control treatments.

Fig. 3. Cellular O₂⁻ generation during continuous ARG exposure for 7 days in culture medium followed by 2 h challenge in Locke’s Buffer. L-NAME and DPI significantly attenuated O₂⁻ generation (*, p <0.05) during continuous ARG treatment. All indicative values are mean ± SE (n = 6).
Fig. 4. Characterization of competitive binding and of BH₄ to eNOS. All binding studies were done at 22°C. Points are mean ± SE of six determinations.

Fig. 5. Kinetics of dissociation efficacy of [³H] BH₄ based on competitive binding of unlabeled pterin to eNOS. Unlabeled BH₃ shows similar binding efficacy to eNOS as BH₄, while PH₄, which lacks the 6-position dihydroxy-propyl side chain, shows >1000-fold lower affinity versus BH₄ or BH₂. All binding studies were done at 22°C. Points are mean ± SE of six determinations.

Fig. 6. Binding avidity of pterins. BH₂ shows greater binding avidity to eNOS in forming a complex than BH₄ when exposed to >10⁻³ M BH₄. Complex formed between [³H] BH₄ and eNOS was lost when exposed to unbound BH₂ of >10⁻⁸ M. (n = 6)

4. DISCUSSION

A wide variety of disease states (Wu and Morris, 1998) are expected to benefit from increased NO bioavailability through increased ARG supply. The development of ARG tolerance (and possible toxicity) upon chronic dosing represents a major hindrance for the use of this important amino acid to benefit patients. Using human endothelial cells, we recently showed (Mohan et al., 2011) that continuous exposure of ARG under in vitro conditions result in eNOS down-regulation, secondary to oxidative stress and induced glucose accumulation. Concurrently, several studies in whole animals indicate that ARG supplementation is associated with oxidative stress (Chen et al., 2003; Huang et al., 2008; Simonet et al., 2004) and the specific formation of O₂⁻ from ARG was implicated (Simonet et al., 2004). The O₂⁻ formed in association with continuous ARG exposure, scavenge the available NO, resulting in the formation of peroxynitrite formation, thereby compromising NO bioactivity by promoting the oxidation of BH₄, leading to eNOS uncoupling (Bitar et al., 2005; Schmidt and Alp, 2007). Here we extend these preliminary literature findings to show the increase in the rate of BH₂ binding to eNOS, alteration to intracellular BH₄ to BH₂ ratio and the greater binding avidity of eNOS with BH₂ than BH₄, as the key determinants in initiating the various tolerance sparing events observed during continuous ARG supplementation, rather than simply a consequence of BH₄ oxidation.

Cells continuously exposed to ARG in the presence of inhibitors of the mitochondrial electron transport complexes I and II (2 µM rotenone and 5 µM TTFA, respectively) abolished BH₄ oxidation and O₂⁻ generation, restored eNOS expression and activity, but had no effect in preventing glucose accumulation, as shown in Table 2. The concentrations of rotenone and TTFA were chosen based on prior identification as their lowest limit of inhibition in our endothelial cell culture (data not shown).
We showed during continuous ARG supplementation that the accumulation of BH\textsubscript{4} in endothelial cells to increase reciprocally towards subsequent decrease in BH\textsubscript{4} level, with concomitant increases seen in glucose accumulation and \textit{O}\textsubscript{2}\textsuperscript{\dot{}}, production, while compromising ARG consumption by eNOS for NO generation. If BH\textsubscript{4} oxidation is the primary basis of eNOS uncoupling, supplementation of BH\textsubscript{4} would rapidly reestablish the synthesis of NO and eNOS activity. This prediction was based on multiple studies conducted with in vitro and in vivo models showing that acute administration of BH\textsubscript{4} to enhance eNOS activity and suppress eNOS derived \textit{O}\textsubscript{2}\textsuperscript{\dot{}} generation (Alp and Channon, 2004). Nonetheless, the possibility exists that progressive oxidation of BH\textsubscript{4} would result in BH\textsubscript{2} buildup, which would lead to increase affinity of eNOS binding with BH\textsubscript{2} and a consequent long-term worsening of eNOS uncoupling and endothelial dysfunction. To evaluate the extent to which eNOS couples with oxidized species of BH\textsubscript{4}, the cells pre-incubated with \textit{[H]} BH\textsubscript{4} was exposed to varying concentrations of BH\textsubscript{4}, BH\textsubscript{2} and PH\textsubscript{4}. Our binding studies showed that cells supplemented with BH\textsubscript{2} instead of BH\textsubscript{4}, does not diminish the high affinity level achievable with BH\textsubscript{4} versus BH\textsubscript{2} for eNOS binding and showed the importance of retaining the 6-position dihydroxypropyl side chain of biopterin for achieving high-affinity binding to eNOS. The complexation of eNOS with BH\textsubscript{2} was found to be retained with greater avidity than those achieved with BH\textsubscript{4} during subsequent pterin exposure. These result suggests that the fundamental determinant of NO bioactivity during ARG supplementation to be the balance between the intracellular BH\textsubscript{4} and its primary two-electron oxidation product as BH\textsubscript{2} and not a consequence of BH\textsubscript{4} oxidation alone, as has been generally thought until now.

