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

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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 $O_2^{\bullet}$ 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 $O_2^{\bullet}$ 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 $O_2^{\bullet}$ production. Reciprocally, $O_2^{\bullet}$ 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 (Bednarz *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 (Bednarz *et al.*, 2004). In addition, ARG has been found to improve immunity (Popovic *et al.*, 2007), in patients under critical care (Zhou and Martindale, 2007) and in sickle cell disease (Romero *et al.*, 2002; Vichinsky, 2002). The range of diseases that can be potentially benefited 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 (Bednarz *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 ($O_2^{\bullet-}$) 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 O2• – 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 $O_2^{\bullet-}$ (Veresh *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 increases 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 $O_2^{\bullet}$ 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_4-to-BH_2, 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-[^{3}H(6)]BH_4 (abbreviated as [^{3}H] BH_4) and 7R [^{3}H]BH_2 (abbreviated as [^{3}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 Dubecco'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% CO_2 and passages between 6 and 16 (mean passage number = 9+3) 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 cm^2) were incubated in Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 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 incubating cultured cells with 100 µM ARG in the presence or absence of other reagents in daily refreshed medium consecutively for 7 days, after which cells were washed and challenged with similar treatments in Locke's buffer for 2 h.

After exposure to desired treatment conditions, the cells were washed twice with 1 mL 1X Phosphate Buffered Saline (PBS) and incubated with trypsin EDTA (0.5 mL) for less than 1 min before adding equal amount of F-12K medium (0.5 mL). The cells were centrifuged at 300 x g for 5 min, washed twice with 1 mL of PBS and lysed using 0.5 ml lysis buffer. The lysed mixtures were centrifuged at 13000 x g for 5 min and the supernatant (approximately 150-300 µL) was collected. Protein concentration of the cell lysate sample was determined by the method (Lowry *et al*., 1951), bovine serum albumin as standard.

2.4. Pterin Binding and Dissociation Studies

For binding studies, endothelial cells (HUVEC and Ea.hy926) grown to confluence in their respective medium were washed twice with 1 ml of 1X PBS, before pre-incubating with 50 µM [³H] BH_4 in Locke's buffer (2mL) for 1 h. The cells were again washed twice with 1X PBS (1 mL) to remove any trace [³H] BH_4. The cells were either exposed to 200 fold higher molar concentration dose of unlabeled BH_4, at varying time intervals ranging in 10 min increments, for up to 1 h; or

exposed to varying molar concentrations of BH_2 (10^{-9} to 10^{-4} M) for 1 h. Control cells pre-exposed to 50 µM [³H] BH_4 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 BH_2 and BH_4 after forming a complex with eNOS was tested by initially pre-exposing both cell lines to either 100 µM [³H] BH_2 or [³H] BH_4 for 1 h in Locke's buffer, followed by subsequent incubation in Locke's buffer for 1 h with $10^-9\text{-}10^{-4}$ molar concentrations of BH_4 and BH_2 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] BH_4 oxidation.

2.5. Pterin Quantification by HPLC

Cellular levels of BH_4, BH_2 and the quinonoid isoform of BH_2 (qBH_2) and Biopterin (B) levels were quantified aspreviously described (Heales and Hyland, 1989). The mobile phase consisted of sodium acetate (50 $mmol^{-1}$), citric acid (5 $mmol^{-1}$) pH 5.2, containing EDTA (48 $µmol^{-1}$) and DTT (0.1 $mmol^{-1}$). 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 BH_4 and BH_2 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 nitrite 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 nitrite/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 mU 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).

2.7. Superoxide ($O_2^{\bullet-}$) Measurement

$O_2^{\bullet-}$ 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 $\pm$ 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 $O_2^{\bullet}$ 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, with reciprocal increases in BH_2 content (P < 0.01) was observed, as shown in **Fig. 1** and **Table 1**. The accumulation of BH_2 was found to be almost exclusive, as the quinonoid tautomer qBH_2 and biopterin, were not detected (data not shown). This increase in BH_2 accumulation in endothelial cells occurred progressively with subsequent increases seen in glucose accumulation and $O_2^{\bullet}$ 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 $O_2^{\bullet}$ 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 $O_2^{\bullet}$ production by NOS and other flavoproteins, including NADPH oxidase (data not shown). The significant suppression of $O_2^{\bullet}$ 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 $O_2^{\bullet}$ 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$\pm$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 (PH₄), 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 biopterin 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_2 (thereby forming the eNOS-[³H] BH_2 complex) remained unaltered to subsequent exposures to varying concentrations of $BH_4 \geq 10^{-3}$ 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_2 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 $O_2^{\bullet}$ over NO production.

