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<td>Complete List of Authors:</td>
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Riboflavin and pyrroloquinoline quinone generate carbon monoxide in the presence of tissue microsomes or recombinant human cytochrome P-450 oxidoreductase (CPR); implications for possible roles in gasotransmission.

Dragic Vukomanovic\textsuperscript{a,b}, Zongchao Jia\textsuperscript{a}, Kanji Nakatsu\textsuperscript{a}, and Graeme N. Smith\textsuperscript{a,b} Terence R.S. Ozolinš\textsuperscript{a}

\textsuperscript{a} Department of Biomedical and Molecular Sciences, Queen’s University, Kingston, Ontario, Canada, K7L 3N6

\textsuperscript{b} Department of Obstetrics and Gynaecology, Kingston General Hospital, Kingston, Ontario, Canada, K7L 3N6

**CORRESPONDING AUTHOR:**

Dr. Terence R.S. Ozolinš, Department of Biomedical & Molecular Sciences, Queen’s University, Kingston, ON, Canada

K7L 3N6

Tel 613-533-3306 Facsimile 613-533-6412

ozolinst@queensu.ca

There are no declarations of interest for any of the authors.
ABSTRACT
Carbon monoxide (CO), an endogenously produced gasotransmitter, regulates inflammation and vascular tone, suggesting delivery of CO may be therapeutically useful for pathologies like preeclampsia where CO insufficiency are implicated. Our strategy is to identify chemicals that increase the activity of endogenous CO producing enzymes, including cytochrome P-450 oxidoreductase (CPR). Realizing both riboflavin and pyrroloquinoline quinone (PQQ) are relatively non-toxic, even at high doses, and they share chemical properties with toxic CO activators we previously identified, our goal was to determine whether riboflavin or PQQ could stimulate CO production. Riboflavin and PQQ were incubated in sealed vessels with rat and human tissue extracts and CO generation was measured with headspace-gas chromatography. Riboflavin and PQQ increased CO production ~ 60% in rat spleen microsomes. In rat brain microsomes, riboflavin and PQQ increased respective CO production ~four-fold and two-fold, compared to baseline. CO production by human placenta microsomes increased four-fold with riboflavin and five-fold with PQQ. In the presence of recombinant human CPR, CO production was three-fold greater with PQQ than riboflavin. These observations demonstrate for the first time that riboflavin and PQQ facilitate tissue-specific CO production with significant contributions from CPR. We propose a novel biochemical role for these nutrients in gasotransmission.

Keywords:
- Riboflavin
- Pyrroloquinoline quinone
- Carbon monoxide
- Human placenta microsomes
• Cytochrome P-450 oxidoreductase

**Novelty Bullets:**

- Riboflavin or PQQ stimulated CO generation in a tissue-specific manner
- Recombinant human cytochrome P-450 reductase (CPR) generates CO in the presence of riboflavin or PQQ
- CO production is a novel biochemical function for these nutrients
- Riboflavin or PQQ may increase CO production in conditions of CO insufficiency such as preeclampsia
INTRODUCTION

Carbon monoxide (CO) is an important gasotransmitter critical in certain signalling pathways related to vascular tone and the production of inflammatory mediators (reviewed in Motterlini & Foresti 2017). Thus, the actions of CO may have clinical applications in many diseases associated with vascular dysfunction and/or inflammation, including preeclampsia (Phipps et al. 2019). The development of therapeutic CO delivery is an area of active research and includes exogenous CO delivery methods and the development of CO-releasing molecules (CORMs (Ismailova et al. 2018; Kim & Choi 2018). Another strategy, one that we favour, is to generate CO in situ by activating endogenous CO-producing enzymes.