Our studies show that BH\textsubscript{2} binds with greater avidity to eNOS (than BH\textsubscript{4}) and provokes uncoupling has additional important implications in considering BH\textsubscript{4} supplementation being used for various therapy related to ARG mediated endothelial dysfunction. Previous studies (Gori et al., 2001; Heitze et al., 2000; Higashi et al., 2002; Setoguchi et al., 2002; Stroes et al., 1997) have suggested acute supplementation of high doses of BH\textsubscript{4} to have therapeutic potential to reverse endothelial dysfunction and improve vaso-reactivity. However, these studies have not addressed the more long-term consequences of BH\textsubscript{4} administration in the setting of oxidative stress. The redox sensitive BH\textsubscript{4}, when prone to oxidation during ARG associated \textit{O}\textsubscript{2}\textsuperscript{\dot{}} generation will favor BH\textsubscript{4} accumulation and subsequent complexation with eNOS, resulting in increasing oxidative and nitrosative stress, thereby opposing the desired NO-generating effect.

In addition to our present found importance of eNOS-BH\textsubscript{4} complex to influence endothelial dysfunction and tolerance development during supplementation of ARG, we have also attempted to identify the predominant source that instigates BH\textsubscript{4} oxidation, in the first place. Mitochondrial mediated \textit{O}\textsubscript{2}\textsuperscript{\dot{}} production is considered to provide a trigger for metabolic derangement that mediates diabetic complications. To test whether mitochondrion derived \textit{O}\textsubscript{2}\textsuperscript{\dot{}} plays a role in ARG induced BH\textsubscript{4} oxidation, we assessed whether selective inhibitors of the mitochondrial electron transport chain complexes can provide protection against BH\textsubscript{4} oxidation in endothelial cells. The significant inhibition in BH\textsubscript{4} oxidation during ARG supplementation with inhibitors of the mitochondrial electron transport chain complexes I and II implicate a role for mitochondrion derived \textit{O}\textsubscript{2}\textsuperscript{\dot{}} generation in the genesis of ARG induced BH\textsubscript{4} oxidation, leading to eNOS uncoupling.

While the involvement of mitochondrial electron transport chain inhibitors prevented BH\textsubscript{4} oxidation and subsequent \textit{O}\textsubscript{2}\textsuperscript{\dot{}} generation, it had no influence in attenuating glucose accumulation during short term or long term ARG exposure. The presence of either eNOS specific inhibitor (L-NAME) or DPI, which suppresses \textit{O}\textsubscript{2}\textsuperscript{\dot{}} generation had no impact in avoiding ARG mediated cellular increase in glucose. These results suggest glucose accumulation during ARG supplementation to be not mediated via alteration in cellular BH\textsubscript{4}/to-BH\textsubscript{2} ratio or increase in BH\textsubscript{4} accumulation. The observed glucose accumulation from continuous ARG exposure could alter the intracellular redox-state of cultured cells in several ways: (1) by increasing pro-oxidant enzyme activity, thus increasing \textit{O}\textsubscript{2}\textsuperscript{\dot{}} generation (Srinivasan et al., 2004), (2) by propagating free-radical production (Srinivasan et al., 2004) and (3) by forming mitochondrial derived reactive oxygen species (Mabile et al., 1997; Nishikawa et al., 2000). Thus the \textit{O}\textsubscript{2}\textsuperscript{\dot{}}\textsuperscript{-} generation due to high glucose accumulation (Liu et al., 1997), could also be involved in modulate the activity of eNOS during ARG supplementation. We will be defining this missing mechanistic link of how ARG supplementation increases glucose accumulation and its potential consequences in our forthcoming studies, which will better delineate the ARG tolerance phenomenon.