**Table 1.** Analysis of cellular conditions during continuous ARG exposure

	Units	Control	Treatments	
			Acute ARG (2 h challenge dose)	Chronic ARG (7 Days + 2 h challenge dose)
BH$_4$	pmol/mg protein	93±9.2	88.2±8.4	45.8±7.1*#
BH2	pmol/mg protein	15.3±1.1	16.5±1.4	42.4±5.2*#
NO$_2$ + NO$_3$	pmol/µg protein	3.05±0.37	4.15±0.25 *	1.28±0.3 *#
Glucose	mg/dl	54.1±6.6	70.8±8.1 *	128.3±11.2*#
eNOS	ng/µg protin	0.72±0.15	0.73±0.17	0.51±0.15 *#
O$_2$	Arb. Units	1.64±0.49	2.82±0.15 *	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

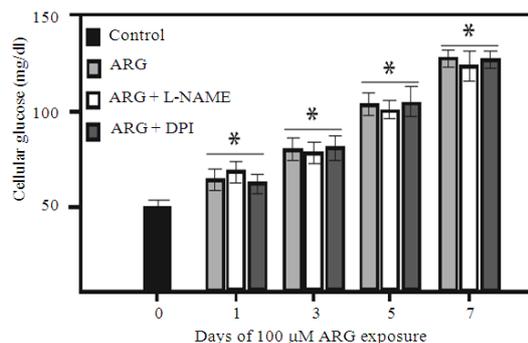|	Units	Control	100µM ARG	7 day Chronic Treatments	
				Chronic ARG 100 µM ARG + 2 µM Rotenone	100 µM ARG + 5 µM TTFA
BH$_4$	pmol/mg protein	93±9.2	45.8±7.1 *	17.4±2.2 #	89.6±10.2 #
BH$_2$	pmol/mg protein	15.3±1.1	42.4±5.2 *	13.4±3.2 #	
NO$_2$ + NO$_3$	pmol/µg protein	3.05±0.37	1.28±0.3 *	2.81±0.26 #	3.32±0.31 #
Glucose	mg/dl	54.1±6.6	128.3±11.2 *	118.7±8.2 *	120.8±6.3 *
eNOS	ng/µg protin	0.72±0.08	0.51±0.11 *	0.69±0.12 #	0.71±0.10 #
O$_2$	Arb. Units	1.64±0.49	3.49±0.32 *	1.56±0.23 #	1.86±0.21 #

* $p < 0.05$ versus control, # $p < 0.05$ versus ARG chronic treatment. (n = 6)
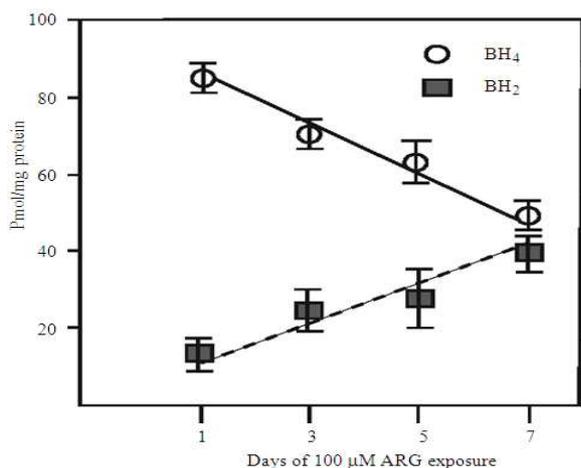
Fig. 1. Continuous ARG exposure in endothelial cells increases oxidation of BH$_4$ to BH$_2$ in a concentration dependent manner. BH$_4$ oxidation was not evident during short term (2 h) exposure to ARG. Points shown in the figure are representing mean ± SE of six determinants

