Potential role of mitochondrial superoxide decreasing ferrochelatase & heme in coronary artery soluble guanylate cyclase depletion by Angiotensin II

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Running Title: Mitochondrial regulation of coronary artery guanyl cyclase

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ABSTRACT

Oxidation of the soluble guanylate cyclase (sGC) heme promotes loss of regulation by nitric oxide (NO) and depletion of sGC. We hypothesized that angiotensin II (AngII) stimulation of mitochondrial superoxide by its AT1 receptor could function as a potential inhibitor of heme biosynthesis by ferrochelatase, and this could decrease vascular responsiveness to NO by depleting sGC. These processes were investigated in a 24hr organoid culture model of bovine coronary arteries (BCA) with 0.1µM AngII. Treatment of BCA with AngII increased mitochondrial superoxide, depleted mitochondrial superoxide dismutase (SOD2), ferrochelatase and cytochrome oxidase subunit-4, and sGC, associated with impairment of relaxation to NO. These processes were attenuated by organoid culture with 8-bromo-cGMP and/or δ-aminolevulinic acid (ALA, a stimulator of sGC by protoporphyrin IX generation and heme biosynthesis). Organoid culture with Mito-TEMPOL, a scavenger of mitochondrial matrix superoxide, also attenuated AngII elicited ferrochelatase depletion and loss of relaxation to NO. Whereas, organoid culture with Tempol, an extra-mitochondrial scavenger of superoxide, attenuated the loss of relaxation to NO by AngII, but not ferrochelatase depletion, suggesting cytosolic superoxide could be an initiating factor in the loss of sGC regulation by NO. The depletion of cytochrome oxidase subunit-4 and sGC (but not catalase), suggests that sGC expression may be very sensitive to depletion of heme caused by AngII disrupting ferrochelatase activity by increasing mitochondrial superoxide. In addition, cGMP-dependent activation of protein kinase G appears to attenuate these AngII stimulated processes through both preventing SOD2 depletion and increases in mitochondrial and extra-mitochondrial superoxide.
Angiotensin II-elicited increases in coronary arterial mitochondrial superoxide caused a loss of ferrochelatase, which appeared to promote a depletion of soluble guanylate cyclase and impaired relaxation to nitric oxide by decreasing heme availability. The heme precursor δ-aminolevulinic acid appeared to prevent these processes by promoting cGMP-stimulation of protein kinase G.
Angiotensin II (Ang II) is thought to be a prominent factor in many aspects of oxidant-associated vascular dysfunction and the progression of hypertension-associated disease processes (5-7, 16-18, 21, 23, 27, 29). There is substantial evidence for angiotensin II increasing the activities of Nox oxidases and mitochondrial superoxide levels through the Ang II type 1 (AT1) receptor in endothelium and vascular smooth muscle. In addition to decreasing the availability of endothelium-derived nitric oxide (NO), Ang II-elicited increases in Nox oxidase activity have been associated with decreasing the responsiveness of vascular smooth muscle to NO through mechanisms including sGC depletion (21). The oxidation of sGC-bound heme in vascular smooth muscle from its normal NO-stimulated Fe²⁺ form to Fe³⁺ is thought to occur in disease processes such as hypertension, and this results in a loss of sGC stimulation by NO, depletion of its heme and a subsequent ubiquitination-activated proteolytic degradation of sGC (28). Peroxynitrite generated from the reaction of superoxide with NO has been observed to oxidize the heme of sGC in vascular tissue (28). Thus, it is well established that impairment of sGC activation by endothelium-derived NO is a major factor in many cardiovascular diseases, including hypertension (6, 16, 21), and modulation of its bound Fe²⁺-heme can be an important contributor to the vascular dysfunction that is observed.

There has been minimal previous consideration of how the biosynthesis of heme influences the function of sGC or is altered in cardiovascular disease processes. It has been proposed that heme biosynthesis is impaired by aging associated with studies observing that subunit 4 of cytochrome C oxidase in the mitochondrial electron transport chain is rapidly depleted when heme biosynthesis by ferrochelatase (FECH) is impaired
A recent study examining the *in vivo* consequences in heterozygous mice deficient in the mitochondrial matrix manganese-containing form of superoxide dismutase (SOD2) in erythrocyte precursor cells detected evidence for decreased activity of FECH (3). Based on evidence for superoxide disrupting iron-sulfur clusters (20) and FECH having an essential iron-sulfur center (4), it was suggested that increased mitochondrial superoxide-elicited impairment of this enzyme’s function was responsible for the observed decrease in hematocrit and hemoglobin that were rescued by an *in vivo* Mito-TEMPOL therapy targeting the increased mitochondrial superoxide (3). Although the actual actions of superoxide on FECH are not well documented, high levels of NO potentially associated with the formation of reactive NO-derived species have been observed to inactivate FECH (8). Since increased mitochondrial superoxide has been detected in vascular disease models (15) and in the actions of angiotensin II on vascular smooth muscle (6), we hypothesized that increases in mitochondrial superoxide could impair mitochondrial ferrochelatase and disrupt mitochondrial heme biosynthesis and its ability to maintain the heme needed for sGC regulation.

