Protoporphyrin IX generation from δ-aminolevulinic acid elicits pulmonary artery relaxation and soluble guanylate cyclase activation

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Mingone, Christopher J., Sachin A. Gupta, Joseph L. Chow, Mansoor Ahmad, Nader G. Abraham, and Michael S. Wolin. Protoporphyrin IX generation from δ-aminolevulinic acid elicits pulmonary artery relaxation and soluble guanylate cyclase activation. Am J Physiol Lung Cell Mol Physiol 291: L337–L344, 2006; doi:10.1152/ajplung.00482.2005.—Protoporphyrin IX-elicited activation of sGC resulted in the discovery that the iron-free biosynthetic precursor to heme, protoporphyrin IX, activated sGC (19). The protoporphyrin IX–activated form of sGC showed changes in enzyme kinetic properties (e.g., $K_M$ for Mg-GTP and maximum velocity) which resembled the NO-stimulated form of sGC (34). This observation resulted in the proposal that NO stimulated sGC by binding the iron of the heme of sGC, resulting in a disruption of the bond normally present between an amino acid of sGC and the iron of the sGC heme (34). This amino acid was subsequently identified as a histidine residue (5, 31). Heme oxygenase-derived carbon monoxide has been reported to activate guanylate cyclase (4, 14) and causes vascular relaxation associated with increases in cGMP (14). Cleavage of the histidine bond to heme by NO distinguishes the marked stimulation of sGC activity by NO from a modest activation by CO (31), which does not result in the cleavage of the iron-histidine binding interaction (5). None of the other biosynthetic precursors of heme other than protoporphyrin IX were found to alter sGC activity (19), and heme was observed to be a competitive inhibitor of the activation of sGC by protoporphyrin IX, suggesting both porphyrins bound to the same site on sGC (34). Although drugs that mimicked human diseases of porphyrin metabolism associated with increased hepatic levels of protoporphyrin IX were observed to increase cGMP levels in the liver (20), minimal work has been done to investigate the biological significance of this mechanism of regulating sGC.

The accumulation of protoporphyrin IX generated from the heme precursor δ-aminolevulinic acid (ALA) has been extensively investigated as an approach to detect tumor cells based on its fluorescence and as a phototherapy. Although measurements of protoporphyrin IX fluorescence in tumor and normal cells also detect fluorescence from its biosynthetic precursors uroporphyrin III and coproporphyrin III, the ratios of these porphyrins appear to be similar across a variety of cell types (27). Thus tissue protoporphyrin IX fluorescence after treatment with ALA has been demonstrated in many studies to be closely associated with measurements of its levels in tissue extracts (30). The photosensitizing action of the protoporphyrin IX accumulated during exposure to ALA has also been developed as a phototherapy approach to kill tumors and other proliferating cells. A reduction in systemic and pulmonary porphyrin metabolism associated with increased hepatic levels of protoporphyrin IX were observed to increase cGMP levels in the liver (20), minimal work has been done to investigate the biological significance of this mechanism of regulating sGC.

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artery pressure was reported as a factor that limited the doses of ALA that could be used in humans in this approach (13). However, the mechanism by which ALA directly or indirectly mediates these effects has not been elucidated. In addition, phototherapy using ALA administration to generate protoporphyrin IX has been investigated as an approach to treat restenosis (24, 28). Studies examining the treatment of animals with ALA for restenosis phototherapy have documented that ALA increases protoporphyrin IX fluorescence levels in the smooth muscle region of the arterial wall (21, 28). Treatment of isolated skeletal muscle arterioles with ALA has been used in studies on vascular regulation by the heme oxygenase system as a method of increasing heme availability for the production of carbon monoxide (22). However, there do not appear to be studies in the literature examining the regulation of sGC resulting from protoporphyrin IX generation by ALA in vascular tissue or any other cellular system.