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 supress glucose accumulation during continuous ARG exposure. All indicative values are mean ± SE (n = 6). *, $p < 0.05$ versus control treatments
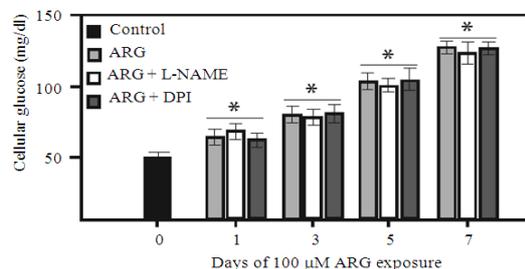
Fig. 3. Cellular O2$^{\cdot-}$ generation during continuous ARG exposure for 7 days in culture medium followed by 2 h challenge in Locke's Buffer. L-NAME and DPI significantly attenuated O2$^{\cdot-}$ generation (*, $p <0.05$) during continuous ARG treatment. All indicative values are mean ± SE (n = 6)

3.3. Role of Mitochondrial Electron Transport Chain in ARG Induced O$_2^{\cdot}$ Generation and BH$_4$ Oxidation
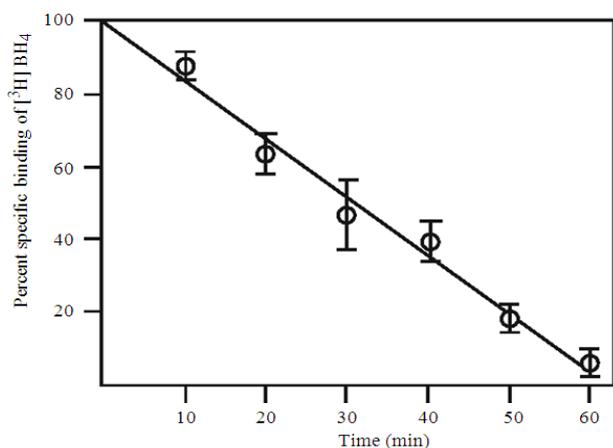
The mitochondrion electron transport chain is notably a predominant source of O$_2^{\cdot-}$ generation in normal respiring cells. Since eNOS derived O$_2^{\cdot}$ was found to be necessary for oxidation of BH$_4$ in endothelial cells, we asked the question as to whether O$_2^{\cdot-}$ produced from mitochondrion during initial ARG exposure is key to originate the BH$_4$ oxidation and subsequent eNOS uncoupling.

Fig. 4. Characterization of competitive binding and of BH_4 to eNOS. All binding studies were done at 22°C. Points are mean ± SE of six determinations
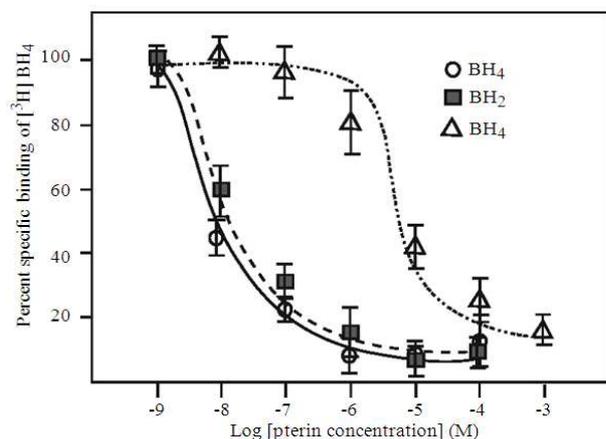
Fig 5. Kinetics of dissociation efficacy of [^{3}H] BH4 based on competitive binding of unlabeled pterin to eNOS. Unlabeled BH_2 shows similar binding efficacy to eNOS as BH4, while PH4, which lacks the 6-position dihydroxy-propyl side chain, shows >1000-fold lower affinity versus BH_4 or BH_2. All binding studies were done at 22°C. Points are mean ± SE of six determinations

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 BH_4 oxidation and $O_2^{\cdot}$ 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).
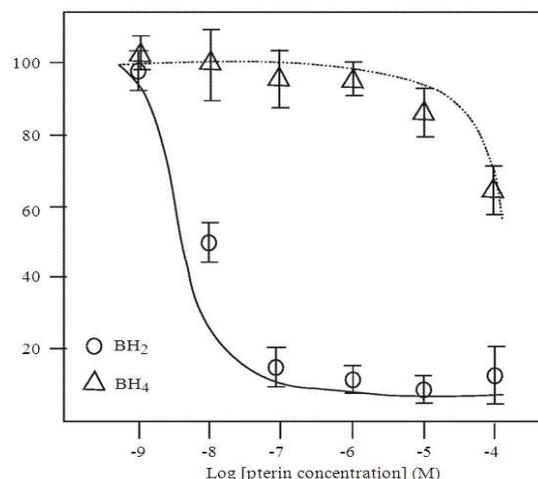
Fig. 6. Binding avidity of pterins. BH_2 shows greater binding avidity to eNOS in forming a complex than BH4 when exposed to >10^{-3}M BH_4. Complex formed between [^{3}H] BH_4 and eNOS was lost when exposed to unbound BH_2 of >10^{-8}M. (n =6)