The primary endogenous source of CO is from heme oxygenase during heme degradation (Motterlini & Foresti 2017). There are two primary forms of mammalian heme oxygenases (HO). HO-2 is predominantly expressed in brain and testes, and although HO-1 is highly inducible following oxidative stress, the spleen is a rich source of its constitutive expression (Vukomanovic et al. 2014). Recently, we demonstrated CO is also produced by cytochrome P-450 oxidoreductase (CPR) (Vukomanovic et al. 2017), indicating at least three significant enzymatic sources of CO. The CO-producing enzymes HO-1, HO-2 and CPR reside in the endoplasmic reticulum (Riddick et al. 2013; Motterlini and Foresti 2017), and when cells are disrupted, the endoplasmic reticulum forms heterologous vesicles, which after differential centrifugation yield a highly enriched source of endoplasmic reticulum enzymes, i.e. microsomes. We use microsomes as an in vitro screening tool to understand the important structure activity relationships (SAR) necessary to stimulate CO production (Vukomanovic et al. 2011; Vukomanovic et al. 2014). Briefly, we came to realize that the capacity of a compound to
enhance CO production was not associated with a traditional pharmacophore, but instead appeared related to redox activities inherent to the structure; a potent prototypic CO generator is menadione, a synthetic analogue of vitamin K (K₃). Using menadione, a subsequent series of studies identified a novel enzymatic source of CO production. Briefly, the use of specific HO-2 inhibitors with rat brain microsomes, and recombinant forms of HO-2 and CPR allowed us to conclude that the menadione-mediated CO production that we had previously noted in rat brain extracts was primarily the result of CPR, not HO-2 (Vukomanovic et al. 2017).

We have identified a number of synthetic isoalloxazine derivatives as potent CO producers (unpublished data). Isoalloxazine is a tricyclic heterocycle from which flavins are derived, and we reasoned that a biochemical source of flavin may provide an acceptable safety profile. Riboflavin is the most abundant biochemical source of flavin and although the riboflavin recommended daily allowance (RDA) is 1.3 milligrams daily for men and 1.1 mg for women, it may be consumed orally as high as 400 mg/day to treat migraine headaches with few adverse side effects (Powers 2003; Thakur et al. 2016). Thus, there is compelling rationale to test whether riboflavin stimulates CO generation.

Naphthoquinones, are also potent CO generators (Vukomanovic et al. 2011), but the dose of menadione required to increase CO levels in vivo was toxic (Odozor et al. 2018). Recognizing the need to identify less toxic naturally occurring naphthoquinones, we investigated vitamin K₁ (phylloquinone) and K₂ (menaquinone; MK-4), but disappointingly, neither was effective at producing CO in spleen or brain microsomes (Vukomanovic et al. 2014). This prompted a search for alternate compounds with a quinone or quinone-like nucleus, and evidence of clinical safety. Pyrroloquinoline quinone (PQQ; 4,5-dioxo-4,5-dihydro-1H-pyrrolo[2,3-
f]quinoline-2,7,9-tricarboxylic acid), an aromatic tricyclic o-quinone, is an important nutrient, found in a variety of foods, although its role as a vitamin remains controversial (reviewed in Misra et al. 2012; Akagawa, et al. 2016; Ames 2018). PQQ has a number of proposed health benefits including reduction of pro-inflammatory markers (Harris et al. 2013), a pathophysiological factor in preeclampsia. Together, these observations, and its limited toxicity when taken orally (Akagawa et al., 2016), made PQQ a promising candidate for CO production.

The goal of our study was to determine whether riboflavin and PQQ promote CO production in microsomes from several tissue sources, including human placenta, the organ in which CO insufficiency has been implicated in the etiology of preeclampsia (Levytska et al. 2013; Venditti and Smith, 2014).

MATERIAL AND METHODS

Preparation of Riboflavin and PQQ

A 10.0 mM stock solution of riboflavin 5’-phosphate sodium salt hydrate (Sigma-Aldrich Ltd, Toronto, Canada, catalogue #R7774) was prepared in double distilled water. A 10.0 mM stock solution of PQQ (ChemScene LLC, Monmouth Junction NJ, USA, catalogue # CS-W009276) was prepared in DMSO.