EXPERIMENTAL PROCEDURES

Materials. Spermine-NONOate, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), and cGMP Enzyme Immunoassay (ELISA) kits were purchased from Cayman Chemical. Anti-cGMP-associated vasodilator-stimulated phosphoprotein (VASP), phospho (Ser239)- and anti-VASP antibodies were obtained from Cell Signaling, and anti β-sGC subunit antibodies were obtained from Sigma. Heme was measured using a QuantiChrom heme assay kit purchased from BioAssay Systems. All gasses were purchased from Tech Air (White Plains, NY). All salts used were analyzed reagent grade from Baker Chemical, and all other chemicals were obtained from Sigma Chemical.

Organoid culture isolated arteries for heme modulation. Intralobar BPA were isolated from slaughterhouse-derived bovine calf lungs, cleaned from surrounding tissue, and placed in 37°C Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, penicillin, streptomycin, and fungizone (complete media). The vessels were then depolarized with Krebs containing 123 mM KCl (high K+; balance N2) in the absence of compounds used to modulate BPA before contraction with serotonin. The effects of ODQ on relaxation to NO was determined by first contracting organ cultured control and ODQ-treated BPA with 1 µM 5-HT (after the initial exposure to increasing concentrations of 5-HT and a 30-min equilibration in Krebs).

Determination changes in protoporphyrin IX and heme levels. Methods for the detection of protoporphyrin IX tissue fluorescence developed for the detection in cancerous cells with tissue depths of up to several millimeters (27) were adapted for detecting changes in the endogenous levels of protoporphyrin IX in BPA rings that were organ cultured with ALA. When excited with light in the regions of 505 or 540 nm, protoporphyrin IX has an emission in the region of 635 nm. Studies measuring the ALA-elicited accumulation of protoporphyrin IX in tissues developed for the detection of cancerous cells have consistently found that the observed increase in fluorescence emission at 635 nm was closely associated with the accumulation of protoporphyrin IX in the tissue studied (30). Increases in protoporphyrin IX were measured using an excitation wavelength of 485 ± 20 or 528 ± 20 nm and emission of 620 ± 40 nm in intact vessels. Ring segments of approximately equal size (<4 mm in diameter and length) were centered on the bottom of the ~6-mm diameter wells of a 96-well microplate with 200 µl of Krebs containing with 10 mM HEPES buffer (pH 7.4), and the fluorescence was measured from the bottom surface of the plate using BIOTEK fluorescent microplate reader (model FLX800). Data are reported in the arbitrary fluorescence units measured (AU), after subtraction of the low levels of background fluorescence observed in the absence of BPA.

Heme was quantified in BPA homogenates using a QuantiChrom heme assay kit purchased from BioAssay Systems, and changes in absorbance were measured in a BIOTEK scanning microplate spectrophotometer. In brief, segments were homogenized in 20 mM MOPS + sucrose buffer and diluted to ~5 mg protein/ml. Homogenates were centrifuged at 2,000 g for 5 min, and 50 µl of supernatant obtained were assayed for their heme content based on the manufacturer’s instructions, employing measurements of changes in absorbance at 405 nm. The protein content of the supernatant was measured with Bio-Rad protein assay kit, and tissue heme levels were reported as nmol/mg protein.

Western blot analysis of phosphorylated VASP and sGC expression. Rings from studies on contractile function were snap-frozen in liquid nitrogen, crushed, and homogenized in lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% of a protease inhibitor cocktail (Sigma) and 1% of a phosphatase inhibitor cocktail (Sigma)]. The protease inhibitor cocktail included 4-(2-aminoethyl)benzamidesulfonfluoride hydrochloride, aprotinin, bestatin hydrochloride, N-(trans-epoxysuccinyl)-l-leucine-4-guanidinobutylinamide, leupeptin hemisulfate, pepstatin, and the phosphatase inhibitor cocktail included sodium orthovanadate, sodium molybdate, sodium tartarate, and imidazole. Protein concentration was determined and 30 µg of protein samples were prepared in electrophoresis sample buffer containing 3% SDS and 3% 2-mercaptoethanol and were separated using a 12% gel SDS-PAGE electrophoresis. Membranes were blocked for 1 h in Tris-buffered saline plus Tween-5% milk and incubated overnight with phospho-VASP antibodies (diluted 1:1,000). Membranes were exposed to a secondary horseradish peroxidase-linked antibody and visualized with an ECL kit (Amersham). The membranes were subsequently exposed to X-Omat Autoradiography paper (Kodak). Membranes were stripped with Tris-HCl buffer with 100 mM 2-mercaptoethanol at 50°C for 30 min. Membranes were subsequently washed, blocked, and detected for total VAP. Each measurement of phosphorylated VASP was normalized to the total level of VASP, and data are reported as the percent of the levels of phosphorylated VASP seen in control arteries not exposed to agents that modulate the activity of sGC. Expression of sGC was detected with β-sGC subunit antibodies (diluted 1:2,000), employing anti-rabbit secondary antibodies, and a protocol where membranes were
blocked for 2 h. After analyzing for sGC protein expression, blots were stripped and reprobed for the detection of α-actin protein levels, which were used for normalization of data for statistical analysis based on protein loading.