5. CONCLUSION

Together, our findings suggest the following model for the initiation of endothelial tolerance and dysfunction during continuous ARG supplementation.
Endothelial exposure to ARG triggers O$_2^-$ overproduction via electron transport leak from the mitochondria. The reaction between O$_2^-$ and eNOS derived NO results in peroxynitrite synthesis, which promotes BH$_4$ oxidation and hence accumulation of BH$_3$. The accumulated BH$_2$ binds with greater avidity than BH$_4$ to eNOS forming the complex, which initiates eNOS uncoupling, oxidant formation and perpetuating additional BH$_4$ oxidation, thereby initiating the vicious cycle favoring endothelial dysfunction. Thus, diminished BH$_4$-to-BH$_2$ ratio is likely to be the fundamental molecular link between oxidative stress and endothelial dysfunction during long-term ARG supplementation.

6. ACKNOWLEDGEMENT

We thank the University of New England (UNE) for helping the College of Pharmacy to promote independent research opportunities to their students. This project was funded in part from UNE start-up funds, as well as the UNE-mini-grant and Emily Jane Etherton Charitable Lead Trust contribution.

7. REFERENCES

Alp, N.J. and K.M. Channon, 2004. Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. Arterioscler. Thromb. Vasc. Biol., 24: 413-420. DOI: 10.1161/01.ATV.0000107859.6039.f6

Bednarz, B., R. Wolk, T. Chamiec, K. Herbaczyńska-Cedro and D. Winek et al., 2000. Effects of oral L-arginine supplementation on exercise-induced QT dispersion and exercise tolerance in stable angina pectoris. Int. J. Cardiol., 75: 205-210. DOI: 10.1016/S0167-5273(00)00324-7

Bednarz, B., T. Jaxa-Chamiec, J. Gebalska, K. Herbaczyńska-Cedro and L. Ceremuzynski, 2004. L-arginine supplementation prolongs exercise capacity in congestive heart failure. Kardiol. Pol., 60: 348-353. PMID: 15226784

Bednarz, B., T. Jaxa-Chamiec, P. Maciejewski, M. Szpajer and K. Janik et al., 2005. Efficacy and safety of oral L-arginine in acute myocardial infarction. Results of the multicenter, randomized, double-blind, placebo-controlled ARAMI pilot trial. Kardiol. Pol., 62: 421-427. PMID: 15928719

Bitar, M.S., S. Wahid, S. Mustafa, E. Al-Saleh and G.S. Dhaunsi et al., 2005. Nitric oxide dynamics and endothelial dysfunction in type II model of genetic diabetes. Eur. J. Pharmacol., 511: 53-64. DOI: 10.1016/j.ejphar.2005.01.014

Chen, J., P. Kuhlen TR, F. Urano, H. Ichinose and J. Astern et al., 2003. Effects of chronic treatment with L-arginine on atherosclerosis in apoE knockout and apoE-inducible NO synthase double-knockout mice. Arterioscler. Thromb. Vasc. Biol., 23: 97-103. DOI: 10.1161/01.ATV.0000040223.74255.5A

Gori, T., J.M. Burstein, S. Ahmed, S.E.S. Miner and A. Al-Hesayen et al., 2001. Folic acid prevents nitroglycerin-induced nitric oxide synthase dysfunction and nitrate tolerance: A human in vivo study. Circulation, 104: 1119-1123.

Gross, S.S., E.A. Jaffe, R. Levi and R.G. Kilbourn, 1991. Cytokine-activated endothelial cells express an isotype of nitric oxide synthase which is tetrahydrobiopterin-dependent, calmodulin-independent and inhibited by arginine analogs with a rank-order of potency characteristic of activated macrophages. Biochem. Biophys. Res. Commun., 178: 823-829. DOI: 10.1016/0006-291X(91)90965-A

Ha, Y.H. and J.A. Milner, 1979. Urea cycle operation in the arginine deficient rat. Biochem. Med., 22: 149-155. DOI: 10.1016/0006-2944(79)90001-2

Heales, S. and K. Hyland, 1989. Determination of quinonoid dihydrobiopterin by high-performance liquid chromatography and electrochemical detection. J. Chromatography B: Biomed. Sci. Appli., 494: 77-85. DOI: 10.1016/S0378-4347(00)82658-4

Heitzer, T., K. Krohn, S. Albers and T. Meine rzt, 2000. Tetrahydrobiopterin improves endothelium-dependent vasodilation by increasing nitric oxide activity in patients with Type II diabetes mellitus. Diabetologia, 43: 1435-1438. DOI: 10.1007/s001250051551

Higashi, Y., S. Sasaki, K. Nakagawa, Y. Fukuda and H. Matsuura et al., 2002. Tetrahydrobiopterin enhances forearm vascular response to acetycholine in both normotensive and hypertensive individuals. Am. J. Hypertension, 15: 326-332. DOI: 10.1016/S0895-7061(01)02317-2