Preparation of rat spleen and brain microsomal fractions
Male rats were obtained from Charles River Ltd (St-Constant, Canada) and the organs were harvested under approved protocols and in accordance with Queen’s University Animal Care Committee and the Canadian Council on Animal Care’s Guide to the Care and Use of Experimental Animals. The microsomal fractions of rat spleen (Braggins et al. 1986) and rat brain (Trakshel et al. 1988), were prepared by differential centrifugation of tissue homogenate as previously described (Appleton et al. 1999; Kinobi et al. 2006). Protein concentration of the microsomal fraction was determined by a modification of the Biuret method (Cook et al. 1995). The microsomal fraction was resuspended in 100 mM phosphate buffer/20% (v/v), glycerol solution containing 10 mM EDTA and stored at −80 °C until used (Kinobi et al. 2006).

Collection and perfusion of human placentas

Term human placentas (>37 weeks) from uncomplicated pregnancies were obtained following elective Caesarean section at the Kingston General Hospital (Kingston, Ontario), under the approval of the Health Sciences Research Ethics Board at Queen’s University (OBGY-290-16). Maternal identifiers were not collected in order to maintain confidentiality and anonymity. Following removal from the uterus, the umbilical cord remained clamped to maintain blood pressure and the placenta was placed on ice for transport to the laboratory.

After removal of the amnion on the placental surface, a peripheral chorionic plate artery was cannulated and continuously perfused using a peristaltic pump (Gilson Minipuls 3, Gilson Inc. USA, Middleton WI) with ice cold Krebs’ buffer (Ahmed et al. 2005) at the rate of 20 mL/min. Perfusate and blood were allowed to drain from the paired vein during perfusion of the cotyledon. When the cotyledon on the fetal side became pale, perfusion was initiated on the
maternal side by inserting a catheter into the cotyledon until the entire cotyledon appeared bloodless. The perfused cotyledon was then excised, and the rest of the placenta disposed.

Preparation of human placenta microsomal fractions

The microsomal fractions from perfused human placenta tissue were prepared by differential centrifugation of tissue homogenate as previously described (Vukomanovic et al. 2010). Protein concentration of the microsomal fraction was determined using a modification of the Biuret method, as we described previously (Cook et al. 1995). The microsomal fraction was resuspended in a 100 mM phosphate buffer/20% (v/v), glycerol solution containing 10 mM EDTA and stored at -80 °C.

Preparation of recombinant human CPR (rh-CPR)

The hCPR-pET22b vector encoding full-length human CPR was a generous gift from Dr. Paul Ortiz de Montellano (University of San Francisco). Expression and purification were based on protocols previously described (Dierks et al. 1998). In brief, the plasmid was transformed into BL21 (DE3) pLysS cells that were cultured in Terrific Broth (TB) supplemented with trace elements and riboflavin in the presence of ampicillin and chloramphenicol. Induction of expression was initiated with 0.4 M isopropyl-β-D-thiogalactopyranoside (IPTG). When the absorbance (A) at 600 nm had reached ~0.4 the cells were allowed to grow further overnight at 30°C. Spheroplasts were generated from the harvested cells prior to lysis by sonication. Lysates were centrifuged at 10,000 r/min (Ti45 rotor) and the supernatant further centrifuged at 35,000 r/min (Ti45 rotor) to pellet the membrane fraction which was subsequently stored at –80°C for future purification.
Membrane pellets were thawed, then solubilized in 20 mM potassium phosphate (pH 7.4), 20% (v/v) glycerol, 0.2% Triton X-100, 0.1 mM EDTA, 1 mM PMSF, 0.2% sodium cholate with one Roche EDTA-free protease cocktail tablet. After clarification, the supernatant was purified over a 2′5′-ADP-Sepharose column, which had been equilibrated with Buffer A (20 mM potassium phosphate (pH 7.4), 20% glycerol, 0.1 mM EDTA, 0.2% sodium cholate). The column was washed with Buffer A, followed by washes in the presence of 250 mM NaCl, and in the presence of 2 mM adenosine. Elution of the rh-CPR protein was performed with 5 mM 2’AMP. Purified protein was dialyzed against 40 mM potassium phosphate, 20% glycerol overnight at 4°C, concentrated, and quantified by Bradford assay using bovine serum albumin (Sigma-Aldrich USA Inc.) as a standard. Protein was aliquoted, flash-frozen in liquid nitrogen and stored at –80°C.