Assay of sGC activity in BPA homogenates. Guanylate cyclase activity of homogenates of BPA was measured by enzyme immunoassay of the amounts of cGMP generated. In brief, BPA were pulverized in liquid nitrogen and homogenized in MOPS buffer 1:3 wt to buffer ratio at 5°C. Reaction mixture (0.2 ml final volume) contained 20 mM MOPS-KOH (pH 7.4), 0.1 mM GTP, 2 mM MgCl2, 0.3 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, a GTP-regenerating system consisting of 10 mM phosphocreatine and 150 U/ml creatine phosphokinase, and 0.1 ml of homogenate (11, 15), and test agents, as indicated. Assays of sGC activity were initiated by the addition of BPA homogenate. Incubations were conducted for 10 min at 37°C, and they were terminated by the addition of 0.1 ml of preheated 12 mM EDTA. This was followed by boiling the assay mixtures for 10 min and a subsequent addition of 5% trichloroacetic acid. The precipitate was removed by centrifugation, and the supernatant was washed three times with water-saturated diethyl ether. Residual ether was removed by incubation at 70°C for 5 min, before measurement of cGMP by ELISA (Cayman), as described in the manual supplied by manufacturer of the ELISA kit.

Statistical analysis. Data are reported as means ± SE with n describing the number of BPA from different animals. Statistical analyses were performed by a one-way ANOVA with a post hoc Bonferroni correction for comparison between multiple groups.

RESULTS

Effect of ALA on vascular contraction to 5-HT and the detection of protoporphyrin IX. Organ culture of BPA with 100 μM ALA was observed to increase in tissue fluorescence potentially originating from protoporphyrin IX after 16 and 24 h of incubation (see Fig. 1A). When examined after 24 h of organ culture in studies examining contractile function to 0.1–10 μM 5-HT in the absence of ALA, exposure to 100 μM ALA was observed to markedly inhibit force generation to doses of 5-HT that induce submaximal contraction (Fig. 1B). The data in Fig. 1B show that the organ cultured Control BPA contracted to 5-HT, and organ culture with the 10 μM dose of ALA appeared to depress force generation at higher doses of 5-HT. However, these changes did not reach statistical significance. Statistically significant decreases in force generation to 5-HT in the range of 50–90% were observed in the presence of the 50 μM and 100 μM doses of ALA. As shown in Fig. 1C, the 10 μM dose of ALA did not cause a detectable increase in fluorescence potentially originating from protoporphyrin IX in intact vessels, whereas 50 μM and 100 μM ALA caused increases in fluorescence of up to twofold greater than the background fluorescence of BPA. The increase in BPA fluorescence caused by 100 μM ALA was similar to the fluorescence produced by a 3 μM solution of protoporphyrin IX under the conditions examined. These data are consistent with organ culture with 50–100 μM ALA resulting in increases in levels of protoporphyrin IX detected by fluorescence associated with decreased in force generation when exposed to 5-HT.