Initial studies were conducted to investigate if Ang II induced mitochondrial superoxide causes a loss of FECH, and it was observed that organoid culture of endothelium-denuded bovine coronary arteries for 24 hours with 0.1µM Ang II decreased FECH activity in a manner that was inhibited by organoid culture with Mito-TEMPOL. We then investigated if this exposure to Ang II showed evidence for depletion of heme and sGC. The depletion of subunit 4 of cytochrome oxidase (COx4), but not catalase, was used as an indicator to detect conditions of heme depletion on proteins whose expression is very sensitive to low heme (2). In addition, since we previously reported that 24-hour
organoid culture of bovine arteries with the heme precursor δ-aminolevulinic acid (ALA)
caused an increase in sGC activity by generating protoporphyrin IX (PpIX) (19), as well as
finding that ALA attenuated endothelin-1 (ET-1) induced mitochondrial superoxide in
bovine pulmonary arteries associated with prevention of pulmonary hypertension (PH)
(1), we also investigated if ALA and cGMP mechanisms could protect the function of
FECH through lowering mitochondrial superoxide generation.

**Materials and Methods**

**Materials:** All physiological buffers were prepared using analytical grade reagent salts
purchased from J. T. Baker Chemical. All other chemicals were obtained from Sigma
Chemical unless otherwise mentioned. Catalase, SOD-2 and ferrochelatase antibodies
were purchased from Abcam. Phospho-vasodilator-stimulated phosphoprotein (VASP),
Total-VASP and voltage dependent anion channel (VDAC) antibodies were purchased
from Cell Signaling (Beverly, MA). β-actin antibody was purchased from Sigma
Chemicals. ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) and Spermine
NONOate was purchased from Cayman Chemical (Ann Arbor, MI). Mito-TEMPOL
(2,2,6,6-Tetramethyl-4-[[5-(triphenylphosphonio)pentyl]oxy]-1-piperidinyloxy bromide)
was obtained from Abcam. Cytochrome oxidase subunit-4 (COx4) antibody, MitoSox
and dihydroethidium (DHE) were purchased from Life Technologies. All gases were
obtained from Air Gas (Allentown, PA).
**Tissue Preparations:** Bovine hearts were obtained from a slaughterhouse in ice-cold PBS. Left anterior descending arteries were used for bovine coronary artery (BCA) experiments. BCAs were cleaned of their connective tissue and then cut into rings of 2–3 mm in diameter and width. The endothelium was removed by rubbing the lumen. Organoid cultured BCA rings were used in experiments for vascular reactivity, superoxide measurements, PpIX fluorescence, FECH activity and Western blot protein analysis. As indicated in *Results*, organoid cultures were performed (22) with BCA rings in the absence and presence of 0.1 µM Ang II, 100 µM ALA, 50µM gp91dstat, 100 µM 8-Bromo-cGMP, 1mM Mito-TEMPOL and 1mM Tempol with DMEM media containing 10% FBS and 1% antibiotics (penicillin, streptomycin, and amphotericin B) for 24 hrs at 37°C with 5% CO₂.

**Ferrochelatase Activity Assay:** As published previously (1) BCA rings were pulverized in liquid nitrogen, incubated on ice for 45 minutes with 50 µL of buffer (0.25 M Tris-HCl buffer, pH 8.2, containing 1% Triton X-100 and 1.75 mM of palmitic acid), and then sonicated on ice for 10 seconds. Samples were then centrifuged and the supernatant was assayed for protein content by the Bradford method. The amount of FECH activity present after 60 minute incubation was assayed by the accumulation of Zinc PpIX (ZnPpIX) from 67µM PpIX and 42µM zinc acetate, using 20µg protein in a final volume of 30µl, as previously described (30). At the end FECH reaction was stopped by using dimethyl sulfoxide: methanol (30:70) solution. HPLC measurement of the amount of ZnPpIX formation was used to determine the FECH activity in each sample. At the beginning of the experiment, to generate a standard curve for ZnPpIX,
increasing concentrations of ZnPpIX were loaded into the column. We used an Agilent 1100 HPLC system using a normal phase a Phenomenex column (Luna 5µ Silica-2 100A, 250 x 4.6 mm) with acetone:methanol:water:formic acid (560:240:200:2) and 1 mL/min, as the mobile phase. ZnPpIX was detected based on the amount of fluorescence observed with excitation and emission wavelengths of 415 nm and 580 nm, respectively.