**Enzymatic assay for CO generation**

CO generation by human placenta microsomal fractions was determined with a gas chromatographic method using headspace-gas analysis as described by (Vukomanovic et al. 2014). The incubation conditions had been previously optimized with respect to concentrations of reaction components and the duration of incubation (Vukomanovic et al. 2017). Briefly, a reaction mixture (150 μL) containing 100 mM phosphate buffer (pH 7.4), 50 μM methemalbumin and enzyme (1 mg microsomal protein or 10 μM recombinant protein), was pre-incubated with an riboflavin or PQQ concentration ranging from 0.01 to 100 μM, for 10 min at 37 °C in a shaking water-bath and then each vial’s head space was purged with CO-free air for 15s. The reaction was initiated by adding nicotinamide adenine dinucleotide phosphate
(NADPH; Sigma-Aldrich Ltd, Toronto, Canada) to a final concentration of 1.0 mM, and was continued for 15 min at 37 °C. The reaction was stopped by instantly freezing the reaction mixture on dry ice. The generated CO in the head-space was quantified using a specially designed gas-chromatograph for CO analysis (ta 3000R Process Gas Analyzer; Ametek Inc. USA, Newark, DE) which is sensitive and selective for CO by virtue of headspace sampling, size exclusion gas chromatography and mercuric oxide reduction detection (Vreman and Stevenson, 1988). Peak heights and areas were used to correlate detector response between standards and unknowns in the linear range. CO production by samples and blanks, analyzed in triplicate, was calculated and HO activity is presented as the net difference between total and blank reactions expressed as pmoles of CO. Controls in which methemalbumin, NADPH or enzymes were omitted have been reported previously (Vukomanovic et al. 2011).

Western Blot Analysis

The rh-CPR preparation (5 μg of protein) was run on a 10% SDS-PAGE gel, and stained with Coomasie Brilliant Blue. A molecular weight ladder used to confirm protein identity (Precision Plus Protein™ Standards; Biorad Ltd., Mississauga Canada, cat#161-0373).

Statistical analysis

Data were expressed as the mean ± SD and analyzed by one-way ANOVA, followed by a Newman-Keuls multiple comparison test for a significant F statistic (P < 0.05) (Prism 7; GraphPad Software, San Diego CA). A P value less than 0.05 was considered to be significant.

RESULTS
All microsomal preparations showed significant baseline CO production of approximately 70 pmol (Figs.1-3).

Riboflavin and PQQ are activators of CO production in spleen

At concentrations below 25 µM, riboflavin was a more potent activator of CO production than PQQ. For example, riboflavin stimulated increased CO production at concentrations as low as 100 nM, whereas the threshold for PQQ-mediated CO production was 25 µM (Fig 1). At 100 µM, the highest concentration used, riboflavin and PQQ increased CO production to 169±10 and 157±15 respectively, reflecting an approximately two-fold over baseline. Rat spleen microsomes contain CPR and HO-1, but comparatively little HO-2, implicating the former two enzymes at the catalytic source of CO.

PQQ is superior to riboflavin at activating CO production in brain

The baseline for CO generated by rat brain microsomes was approximately 75 pmol (Fig. 2). The addition of PQQ over the range of 0.01 to 1 µM did not increase CO production significantly. After the addition of 25 µM of PQQ the production of CO increased to 148±12 pmol, and the presence of 100 µM PQQ, increased CO to 303±70 pmol (Fig. 2). The CO generation curve for riboflavin was similar to that of PQQ except at the highest concentration (100 µM) riboflavin increased CO production to 175±9 pmol.

Riboflavin and PQQ stimulate CO production in human placenta

Having demonstrated CO production by riboflavin and PQQ in rodent tissue, we tested whether human tissue contained similar biochemical machinery by preparing placental microsomes from
healthy infant deliveries. The baseline for CO generated by human placenta microsomes was approximately 70 pmol, and addition of 1 µM PQQ increased CO production to 112±18 pmol (Fig. 3). The addition of 10 and 25 µM PQQ increased CO production to 210±35 and 299±48, respectively. The CO production curve of riboflavin was shifted approximately one order of magnitude to the right with 10 µM producing no detectable increase in CO and the addition of 25 µM riboflavin only yielding 129±24 pmol CO production. At 100 µM PQQ and riboflavin increased CO generation to 370±27 and 280±62 pmol, reflecting respectively, an approximate five- and four-fold change from 10 nM.