Effect of Fe on inhibition of vascular reactivity and increased protoporphyrin IX-associated fluorescence caused by ALA. Because the availability of Fe may influence protoporphyrin IX levels by serving as a cofactor for its conversion to heme, the influence of the presence of 100 μM Fe during organ culture was examined. As shown in Fig. 2A, the presence of organ culture with Fe did not alter force generation by 5-HT. However, Fe reversed the force attenuating effects of organ culture with 100 μM ALA. Data in Fig. 2B show that organ culture with Fe did not alter the background fluorescence of ALA.
Influence of organ culture with combinations of 100 μM ALA and 100 μM Fe on BPA force generation by 5-HT (A) and PIX-associated (B) fluorescence. The decreased level of force generation in ALA-treated vessels was improved in the presence of Fe, whereas the ALA-elicted increase in fluorescence associated with PIX detection was attenuated by Fe (*P < 0.05 vs. Control, n = 5–8).

BPA; however, it reversed the increase in protoporphyrin IX fluorescence seen in the presence of 100 μM ALA. These data are consistent with Fe’s lowering the levels of protoporphyrin IX in BPA associated removing its force depressing or relaxing effects on the contraction to 5-HT.

Influence of organ culture in the absence and presence of ALA and Fe on BPA sGC activity and heme levels. The enzyme activity of sGC in BPA was examined to detect whether organ culture in the absence and presence of combinations of 100 μM ALA and Fe influenced sGC activity. As shown in Fig. 3A, an increase in BPA homogenate sGC activity was seen in the presence of ALA, and organ culture with Fe alone or ALA + Fe did not alter sGC activity, suggesting that Fe was reversing the effects of ALA on sGC activity. Changes in the expression of sGC did not appear to be the cause of increased sGC activity because based on Western analysis, BPA organ cultured with 100 μM ALA had 71 ± 19% (n = 4) of the levels of sGC detected in BPA organ cultured in the absence of ALA. The levels of heme were measured in the absence and presence of combinations of 100 μM ALA and Fe to determine influence of these conditions on heme biosynthesis. The data in Fig. 3B show that while Fe or ALA alone appeared to increase BPA heme levels under the conditions examined, the changes in heme did not reach statistical significance. However, organ culture with 100 μM ALA + Fe resulted in a marked increase in heme, which was statistically significant. These data are consistent with sGC activity being stimulated under conditions where an elevated level of protoporphyrin IX was detected (100 μM ALA), and with elevated levels of heme (100 μM ALA + Fe) not appearing to influence basal sGC activity.

Fig. 3. Effects of organ culture with combinations of 100 μM ALA and 100 μM Fe on BPA soluble guanylate cyclase (sGC) activity (A) and heme content (B). ALA increased sGC activity under conditions where elevated levels of PIX-associated fluorescence was detected. Fe treatment with ALA increased heme and prevented the increase in sGC activity (n = 7, *P < 0.05).

Influence of the sGC heme oxidant ODQ on decreases in BPA force generation resulting from organ culture with ALA. The actions of ODQ, an oxidant of the ferrous form of the heme of sGC that inhibits of sGC activation by NO and carbon monoxide (CO) (8, 29), on the decrease in force elicited by organ culture of BPA in the presence of ALA were examined to characterize the role of heme-binding dependent mechanisms of sGC activation in decrease in force that was observed under these conditions. The data in Fig. 4A show that 10 μM ODQ did not alter force generation to 5-HT or the decrease in contraction to 5-HT observed in the presence of 100 μM ALA. Organ cultured BPA relaxed to the NO donor 10 μM spermine-NONOate (see Fig. 4B), and pretreatment with 10 μM ODQ markedly attenuated relaxation to this NO donor. Thus the depression of force observed in BPA organ cultured with ALA does not appear to be mediated through a mechanism dependent of the ferrous heme form of sGC.

Organ culture of BPA with ALA increases the cGMP-associated phosphorylation of VASP. Increases in the phosphorylation of serine-239 on VASP in BPA organ cultured in the absence and presence of 100 μM ALA was examined as an indicator of cGMP-dependent protein kinase activation. BPA organ culture with 100 μM ALA showed an increase in VASP phosphorylation (see Fig. 4C), which was similar in magnitude to the increase caused during relaxation of organ cultured BPA when they were exposed to a 10 μM dose of the NO donor spermine-NONOate (Fig. 4D). As shown in Fig. 4, the heme oxidant inhibitor of sGC ODQ did not significantly alter the increase in serine-239 VASP phosphorylation caused by organ culture with ALA under conditions where it was observed to attenuate the increase caused by the NO donor. Thus organ culture with 100 μM ALA appears to be promoting...
increases in VASP phosphorylation associated with cGMP-dependent protein kinase activation in a manner similar to 10 μM spermine-NONOate; however, this effect of ALA does not appear to be dependent on sGC being stimulated through a ferrous heme-dependent mechanism.