**Detection of changes in mitochondrial and extra-mitochondrial superoxide:** HPLC measurement of the superoxide-specific hydroxylated products of MitoSox and dihydroethidium were employed for quantifying changes in mitochondrial matrix and extra-mitochondrial matrix superoxide, using previously described methods (32). At the beginning of the experiment, a standard curve was generated by injecting increasing concentrations of Mito-2-hydroxyethidium or 2-hydroxyethidium into the column. After 24 hrs of organoid culture, BCA rings were incubated with either 5 µM MitoSox or dihydroethidium (DHE) for one hour in the dark (1, 9) under conditions described in the Results section, to measure mitochondrial and extra-mitochondrial superoxide, respectively. They were washed several times with Krebs solution buffered with 10mM HEPES-NaOH (pH 7.4), and then flash frozen with liquid nitrogen. Tissues were first weighed and then pulverized in the presence of liquid nitrogen, dissolved in a solution of 100% acetonitrile (HPLC grade). These samples were incubated at -20°C for 1 hr. After 1 hr samples were centrifuged and the supernatant was used for HPLC analysis of the superoxide-specific hydroxylated product of MitoSox (Mito-2-hydroxyethidium) or of dihydroethidine (2-hydroxyethidium) using an HPLC system with a Jasco FP-1520
fluorescence detector and a Beckman ultrasphere reverse column (C18) (5µ, 250 x 4.6 mm).

**Measurement of protoporphyrin IX fluorescence:** PpIX fluorescence from BCA rings was measured by a method described previously by our laboratory (19). Increases in protoporphyrin IX fluorescence were measured using an excitation wavelength of 485 ± 20 and emission of 620 ±40 nm in 24 hr organoid cultured BCA rings with or without Ang II and ALA. Arterial rings were placed in the bottom of the ~6-mm diameter wells of a sterile 96-well microplate with 200 µl of Krebs containing 10 mM HEPES buffer (pH 7.4). PpIX fluorescence was measured from the bottom surface of the plate using BIOTEK fluorescent microplate reader (model FLx800i), as previously described (19). Data are reported in the arbitrary fluorescence units measured (AU) after subtraction of the low levels of background fluorescence observed in the absence of BCA rings.

**Western blot analysis:** Frozen BCAs were pulverized and then homogenized in lysis buffer containing protease and phosphatase inhibitors, as previously described (25). Bradford method was used for protein quantification assay, and samples were prepared for gel electrophoresis. Proteins were separated using a 10% SDS-polyacrylamide gel under reducing and denaturing conditions. Gels were transferred to PVDF membranes, and the membranes were blocked with Tris-buffered saline with Tween-20 + 5% milk for 1 hr. After this the membranes were exposed to primary and secondary antibodies as per the manufacturer’s protocol. Protein bands were visualized with an enhanced chemiluminescence kit (Pierce, Rockford, IL) on X-OMAT autoradiography paper.
(Kodak, Rochester, NY) in a dark room. Protein levels were measured using densitometry analysis with the UN-SCAN-IT gel software by Silk Scientific (Orem, UT).

Molecular weights (kDa) of different proteins are as follows: SOD-2: 25; sGCβ1: 70; FECH: 50; Catalase: 60; COx4: 15. For detection of changes in the expression of mitochondrial proteins (SOD-2, FECH and COx4), the mitochondrial protein VDAC was used as a loading control. For detection of changes in the expression of sGCβ1 and Catalase, β-actin was used as a loading control. The expression of these loading controls was not altered under the conditions examined in the Results. Thiol-reducing conditions were avoided in the samples analyzed for PKG1α dimmer (22).

Measurement of vascular reactivity in BCA: Endothelium-rubbed 24 hr organoid cultured BCA rings were mounted on Grass FT-03 or Coulborne Instruments force displacement transducers for recording isometric force development through a Powerlab data acquisition system obtained from ADInstruments (Colorado Springs, CO), as previously described (25). Arterial rings were incubated at 37°C in Krebs-bicarbonate buffer (pH 7.4) containing 118 mM NaCl, 4.7 mM KCl, 1.5 mM CaCl₂, 25 mM NaHCO₃, 1.1 mM MgSO₄, 1.2 mM KH₂PO₄, and 5.6 mM glucose under an atmosphere of 21% O₂-5% CO₂-74% N₂ (pH 7.4) for 1 hr under resting tension of 5 g. In all studies, arterial rings were depolarized with 123 mM KCl containing Krebs bicarbonate buffer, and the rings were then reequilibrated with Krebs-bicarbonate buffer for 30 min and subsequently contracted with Krebs bicarbonate containing 30 mM KCl.
Arterial rings were either relaxed to increasing cumulative concentrations of spermine-
NONOate (10^{-9} M to 10^{-5} M) or Isoproterenol (10^{-10} M to 10^{-5} M).