**PQQ produces significantly more CO than riboflavin in the presence of rh-CPR**

To determine the extent to which CPR may contribute to CO production in the human placenta, experiments were conducted where riboflavin and PQQ were incubated in the presence of recombinant human CPR (rh-CPR) and the necessary substrates and cofactors; methemalbumin and NADPH, respectively. Unlike the microsomal preparations, baseline levels of CO production were nearly undetectable in the reconstituted system (Fig. 4A). In the presence of 1 µM PQQ, 9±3pmol of CO was generated while 10 µM PQQ increased this to 75±17 pmol CO. The further escalation to 100 µM increased CO production to 159 ± 17 pmol. Relative to the PQQ CO generation curve, the riboflavin CO generation curve was shifted approximately two orders of magnitude to the right. Here, the first significant increase in CO generation occurred at 100 µM riboflavin (47±15 pmol CO; Fig 4A). The rh-CPR containing preparation used in Fig. 4 was run on 10% SDS-PAGE (Fig. 4B). A very dense over-saturated band corresponding to rh-CPR was identified at approximately 75 kDa with several fainter minor contaminants identified at higher and lower molecular weights.
DISCUSSION

There were two critical observations in this study. First, both riboflavin and PQQ stimulated significant CO production in the presence of microsomes prepared from rat spleen, rat brain or human placenta. The second critical observation is that riboflavin and PQQ also stimulated the generation of CO in the presence of purified rh-CPR.

The preparation containing rh-CPR had as its major component an oversaturated band at approximately 75 kDa corresponding closely to the previously reported molecular weight of approximately 76.5 kDa (Uniprot.org). Although faint bands of contaminating proteins were present it is unlikely they contributed to CO generation. First, by comparison rh-CHR was present at exponentially greater quantity than all the contaminants combined. Second, although HO have been reported in a number of bacteria, only one has been reported in *E. Coli*, the species from which the BL21 (DE3) pLysS cells, used to grow the expression vector, are derived. In *E. Coli*, the HO is called ChuS (Maharshak *et al*. 2015) and is reported to run at approximately 39 kDa (Uniprot.org). There is a faint shadow corresponding to this approximate molecular weight (Fig 4B) but is many orders of magnitude less abundant than rh-CPR suggesting it does not contribute to CO production.

In view of CO’s role as an important second messenger gas (reviewed in Motterlini and Foresti 2017), these observations suggest that in addition to their well-described functions as enzymatic co-factors (reviewed in Ames 2018; Powers 2003; Thakur *et al*. 2017), riboflavin and PQQ may have hitherto unreported roles in the regulation of CO-dependent physiological signalling. All microsomal preparations in the current study showed a significant baseline level
of CO production approximating 70 pmol. In the presence of menadione, we demonstrated baseline CO arises from contributions by HO, whereas the asymptotic component of the CO generation curve is primarily the result of CPR (Vukomanovic et al. 2017). Therefore, we ascribe the CO production observed at higher concentrations of riboflavin and PQQ to also be predominantly CPR. It has long been held that CO production in mammalian systems is the domain of heme-oxygenase enzymes (reviewed in Maines 1997), but these current observations further underscore our recent studies showing the importance of CPR in physiological CO production (Vukomanovic et al. 2017). CO generation occurred in persistent tissues such as spleen and brain, as well as the placenta which exists transiently, suggesting that significant CO generation may occur in any tissue containing CPR.