Effect of the iron chelator deferoxamine on vascular contraction and protoporphyrin IX detection resulting from organ culture of BPA with a low dose of ALA. Force generation to 5-HT and increases in the detection of protoporphyrin IX fluorescence were not significantly altered by the low dose of ALA (10 μM) examined in Fig. 1. Thus the effect of organ culture with the iron chelator deferoxamine was examined to determination if decreasing the availability of iron enhanced protoporphyrin IX levels and its effects on decreasing force. As shown by the data in Fig. 5A, organ culture with 100 μM deferoxamine did not alter contraction to 5-HT, but the presence of this iron binding agent during organ culture markedly reduced the contraction to 5-HT in the presence of 10 μM ALA. Organ culture with the iron chelator deferoxamine did not alter the basal levels of protoporphyrin IX-associated fluorescence. However, as shown in Fig. 5B, organ culture with 10 μM ALA in the presence of 100 μM deferoxamine caused a detectable increase in protoporphyrin IX-associated fluorescence, suggesting that lowering of the availability of iron, permits the accumulation of protoporphyrin IX and a reduction in the contraction to 5-HT. The presence of 100 μM deferoxamine did not appear to accelerate the time course of protoporphyrin IX accumulation or the amount formed from 100 μM ALA. After 8 h of organ culture, the BPA fluorescence in the presence of ALA (1,015 ± 79 AU) was not significantly increased from BPA exposed to 100 μM ALA + 100 μM deferoxamine (939 ± 137 AU, n = 8), and the increased amount of ALA fluorescence observed after 24 h (1,658 ± 122 AU) was not significantly increased from the low dose of ALA alone (1,015 ± 79 AU).

Fig. 4. The effects of organ culture with 100 μM ALA (A, C) or relaxation of organ cultured BPA by 10 μM of the NO donor spermine-NONOate (B, D) on force generation to 5-HT (A, B) and VASP serine-239 phosphorylation (C, D) associated with cGMP-dependent protein kinase activation in the absence and presence of 10 μM 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ). The sGC heme oxidant inhibitor ODQ did not significantly alter the decreased levels of 5-HT contraction seen in BPA exposed to ALA (n = 6). ALA and spermine-NONOate (NO) increased VASP phosphorylation in a similar manner; however, only NO-induced VASP-phosphorylation is decreased in the presence of ODQ (n = 5, P < 0.05). Data are normalized to total VASP. Whereas ODQ appeared to decrease VASP phosphorylation and NO appeared to increase VASP phosphorylation in the presence of ODQ, these changes did not reach statistical significance.

Fig. 5. Effects of organ culture with a low dose of ALA (10 μM) on BPA force generation by 5-HT (A) and PIX-associated (B) fluorescence. The low dose of ALA did not significantly alter force generation or PIX-associated fluorescence. The iron chelator deferoxamine attenuated force generation and increased PIX-associated fluorescence in the presence of the low dose of ALA compared with the Control and ALA-treated BPA (P < 0.05, n = 8–12).
This study provides evidence that a previously identified activator of sGC, protoporphyrin IX, can function as an endogenous regulator of vascular function through cGMP. Whereas most previous work with protoporphyrin IX was performed on purified sGC (19, 20, 34), there is an absence of evidence regarding relationships between protoporphyrin IX production in intact vascular tissue and how this pathway could function to regulate sGC. Exposure of isolated BPA under organ culture conditions to 50–100 μM doses of ALA increased protoporphyrin IX levels to amounts that decreased force in a manner that was associated with increased sGC activity and phosphorylation of VASP on the serine-239 site phosphorylated by cGMP-dependent protein kinase. The availability of Fe appeared to have a major role in controlling arterial levels of protoporphyrin IX and the ability of organ culture with ALA to suppress force generation. A model showing how ALA appears to control BPA force through generating a protoporphyrin IX-bound activated form of sGC is shown in Fig. 6. This model compares the actions of protoporphyrin IX with the NO-binding Fe2+ form of heme-containing sGC, because it appears to be the major form of sGC present in BPA under basal conditions.