Statistical Analysis: Data values are means ± SE of the number of arterial segments (n) from different animals. Statistical analyses between two groups were performed with paired and unpaired Student’s t-test, and a one-way ANOVA with Newman Keuls correction were used for comparison between multiple groups. A value of \( P < 0.05 \) was used to establish statistical significance.

RESULTS

Organoid culture of BCA with Ang II decreases ferrochelatase activity through processes prevented by scavenging mitochondrial superoxide and by ALA:

Endothelium-denuded BCA were organoid cultured with 0.1\( \mu \)M Ang II for 24 hours to examine the direct effects of Ang II on coronary arterial smooth muscle in the absence of its effects on endothelium and changes in the influence of endothelium-derived mediators. Initial experiments for developing this study shown in Figure 1 suggested that decreased ferrochelatase (FECH) activity was readily detected in BCA exposed to the Ang II-organoid culture conditions. As shown in figure 1, Ang II decreased FECH activity in a manner which was prevented by organoid culture in the presence of Mito-TEMPOL, a mitochondrial superoxide scavenger, ALA and the Ang II type-1 receptor antagonist losartan. The NADPH oxidase (Nox oxidase) inhibitor gp91ds-tat (11) or a relatively large
1 mM dose of the extra-mitochondrial superoxide scavenger Tempol did not have a
significant effect in preventing the inhibition of FECH by Ang II.

Ang II induces increases in generation of mitochondrial superoxide and extra-
mitochondrial superoxide through processes attenuated ALA: Organoid culture of
BCA with Ang II caused increases in mitochondrial superoxide (Figure 2A) based on the
detection of a MitoSox-derived superoxide specific product by HPLC. Under these
conditions, Ang II also increased extra-mitochondrial superoxide (Figure 2B) based on the
detection of a dihydroethidine-derived superoxide specific product by HPLC. The increase
in mitochondrial superoxide elicited by Ang II was significantly attenuated by ALA (Figure
2A). The conditions of ALA co-treatment with Ang II did not appear to show a significant
increase in extra-mitochondrial superoxide compared to the Control. While this suggests
that ALA attenuates the Ang II induced increase in extra-mitochondrial superoxide
generation, the levels of superoxide detected under these conditions was not significantly
different from those observed with Ang II in the absence of ALA (Figure 2B). Thus, the
influence of ALA on the detection of changes in superoxide are consistent with Ang II
promoting a loss of FECH activity under conditions where it is increasing mitochondrial
superoxide.

Effects of Ang II and ALA during 24 hour organoid culture of BCA on the
expression of mitochondrial matrix SOD-2: Treatment of BCAs with Ang II decreased
SOD-2 (Figure 3A), expression under conditions where mitochondrial superoxide was
elevated. Treatment of BCA with ALA under organoid culture conditions did not
significantly alter the expression SOD-2. However, co-treatment with Ang II together with ALA prevented depletion of SOD-2 by Ang II.

Effects of Ang II and ALA during 24 hour organoid culture of BCA on the expression of sGCβ1 and the influence of Ang II on PKG activation: Treatment of BCAs with Ang II decreased sGCβ1 (Figure 3B) expression under conditions where both mitochondrial and extra-mitochondrial superoxide were elevated. Treatment of BCA with ALA under organoid culture conditions did not significantly alter the expression of sGCβ1. However, co-treatment with Ang II together with ALA prevented depletion of sGCβ1 by Ang II. The treatment of BCA with Ang II significantly decreased endogenous protein kinase G activity as documented by a decrease in Serine\textsuperscript{239} phosphorylation of VASP (Figure 3C). These observations suggest that Ang II is promoting a depletion of sGC associated with a decrease in PKG activity detected through the decreased in its phosphorylation of VASP.

Effects of organoid culture of BCA with Ang II and ALA on FECH expression and protoporphyrin IX fluorescence: The expression of FECH was measured in BCA to determine if changes in the activity of FECH shown in Figure 1 were associated changes in the expression of this protein. Treatment of BCAs with Ang II decreased FECH expression (Figure 4A) under conditions where mitochondrial superoxide was elevated. Measurements of changes in the surface fluorescence of PpIX (19) were examined to detect if Ang II influences BCA levels of PpIX in the absence and presence of promoting PpIX accumulation with ALA. While Ang II did not significantly alter the levels of PpIX,
ALA increased PpIX levels in 24 hr organoid cultured BCAs both in the absence or presence of Ang II (Figure 4B). While the increase in PpIX fluorescence elicited by ALA together with Ang II appeared slightly greater than ALA alone, this difference was not statistically significant.