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 $O2^{\cdot-}$ from ARG was implicated (Simonet *et al*., 2004). The $O_2^{\cdot}$ formed in association with continuous ARG exposure, scavenges the available NO, resulting in the formation of peroxynitrite formation, thereby compromising NO bioactivity by promoting the oxidation of BH_4, 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 BH_2 binding to eNOS, alteration to intracellular BH_4 to BH_2 ratio and the greater binding avidity of eNOS with BH_2 than BH_4, as the key determinants in initiating the various tolerance sparing events observed during continuous ARG supplementation, rather than simply a consequence of BH_4 oxidation.

We showed during continuous ARG supplementation that the accumulation of BH_2 in endothelial cells to increase reciprocally towards subsequent decrease in BH_4 level, with concomitant increases seen in glucose accumulation and $O_2^{\cdot}$ production, while compromising ARG consumption by eNOS for NO generation. If BH_4 oxidation is the primary basis of eNOS uncoupling, supplementation of BH_4 would rapidly reinstate 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_4 to enhance eNOS activity and suppress eNOS derived $O_2^{\cdot}$ generation (Alp and Channon, 2004). Nonetheless, the possibility exists that progressive oxidation of BH_4 would result in BH_2 buildup, which would lead to increase affinity of eNOS binding with BH_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_4, the cells pre-incubated with [^{3}H] BH_4 was exposed to varying concentrations of BH_4, BH_2 and PH_4.

Our binding studies showed that cells supplemented with BH_2 instead of BH_4, does not diminish the high affinity level achievable with BH_4 versus BH_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_2 was found to be retained with greater avidity than those achieved with BH_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_4 and its primary two-electron oxidization product as BH_2 and not a consequence of BH_4 oxidation alone, as has been generally thought until now.

Our studies show that BH_2 binds with greater avidity to eNOS (than BH_4) and provokes uncoupling has additional important implications in considering BH_4 supplementation being used for various therapy related to ARG mediated endothelial dysfunction. Previous studies (Gori *et al*., 2001; Heitzer *et al*., 2000; Higashi *et al*., 2002; Setoguchi *et al*., 2002; Stroes *et al*., 1997) have suggested acute supplementation of high doses of BH_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_4 administration in the setting of oxidative stress. The redox sensitive BH_4, when prone to oxidation during ARG associated $O_2^{\cdot}$ generation willfavor BH_2 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_2 complex to influence endothelial dysfunction and tolerance development during supplementation of ARG, we have also attempted to identify the predominant source that instigates BH_4 oxidation, in the first place. Mitochondrial mediated $O_2^{\cdot}$ production is considered to provide a trigger for metabolic derangement that mediates diabetic complications. To test whether mitochondrion derived $O_2^{\cdot}$ plays a role in ARG induced BH_4 oxidation, we assessed whether selective inhibitors of the mitochondrial electron transport chain complexes can provide protection against BH_4 oxidation in endothelial cells. The significant inhibition in BH_4 oxidation during ARG supplementation with inhibitors of the mitochondrial electron transport chain complexes I and II implicate a role for mitochondrion derived $O_2^{\cdot}$ generation in the genesis of ARG induced BH_4 oxidation, leading to eNOS uncoupling.

While the involvement of mitochondrial electron transport chain inhibitors prevented BH_4 oxidation and subsequent $O_2^{\cdot}$ 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 $O_2^{\cdot}$ 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_4-to-BH_2 ratio or increase in BH_2 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 $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ overproduction via electron transport leak from the mitochondria. The reaction between $O_2^{\cdot}$ and eNOS derived NO results in peroxynitrite synthesis, which promotes BH_4 oxidation and hence accumulation of BH_2. 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.

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7. REFERENCES

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