Fig. 6. This model shows how ALA promotes the biosynthesis PIX in a manner controlled by the availability of Fe and how PIX increases cGMP production and vascular relaxation through binding the heme site of sGC. Because ODQ is an oxidant of the heme of sGC, it attenuates increases in cGMP and relaxation caused by NO but not responses caused by PIX.
in BPA organ-cultured with 10 μM ALA and a significantly inhibited level of force generation to 5-HT. These data suggest that Fe present in BPA under organ culture conditions was probably promoting the conversion of protoporphyrin IX synthesized from exogenous ALA to heme. The absence of detectable changes of heme in the presence of 100 μM ALA implies either that the levels of heme formed under these conditions were below the detection limits of the heme assay or that heme oxygenase further metabolized the heme that was formed. Because conditions of maximal heme generation (100 μM ALA + Fe) were associated with an absence of alterations in the contraction to 5-HT, CO generated by the heme oxygenase reaction was not having a detectible effect on vascular function under the conditions examined. Observations that iron chelation did not increase basal levels of protoporphyrin IX or alter the contraction to 5-HT are also consistent with the absence of endogenous levels of protoporphyrin IX biosynthesis in amounts that could regulate vascular function under the conditions examined. Overall, these observations suggest that the availability of ALA is a key factor in controlling the biosynthesis of protoporphyrin IX in BPA and that Fe availability has a major role influencing the levels of protoporphyrin IX that are observed as a result of its role in enabling the biosynthesis of heme. The influence of Fe availability on controlling the levels of protoporphyrin IX were closely associated with its effects on force generation by 5-HT, which further supports a role for protoporphyrin IX in controlling vascular function under the conditions examined.

The data in this study are consistent with increasing protoporphyrin IX biosynthesis from ALA resulting in sGC activation and a cGMP-elicited decrease in force generation. This mechanism may be a contributing factor to the decrease in systemic and pulmonary artery pressure seen in humans treated with ALA (13), because animal studies on the use of ALA in phototherapy for restenosis have shown (28) that protoporphyrin IX accumulates in the smooth muscle of arteries on a time course that is similar to its effects on blood pressure seen in the human study. The availability of iron is a major factor in determining whether protoporphyrin IX is allowed to accumulate or whether it is converted to heme through the ferrochelatase reaction. Because heme competes with protoporphyrin IX for its binding site on sGC with a binding affinity for protoporphyrin IX in the low nanomolar concentration range and a binding affinity for heme of ~350 nM (34), endogenous heme could also function to attenuate sGC activation by protoporphyrin IX. In view of the fact that circulating levels of Fe are below the micromolar concentration range (7), cellular mechanisms controlling the biosynthesis of ALA and levels of protoporphyrin IX might be more important factors for identifying the physiological importance of the regulatory mechanism examined in this study. The influence of modulating iron availability in the absence of ALA suggests that endogenous ALA levels are below the amounts that could generate vasoreactive levels of protoporphyrin IX in BPA. Iron levels seem to control the expression of ferrochelatase and the generation of heme (1, 32). Additionally, heme functions as a primary inhibitory regulator of the biosynthesis of ALA (10), and decreases in heme biosynthesis are associated with diseases of aging (3). Thus genetic and/or metabolic conditions that disrupt the regulatory mechanisms of heme biosynthesis might allow the accumulation of protoporphyrin IX in amounts that alter multiple aspects of vascular function through stimulating sGC. Therefore, the regulation of vascular function through protoporphyrin IX could potentially be a physiologically important regulatory mechanism under conditions where this heme precursor is allowed to accumulate. In addition, promoting protoporphyrin IX accumulation could also be a therapeutic target for the treatment of vascular disease.

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REFERENCES