8-bromo-cGMP co-treatment reverses Ang II induced increases in generation of mitochondrial superoxide and extra-mitochondrial superoxide: The PKG activator 8-bromo-cGMP was used to examine if cGMP/PKG signaling functions to attenuate increases in mitochondrial superoxide and potential consequences of its actions on FECH that were observed with ALA. These experiments were designed to help define processes potentially regulated by cGMP as a result of PpIX generated from ALA stimulating sGC (19). Ang II-elicited increases in mitochondrial superoxide (Figure 5A) and extra-mitochondrial (cytosolic) (Figure 5B) superoxide observed in organoid cultured BCA were significantly attenuated by the protein kinase G (PKG) activator, 8-bromo-cGMP, suggesting that cGMP may have a major role in superoxide regulation and the actions of ALA.

Effects of Ang II and 8-bromo-cGMP during 24 hour organoid culture of BCA on expression of the heme-containing protein subunits sGCβ1, COX4 and Catalase: Organoid culture of BCA with Ang II also depleted sGCβ1 (Figure 6A) and COX4 (Figure 6B), but not catalase (Figure 6C), suggesting that Ang II depletes heme, and that sGC expression may be very sensitive to the depletion of heme caused by Ang II. These
effects of Ang II were significantly attenuated by co-organoid culture with the PKG activator 8-bromo-cGMP.

**Effects of Ang II during 24 hour organoid culture of BCA on relaxation to a NO donor and Isoproterenol:** Spermine-NONOate was used in this study as an NO donor, under conditions where NO-mediated relaxation functions primarily through a Fe$^{2+}$ heme-dependent stimulation of sGC (13). As shown in figure 7, the 24 hr organoid culture of Ang II significantly reduced relaxation to NONOate at doses of $10^{-7}$ M, $10^{-6}$ M and $10^{-5}$ M. ALA (Figure 7A), Mito-TEMPOL (Figure 7B) and Tempol (Figure 7C) co-treatment significantly reversed the effects of Ang II. Acute treatment with 10 μM ODQ markedly inhibited NONOate-induced relaxation (Figure 7D) at doses of $10^{-7}$ M, $10^{-6}$ M and $10^{-5}$ M in control organoid cultured BCA rings. While acute treatment with 10 μM ODQ inhibited NONOate-induced relaxation at $10^{-5}$ M dose in BCA rings organoid cultured with Ang II, Ang II did alter the minor relaxation to NONOate observed in the presence of ODQ. These data suggest that the increased mitochondrial and extra-mitochondrial superoxide elicited by Angiotensin II contributes to the observed loss of NO-stimulated vasodilation. Isoproterenol, the β-adrenergic receptor agonist of cAMP-mediated relaxation, was used as a sGC-independent relaxing agent. As shown in Figure 8, relaxation of BCA to isoproterenol was not altered by Ang II or by any other co-treatments in organoid treated BCAs. None of these treatments had a significant effect on the force generated by 30 mM KCl used to examine relaxation responses to NONOate or isoproterenol under any of the conditions examined in these studies (not shown). Thus, the actions of Ang II
appeared to selectively attenuate cGMP-associated relaxation to a NO donor, without
altering cAMP-associated relaxation to isoproterenol.

**DISCUSSION**

The data in this study provide evidence that organ culture of endothelium-
denuded bovine coronary arteries with Ang II increases mitochondrial and extra-
mitochondrial superoxide generation in a manner that appears to be associated with a
depletion of sGC. Under these conditions, Ang II appeared to selectively attenuate
cGMP-associated relaxation to a NO donor, without altering cAMP-associated
relaxation to isoproterenol. Since this loss of relaxation to a NO donor resulting from
organoid cultured BCAs with Ang II was attenuated by organoid culture in the presence
of Tempol or Mito-TEMPOL (Figure 7), both mitochondrial and extra-mitochondrial
superoxide appear to be contributing to the observed loss of relaxation to NO. Organoid
culture of BCA with Ang II also caused a loss of SOD2 expression, ferrochelatase
expression and activity and sGC expression, and decreased PKG phosphorylation of
VASP, under conditions that appeared to be associated with a depletion of heme based
on the observed depletion of subunit 4 of cytochrome oxidase. These data suggest that
sGC expression and its ability to participate in promoting relaxation by NO appear to be
very sensitive to actions of superoxide, potentially through superoxide influencing the
availability of heme and interactions of NO and heme with sGC.