Our interest in identifying agents that stimulate CO generation in vivo stems from a growing body of work implicating CO insufficiency in the etiology of preeclampsia (Bainbridge et al. 2005; Levytska et al. 2013; George et al. 2014; Ramma and Ahmed 2014; Venditti and Smith 2014). Indeed, a mouse model of preeclampsia was used to demonstrate, that direct inhalation of CO augments uterine artery blood flow and uteroplacental vascular growth (Vendetti et al. 2013) and protected against associated maternal organ damage (Venditti et al. 2014). The present study demonstrating that riboflavin stimulates CO generation in human placenta microsomes may provide a mechanistic link to the observation that riboflavin deficiency is significant risk factor for preeclampsia (Wacker et al. 2000). A subsequent riboflavin intervention study was unable to demonstrate a clinical benefit for the treatment of preeclampsia (Neugebauer et al. 2006); however, we speculate the “low dose” 15 mg/day used in the aforementioned study was insufficient. For instance, multiple migraine intervention trials have
used “high dose” 400 mg/day riboflavin to effectively reduce the incidence and severity of
migraines (reviewed in Thakur et al. 2017). Our current studies demonstrating significant CO
production from brain microsomes, lead us to propose that the therapeutic efficacy of riboflavin
against migraines may be, in part, CO-dependent; recalling that CO is an important mediator of
inflammation and vascular tone (Motterlini and Foresti 2017), both of which are believed to be
dysregulated in migraine (Conti et al. 2019). A role for targeted riboflavin supplementation in
the prevention of hypertension has also been identified in patients with a specific defect in
methylenetetrahydrofolatereductase (MTHFR) (reviewed in McNulty et al. 2017). These
randomized trials showed that riboflavin has a genotype-specific role in lowering blood pressure
by an average of 6-13 mmHg independent of the effect of antihypertensive drugs. The
mechanism of action that has been proposed is that riboflavin supplementation in patients with
MTHFR677TT genotype might lead to improved endothelial function through increased
bioavailability of nitric oxide via increases in both 5-methyltetrahydrofolate and
tetrahydrobiopterin. Our current findings suggest that CO generation may also contribute to a
portion of riboflavin’s antihypertensive effects. We are unaware of studies linking PQQ with
preeclampsia or migraines, but in a small clinical crossover study, supplementation with PQQ
significantly reduced circulating markers of inflammation (Harris et al. 2013), an effect also
ascribed to CO (reviewed in Motterlini and Foresti 2017). Taken together, our in vitro findings
showing riboflavin and PQQ activation of CO production are consistent with some of the in vivo
clinical observations ascribed to dietary supplementation with these two nutrients.

In the current study, in vitro CO production from human and rat microsomes was
detectable when riboflavin and PQQ approached the μM range, whereas the reported in vivo
tissue concentrations of riboflavin and PQQ are nM (respectively, Bosch et al. 2011; Kumazawa
et al. 1992). Despite this concentration discrepancy our prior studies suggest riboflavin and PQQ will promote CO production in vivo. For instance, in rat brain microsomes menadione increased CO production at μM and not nM concentrations (Vukomanovic et al. 2011), yet when added to the drinking water of mice, menadione significantly increased maternal CO concentrations (Odozur et al. 2018). Although plasma menadione concentrations were not measured in the aforementioned study, it is unlikely that plasma menadione concentrations exceeded the nM range. First, taste aversion reduces water consumption when menadione is added to the drinking water (Odozur et al. 2018), and low μM concentrations have only been reported when menadione was administered i.v. at toxic doses as an adjunct to cancer chemotherapy (Lim et al. 2005).