Huang, H.S., M.C. Ma and J. Chen, 2008. Chronic L-arginine administration increases oxidative and nitrosative stress in rat hyperoxaluric kidneys and excessive crystal deposition. Am. J. Physiol. Renal Physiol., 295: F388-F396. DOI: 10.1152/ajprenal.00405.2007

Kwon, N.S., C.F. Nathan and D.J. Stuehr, 1989. Reduced biop terin as a cofactor in the generation of nitrogen oxides by murine macrophages. J. Biol. Chem., 264: 20496-20501. PMID: 2584226

Landmesser, U., S. Dikalov, S.R. Price, L. McCann and T. Fukai et al., 2003. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J. Clin. Investigat., 111: 1201-1209. DOI: 10.1172/JCI14172

Liu, P., C.E. Hock, R. Nagele and P.Y. Wong, 1997. Formation of nitric oxide, superoxide and peroxynitrite in myocardial ischemia-reperfusion injury in rats. Am. J. Physiol., 272: H2327-2336.
Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Mabile, L., O. Meilhac, I. Escargueil-Blanc, M. Troy and M.T. Pieraggi et al., 1997. Mitochondrial function is involved in LDL oxidation mediated by human cultured endothelial cells. Arterioscler. Thromb. Vasc. Biol., 17: 1575-1582. DOI: 10.1161/01.ATV.17.8.1575

Maxwell, A.J., M.P. Zapien, G.L. Pearce, G. MacCallum Oka, R.K., A. Szuba, J.C. Giacomini and J.P. Cooke, 2005. Hyperglycaemia-induced superoxide production decreases eNOS expression via AP-1 activation in aortic endothelial cells. Diabetologia, 47: 1727-1734. DOI: 10.1007/s00125-004-1525-1

Mohan, S., C.C. Wu, S. Shin and H.L. Fung, 2011. Continuous exposure to L-arginine induces oxidative stress and physiological tolerance in cultured human endothelial cells. Amino Acids, DOI: 10.1007/s00726-011-1173-y

Nishikawa, T., D. Edelstein, X.L. Du, S. Yamagishi and M. Tomiyama et al., 2000. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature, 404: 787-790. DOI: 10.1038/35008121

Oka, R.K., A. Szuba, J.C. Giacomini and J.P. Cooke, 2005. A pilot study of L-arginine supplementation on functional capacity in peripheral arterial disease. Vasc. Med., 10: 265-274. DOI: 10.1191/1358863x05vm637oa

Pieper, G.M., M. Jordan, L. A. Dondlinger, M.B. Adams and A.M. Roza, 1995. Peroxidative stress in diabetic blood vessels. Reversal by pancreatic islet transplantation. Diabetes, 44: 884-889. PMID: 7621992

Popovic, P.J., H.J. Zeh and J.B. Ochoa, 2007. Arginine and Immunity. J Nutr., 137: 1681S-1686S.

Romero, J.R., S.M. Suzuki, R.L. Nagel and M.E. Fabry, 2002. Arginine supplementation of sickle transgenic mice reduces red cell density and Gardos channel activity. Blood, 99: 1103-1108. DOI: 10.1182/blood.V99.4.1103

Schmidt, T.S. and N.J. Alp, 2007. Mechanisms for the role of tetrahydrobiopterin in endothelial function and vascular disease. Clin. Sci., 113: 47-63. DOI: 10.1042/CS20070108

Schulman, S.P., L.C. Becker, D.A. Kass, H.C. Champion and M.L. Terrin et al., 2006. L-Arginine therapy in acute myocardial infarction. The Vascular Interaction With Age in Myocardial Infarction (VINTAGE MI) randomized clinical trial. J. Am. Med. Assoc., 295: 58-64. DOI: 10.1001/jama.295.1.58

Setoguchi, S., Y. Hirooka, K. Eshima, H. Shimokawa and A. Takeshita, 2002. Tetrahydrobiopterin improves impaired endothelium-dependent forearm vasodilation in patients with heart failure. J. Cardiovasc. Pharmacol., 39: 363-368. PMID: 11862115

Shinozaki, K., Y. Nishio, T. Okamura, Y. Yoshida and H. Maegawa et al., 2000. Oral administration of tetrahydrobiopterin prevents endothelial dysfunction and vascular oxidative stress in the aortas of insulin-resistant rats. Circ. Res., 87: 566-573. DOI: 10.1161/01.RES.87.7.566

Simonet, S., A. Rupin, C. Badier-Commander, S. Coumaillau and D. Behr-Roussel et al., 2004. Evidence for superoxide anion generation in aortas of cholesterol-fed rabbits treated with L-arginine. Eur. J. Pharmacol., 492: 211-216. DOI: 10.1016/j.ejphar.2004.03.052