Organoid culture with ALA appeared to reverse many of the effects of Ang II,
including restoring sGC-mediated relaxation to NO. ALA is likely to be functioning under
the organoid culture conditions employed through promoting the biosynthesis of PpIX and/or heme, because increases in PpIX was detected by its fluorescence (Fig. 4B) and the decreased level of COx4 suggesting heme depletion was restored. Since it is known that activators of sGC which function through binding it’s PpIX/heme site prevent sGC degradation once its heme is oxidized (28), this could also be a factor in preventing sGC degradation. However, the observed protection of sGC depletion by the 8-bromo-cGMP activator of PKG suggests additional processes may be involved in controlling the loss of sGC expression caused by Ang II. Since organoid culture of arteries with 8-bromo-cGMP attenuated Ang II-elicited increases in mitochondrial and extramitochondrial (cytosolic) superoxide in a manner similar to ALA, PKG activation as a result of ALA-generated PpIX stimulating cGMP production by sGC (19) appears to be a major factor in the actions of ALA.

Oxidation of the sGC heme and sGC depletion have been observed to be associated with the formation of reactive NO-derived oxidant species (28). However, processes through which superoxide could promote the oxidation of the heme of sGC in an intracellular environment with low levels of NO, such as the organoid culture conditions with endothelium-denuded BCA used in the present study, are not well defined. While the data in Figure 7 are consistent with the presence of Tempol during organoid culture with Ang II attenuating the loss of sGC-dependent relaxation to the NO-donor studied, the high dose of Tempol used leaves open the possibility that it may be functioning through actions that go beyond its intended use as an extra-mitochondrial scavenger of superoxide. In addition, other processes could prevent the depletion of sGC associated with heme oxidation. The Fe^{2+} heme form of sGC appears
to bind PpIX (13, 31) and other activators of sGC that bind its heme site (28), suggesting heme may be released from sGC even in the absence of substantial heme oxidation. Thus, the availability of PpIX, heme and perhaps other porphyrins or activators which bind the heme site of sGC could be a major factor in controlling or maintaining both the expression of sGC by preventing its degradation by proteolysis (26) and the ability of sGC to maintain its stimulation by mediators such as NO. In addition, any process increasing cGMP or promoting PKG activation could function to attenuate the actions of pathophysiological mediators that are associated with increasing superoxide, disrupting heme biosynthesis and/or promoting sGC heme oxidation or depletion.

The inhibition and depletion of ferrochelatase caused by Ang II-elicited increases in mitochondrial superoxide in the present study is potentially a major factor in impairing the availability of heme and its potential influence in maintaining the expression of sGC. The Fe-S cluster of FECH seems to be essential for maintaining its stability and activity (4). It was recently demonstrated that mice with a conditional knockout of mitochondrial matrix SOD2 in hematopoietic stem cells showed evidence of decreased FECH activity, which could originate from superoxide disrupting its Fe-S cluster (20). These animals also showed decreased hemoglobin or hematocrit that was reversed by treatment of the mice with Mito-TEMPOL, suggesting mitochondrial superoxide may disrupt heme biosynthesis and influence other processes potentially related to iron metabolism and oxidant stress (18). Previous studies inhibiting ferrochelatase with N-methyl-PpIX detected a selective depletion of subunit-4 of cytochrome oxidase, but not catalase, suggesting that what is thought to be a small pool of heme, may be needed to maintain
the expression of some heme-containing proteins. Since Mito-TEMPOL, but not Tempol or the Nox oxidase inhibitor gp91dstat, prevented the decrease in FECH activity in coronary arteries organoid cultured with Ang II (Figure 1), it appears that mitochondrial superoxide is a key factor in inhibiting FECH activity, and that other extra-mitochondrial sources of superoxide do not seem to be influencing FECH activity under the conditions examined. The pattern of protective effects of ALA and/or 8-bromo-cGMP suggest that PKG activation promoted by these agents is functioning to prevent the depletion of SOD2 and increased mitochondrial matrix superoxide-mediated inhibition of FECH, and the associated depletion of heme needed for maintaining the expression of COx4 and sGC through hypothesized relationships illustrated in the model shown in Figure 9. Further studies are needed to define the properties of key aspects of this model, such as how cGMP signaling alters the expression of SOD2 and attenuates increased production of mitochondrial superoxide, and to examine how these systems function in vivo under pathophysiological conditions.