The present study showed that 100 μM riboflavin or PQQ increased CO production in human placenta microsomes approximately three- or four- fold, over baseline respectively. This compares favourably with the six-fold increase in CO production in the presence of 100 μM menadione (unpublished data). The manner in which riboflavin and PQQ enhance CO production remains to be elucidated; however, we speculate their mechanism of action may be similar to that of menadione, because these nutrients share a number of electrochemical and structural similarities with the vitamin K analogue. Previously, we demonstrated that the ability of menadione to promote CO production from rh-CPR was reliant upon the generation of reactive oxygen species in the form of superoxide radicals and hydrogen peroxide; addition of superoxide dismutase and catalase significantly reduced CO production (Vukomanovic et al. 2017). The ability of riboflavin and PQQ to act as cofactors free radical formation has been previously reported (respectively, Juárez et al. 2008; Akagawa et al. 2016).
There were tissue-specific differences in CO production noted with both nutrients. The elucidation of the cause of the tissue-specific effects would require further experimentation, but we expect the differences to be, in part, the result of differences in the relative ratios of CPR, HO-1 and HO-2 enzyme activities. For example, we previously demonstrated that menadione stimulates robust CO production in rat brain microsomes, a constitutively rich source of HO-2; however, in spleen an abundant source of HO-1, menadione fails to stimulate CO production (Vukomanovic et al. 2014). In the present study, the addition of riboflavin and PQQ significantly increased CO production in rat spleen microsomes, but the response was greater in brain microsomes suggesting HO-2 and CPR activities are higher in this tissue. Although subtle, riboflavin was the more effective promoter of CO production in the spleen whereas in rat brain and human placenta PQQ was dominant. What is more, in placenta microsomes and with preparations only containing rh-CPR, there was a markedly similar rightward shift of the riboflavin CO production curve relative to that of PQQ. We interpret the shared rightward shift to mean, that with respect to riboflavin and PQQ, CPR rather than HO is the dominant source of CO production in the human placenta. The importance of CPR may have profound clinical implications. For instance, placental HO-1 expression and exhaled CO levels are both lower in women with preeclampsia, consistent with a role for HO-1 as an important source of CO during pregnancy (Levitska et al. 2013). Lower HO activity in cases of preeclampsia presents a profound challenge, recalling that our therapeutic strategy is reliant using a compromised endogenous biochemical process (HO-1) to produce CO. The observation, that CPR is likely the dominant CO generation activity in the human placenta leads us to suggest that riboflavin or PQQ may be used to activate CPR-based CO production to compensate for the HO insufficiency. Additionally, the extraordinary increase of CO production by RB or PQQ becomes even more
significant in the light of Zorn and Wells (2010) pointing out that even a modest enzymatic activity enhancement of 10% is therapeutically useful. Thus, the several-fold increase in placental CO production in the presence of RB or PQQ may have therapeutic potential even if the endogenous process used to produce CO is compromised.

In conclusion, we have described previously that CO production occurs in chorionic villi of human placenta (Ahmed et al. 2005), and the current studies demonstrate for the first time that riboflavin and PQQ increase CO production in human placenta microsomes. Thus, we propose that both nutrients may have novel biochemical roles in gasotransmission. A critical role for CPR in the production of CO was also demonstrated, and the further study of this novel pathway would be warranted because its up- or down-regulation by nutrients may have therapeutic applications.

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ABBREVIATIONS
CO: carbon monoxide
REFERENCES


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FIGURE LEGENDS

Figure 1. Enhanced CO production by rat spleen microsomes in the presence of riboflavin (RB) and pyrolloquinolone-quinone (PQQ). Heme degradation in microsomal fractions (1 mg of protein) prepared from four different male rat spleens (triplicates for each) was quantified by measurement of CO using a headspace gas chromatography method, after 15 minutes of incubation with RB or PQQ and 1.0 mM NADPH in 100 mM phosphate buffer (pH = 7.4) at 37°C. Data are presented as mean ± standard deviation, picomoles of CO (pmol CO). The asterisk denotes a statistically significant difference of at least p< 0.05 from the respective RB or PQQ value at 10 nM.

Figure 2. Enhanced CO production by rat brain microsomes in the presence of riboflavin (RB) and pyrolloquinoline-quinone (PQQ). Heme degradation in microsomal fractions (1 mg of protein) from four different male rat brains (triplicates for each) was quantified by measurement of CO using a headspace gas chromatography method, after 15 minutes of incubation with RB or PQQ and 1.0 mM NADPH in 100 mM phosphate buffer (pH = 7.4) at 37°C. Data are presented as mean ± standard deviation, picomoles of CO (pmol CO). The
asterisk denotes a statistically significant difference of at least p< 0.05 from the respective RB or
PQQ value at 10 nM.

Figure 3. Enhanced CO production by human placenta microsomes in the presence of
riboflavin (RB) and pyrolloquinolone-quinone (PQQ). Heme degradation in microsomal
fractions (1 mg of protein) from four different placentas (triplicates for each) was quantified by
measurement of CO using a headspace gas chromatography method, after 15 minutes of
incubation with RB or PQQ and 1.0 mM NADPH in 100 mM phosphate buffer (pH = 7.4) at
37°C. Data are presented as mean ± standard deviation, picomoles of CO (pmol CO). The
asterisk denotes a statistically significant difference of at least p< 0.05 from the respective RB or
PQQ value at 10 nM.

Figure 4. Pyrolloquinoline-quinone (PQQ) produces significantly more CO than riboflavin
(RB) in the presence of recombinant human CPR (rh-CPR). Panel A); the heme degradation
in the presence of 10 μM rh-CPR (four replicates from the same preparation) was quantified by
measurement of CO using a headspace gas chromatography method, after 15 minutes of
incubation with RB or PQQ and 1.0 mM NADPH in 100 mM phosphate buffer (pH = 7.4) at
37°C. Data are presented as mean ± standard deviation, picomoles of CO (pmol CO). The
asterisk denotes a statistically significant difference of at least p< 0.05 from the respective RB or
PQQ value at 10 nM. Panel B); The SDS PAGE (10%) of the rh-CPR preparation used to
generate CO in panel A) was stained with Coomasie Brilliant Blue. Lane 1 contains a molecular
weight ladder and Lane 2 the rh-CPR-containing preparation. The arrowhead denotes the
location of rh-CPR.
Figure 1. Enhanced CO production by rat spleen microsomes in the presence of riboflavin (RB) and pyrolloquinolone-quinone (PQQ). Heme degradation in microsomal fractions (1 mg of protein) prepared from four different male rat spleens (triplicates for each) was quantified by measurement of CO using a headspace gas chromatography method, after 15 minutes of incubation with RB or PQQ and 1.0 mM NADPH in 100 mM phosphate buffer (pH = 7.4) at 37°C. Data are presented as mean ± standard deviation, picomoles of CO (pmol CO). The asterisk denotes a statistically significant difference of at least p< 0.05 from the respective RB or PQQ value at 10 nM.
Figure 2. Enhanced CO production by rat brain microsomes in the presence of riboflavin (RB) and pyrolloquinoline-quinone (PQQ). Heme degradation in microsomal fractions (1 mg of protein) from four different male rat brains (triplicates for each) was quantified by measurement of CO using a headspace gas chromatography method, after 15 minutes of incubation with RB or PQQ and 1.0 mM NADPH in 100 mM phosphate buffer (pH = 7.4) at 37°C. Data are presented as mean ± standard deviation, picomoles of CO (pmol CO). The asterisk denotes a statistically significant difference of at least p< 0.05 from the respective RB or PQQ value at 10 nM.
Figure 3. Enhanced CO production by human placenta microsomes in the presence of riboflavin (RB) and pyrolloquinolone-quinone (PQQ). Heme degradation in microsomal fractions (1 mg of protein) from four different placentas (triplicates for each) was quantified by measurement of CO using a headspace gas chromatography method, after 15 minutes of incubation with RB or PQQ and 1.0 mM NADPH in 100 mM phosphate buffer (pH = 7.4) at 37°C. Data are presented as mean ± standard deviation, picomoles of CO (pmol CO). The asterisk denotes a statistically significant difference of at least p< 0.05 from the respective RB or PQQ value at 10 nM.

254x190mm (72 x 72 DPI)
Figure 4. Pyrolloquinone-quinone (PQQ) produces significantly more CO than riboflavin (RB) in the presence of recombinant human CPR (rh-CPR). Panel A); the heme degradation in the presence of 10 μM rh-CPR (four replicates from the same preparation) was quantified by measurement of CO using a headspace gas chromatography method, after 15 minutes of incubation with RB or PQQ and 1.0 mM NADPH in 100 mM phosphate buffer (pH = 7.4) at 37°C. Data are presented as mean ± standard deviation, picomoles of CO (pmol CO). The asterisk denotes a statistically significant difference of at least p< 0.05 from the respective RB or PQQ value at 10 nM.  Panel B); The SDS PAGE (10%) of the rh-CPR preparation used to generate CO in panel A) was stained with Coomasie Brilliant Blue. Lane 1 contains a molecular weight ladder and Lane 2 the rh-CPR-containing preparation. The arrowhead denotes the location of rh-CPR.