The results of this study provide documentation that promoting PpIX accumulation, preventing the inactivation of ferrochelatase by mitochondrial superoxide and preserving heme biosynthesis are potentially targets for developing new beneficial therapeutic approaches, especially for maintaining processes such as NO regulation of sGC and aspects of cGMP-mediated regulation (28) such as vasodilation, inhibition of processes involved in inflammatory signaling and vascular remodeling. For example, mitochondrial superoxide scavengers which function in a manner similar to Mito-TEMPOL have the potential for being effective in attenuating the disruption of heme biosynthesis, and Mito-TEMPO has been reported to attenuate vascular dysfunction in
the Ang II infusion model (6, 17). ALA has already been demonstrated to prevent hypoxia-induced pulmonary hypertension in mice (1, 24). Although ALA was not protective in a mouse renal ischemia-reperfusion injury model, when used in combination with ferrous iron, it was protective as a result of generating heme in amounts that appeared to both induce and support the beneficial actions of heme oxygenase (8). The use of ALA for fluorescence detection of cancer cells based on their accumulation of PpIX, and for cancer cell phototherapy targeting PpIX have been extensively studied in humans (11). The actions of Ang II and other pathophysiological mediators that promote increased mitochondrial superoxide and/or reactive NO-derived species in amounts that cause disruption of the function of FECH may allow ALA to promote a selective enhancement of PpIX accumulation in cells with elevated mitochondrial oxidant stress. However, the accumulation of PpIX may then promote processes that reverse these oxidant conditions through promoting a cGMP-mediated inhibition of increased mitochondrial and extra-mitochondrial generation of superoxide.

The most important new aspect of this study appears to be evidence for increased mitochondrial generation of superoxide caused by Ang II (and perhaps other agents) functioning to impair heme biosynthesis by inhibiting ferrochelatase in a manner that results in a loss of the beneficial vascular regulatory effects of heme-dependent expression and/or function of sGC (e.g. stimulation by NO), and perhaps other systems contributing to vascular dysfunction such mitochondrial functions influenced by a heme-dependent depletion of cytochrome oxidase. These processes appear to be prevented by cGMP-stimulation of protein kinase G signaling attenuating both mitochondrial and extra-mitochondrial increases in superoxide. The data in this study also suggest that δ-
aminolevulinic acid supplementation (ALA) could be beneficial in overcoming oxidative inactivation of ferrochelatase and restoring the consequences this has on heme-dependent proteins in a manner that normalizes processes such as vasodilatation.

Acknowledgements

This study was support by NIH grant R01HL115124. Portions of this study were presented at the 2015 Experimental Biology Meeting in Boston, MA (24).
REFERENCES


Figure Legends

Figure 1: Effects of 24 hr organoid culture of BCA with Angiotensin II on ferrochelatase (FECH) activity in presence or absence of 1mM Mito-TEMPOL (a mitochondrial superoxide scavenger), ALA, losartan, gp91dstat (Nox oxidase inhibitor) or Tempol (superoxide scavenger). * P< 0.05 vs. Control; # P < 0.05 vs. Angiotensin II; n=4.

Figure 2: Effects of organoid culture of BCA with Angiotensin II in presence or absence of ALA on (A) mitochondrial superoxide and (B) extra-mitochondrial (cytosolic) superoxide detected by HPLC measurements of the superoxide specific products of MitoSox and dihydroethidium. * P< 0.05 vs. Control; # P < 0.05 vs. Angiotensin II; n=4-6.

Figure 3: Effects of organoid culture of BCA with Ang II in presence or absence of ALA for 24 hr on the expression of (A) SOD2, (B) sGC and (C) VASP-phosphorylation detected by Western Analysis. * P< 0.05 vs. Control; # P < 0.05 vs. Angiotensin II; n=4-6. The mitochondrial protein VDAC as loading control for SOD2.

Figure 4: Effects of organoid culture of BCA with Ang II in presence or absence of ALA on (A) FECH expression detected by Western analysis, (B) protoporphyrin IX (PpIX) levels, detected by its surface fluorescence. * P< 0.05 vs. Control; # P < 0.05 vs. Angiotensin II; n=4-6. The mitochondrial protein VDAC as loading control for FECH.
Figure 5: Effects of organoid culture of BCA with Angiotensin II in presence or absence of 8-bromo-cGMP on (A) mitochondrial superoxide and (B) extra-mitochondrial (cytosolic) superoxide. * P< 0.05 vs. Control; # P < 0.05 vs. Angiotensin II; n= 5-9.

Figure 6: Effects of organoid culture of BCA with Ang II in presence and absence of 8-bromo-cGMP on expressions of the heme containing proteins (A) sGC, (B) COx4 and (C) catalase. * P< 0.05 vs. Control; # P < 0.05 vs. Angiotensin II; n=4. The mitochondrial protein VDAC as loading control for COx4.

Figure 7: Effects of 24 hr organoid cultured BCA rings with or without Angiotensin II in the absence or presence of (A) ALA (B) 1mM Mito-TEMPOL, (C) 1mM Tempol and (D) ODQ on relaxation to increasing cumulative doses of Spermine-NONOate. BCA rings from each group were precontracted with 30mM KCl, and data are reported as percent relaxation of the force generated by this contractile agent. Responses from in the same animals reported in each panel of data to Spermine-NONOate was measured in absence of AngII, ALA, Mito-TEMPOL, Tempol or ODQ in the tissue bath were used for the Controls. * P< 0.05 vs. Control; # P < 0.05 vs. Angiotensin II; n=5-7.

Figure 8: Effects of 24 hr organoid cultured BCA rings with or without Angiotensin II in the absence or presence of (A) ALA (B) Mito-TEMPOL, and (C) Tempol on relaxation to increasing cumulative doses of Isoproterenol. BCA rings from each group were precontracted with 30mM KCl, and data are reported as percent relaxation of the force generated by this contractile agent. Responses from in the same animals reported in
each panel of data to Isoproterenol measured in absence of AngII, ALA, Mito-TEMPOL
or Tempol in the tissue bath were used for the Controls. n=5-7.

Figure 9: Model showing some of the hypothesized actions of Angiotensin II on
vascular smooth muscle cells (VSMC) in the coronary arteries studied that are
potentially contributing to the observed depletion of soluble guanylate cyclase and loss
of relaxation to NO, and the potential origins of detected protective effects of ALA, Mito-
TEMPOL and Tempol. Angiotensin II decreases mitochondrial SOD2 and increases
mitochondrial superoxide (detected by MitoSox), which should both inactivate and
promote depletion of ferrochelatase (FECH). The loss of FECH is expected to impair
heme biosynthesis (as supported by detection of the depletion of cytochrome C oxidase
subunit 4), and this is hypothesized contribute to an acceleration of the depletion of sGC
as its heme is oxidized by processes associated with increased extra-mitochondrial
superoxide derived from systems such as Nox oxidases (NOX) or other oxidant
mechanisms. Based on the actions of the protein kinase G (PKG) activator 8-bromo-
cGMP, PKG functions to lower superoxide and prevent these actions of Ang II. ALA
may function by generating protoporphyrin IX a stimulator cGMP production by sGC,
and perhaps by promoting increased generation of heme.
Figure 1

FECH Activity (pmoles/mg/min)

Control
Angiotensin II
ALA
Tempol
Mito-Tempol
Losartan
GP91

+ Angiotensin II

* # #
Figure 2

A

B

2-OH-Mito-ethidium (pm/gm)

2-OH-ethidium (pm/gm)

Control
Angiotensin II
ALA
ALA + Ang II

Control
Angiotensin II
ALA
ALA + Ang II

* P < 0.05 vs. Control
# P < 0.05 vs. Ang II

N = 5
Figure 3

A

SOD-2
VDAC

M.W. kDa
25
32

B

sGCβ1
β-actin

M.W. kDa
70
42

C

p-VASP
Total-VASP

M.W. kDa
50

Graphs showing percentage of control for SOD-2, sGCβ1, and VASP phosphorylation.
Figure 5

A. 2-OH Mito-ethidium (pm/gram)

- Control
- Ang II
- 8-Bromo-cGMP
- Ang II + 8-Bromo-cGMP

B. 2-OH-ethidium (pm/gram)

- Control
- Ang II
- 8-Bromo-cGMP
- Ang II + 8-Bromo-cGMP

* indicates statistical significance compared to control.
# indicates statistical significance compared to Ang II.
Figure 7

A

Control
Angiotensin II
ALA
Angiotensin II + ALA

% Relaxation

Spermine-NONOate [log M]

B

Control
Angiotensin II
Mito-TEMPOL
Angiotensin II + Mito-TEMPOL

% Relaxation

Spermine-NONOate [log M]

C

Control
Angiotensin II
Tempol
Angiotensin II + Tempol

% Relaxation

Spermine-NONOate [log M]

D

Control
Angiotensin II
ODQ
Angiotensin II + ODQ

% Relaxation

Spermine-NONOate [log M]
Figure 9

Angiotensin II

\[ \text{NOX} \]

\[ \text{O}_2^{\cdot\cdot} \]

\[ \text{Mito-TEMPOL} \]

\[ \text{Tempol} \]

\[ \text{Fe}^{2+} \]

\[ \text{sGC} \]

\[ \text{Fe}^{2+} \text{Heme-sGC} \]

\[ \text{8-bromo-cGMP} \]

\[ \text{cGMP/PKG} \]

\[ \text{Vascular Relaxation} \]

\[ \text{NO} \]

\[ \text{Fe}^{2+} \text{Heme-sGC} \]

\[ \text{PpIX-sGC} \]

\[ \text{ALA} \]

\[ \text{PpIX} \]

\[ \text{sGC} \]

\[ \text{SOD-2} \]

\[ \text{(VSMC)} \]