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In Vivo Inhibition of Renal Heme Oxygenase with an Imidazole-Dioxolane Inhibitor

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Abstract

Recent studies have indentified imidazole-dioxolane based compounds as novel heme oxyenase (HO) inhibitors. While these compounds have been demonstrated to be specific HO inhibitors in vitro, they have yet to be used to inhibit renal HO activity in vivo. The goal of this study was to determine the effectiveness of the imidazole-dioxolane HO-1 inhibitor, QC-13, in the inhibition of renal HO activity in vivo. HO-1 was induced in mice by treatment with cobalt protoporphyrin (CoPP). After 5 days, QC-13 was delivered either by continuous intrarenal medullay interstitial infusion (IRMI) into one kidney at several concentrations for 72 hours or by two intraperitoneal injections over a 48 hour period. IRMI infusion of QC-13 at a concentration of 25 μM resulted in a significant decrease in medullary but not cortical HO activity as compared to CoPP treated kidneys. IRMI infusion of QC-13 at a lower concentration (2.5 μM) had no effect on either medullary or cortical HO activity in CoPP treated mice. In contrast, administration of QC-13 at a higher concentration (250 μM) resulted in a significant decrease in both medullary and cortical HO activity in CoPP treated mice. Systemic administration of QC-13 resulted in significant decrease both renal cortical and medullary HO activity in CoPP treated mice. In contrast to classical porphyrin based HO inhibitors, IRMI infusion of QC-13 did not induce HO-1 protein levels as determined by Western blot analysis of medullary protein samples. Our results demonstrated that imidazole-dioxolane inhibitors are renal HO inhibitors in vivo and can inhibit HO activity independent of HO-1 induction. These inhibitors may be useful tools to elucidate the role of renal HO-1 in numerous physiologic and pathophysiologic conditions.

Keywords

Kidney; Carbon Monoxide; Cobalt Protoporyphrin; Heme Oxygenase-1; Mouse

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Introduction

Heme oxygenase (HO) is the rate limiting enzyme in the degradation of heme to biliverdin in which carbon monoxide (CO) is released [1]. Biliverdin is subsequently converted into bilirubin by biliverdin reductase. The beneficial actions of HO in the cardiovascular system have been demonstrated by studies in which HO-1 levels were induced either chemically or genetically. For example, several studies have demonstrated that induction of HO-1 lowers blood pressure in several rodent models of hypertension [2-5]. Additional studies have demonstrated that induction of HO-1 protects organs from ischemia-reperfusion injury [6-9].

Whereas HO-1 induction studies have revealed important information regarding the biology of HO-1, we have also learned much about the biology of HO-1 from inhibitor studies. The first class of inhibitors and the most widely used are porphyrin compounds. Porphyrins are ring like structures which can bind to metals and form metalloporphyrins which are potent HO inhibitors [10,11]. Metallopophyrin based HO inhibitors have been used widely to elucidate the function of HO-1; however, they have several limitations with their use *in vivo*. First, most metalloporphyrins are photosensitive and can breakdown upon exposure to light which makes working with them difficult [12]. Second, the specificity of these compounds can be an issue. Several studies have demonstrated that metalloporphyrins can inhibit other proteins such as nitric oxide synthase and soluble guanylyl cyclase especially when used at higher concentrations [13-15]. Lastly, while these compounds are inhibitors of HO activity, they are also very potent inducers of HO-1 due to the metal group associated with the porphyrin ring [16,17]. Imidazole-dioxolane HO inhibitors are a new class of compounds which have recently been reported to be specific inhibitors of HO *in vitro* [18,19]. These compounds offer a potential advantage in that they do not resemble heme, the natural HO substrate, and they do not contain metals which may induce HO-1. Recently, the effectiveness and specificity of imidazole-dioxolane inhibitors was demonstrated in cultures of proximal tubule cells [20]. Whether these compounds are effective HO inhibitors in the kidney has yet to be tested. In the present study, we determined for the first time if the imidazole-dioxolane HO inhibitor, QC-13, is an effective *in vivo* inhibitor of renal HO activity.

Methods

Animals

Studies were performed on 16-20 week male C57BL/6J mice (Jackson Labs, Bar Harbor, ME). All studies were performed in accordance with the approval of the University of Mississippi Medical Center Institutional Animal Care and Use Committee (IACUC) and in line with NIH guidelines. Mice were treated with cobalt protoporphyrin (CoPP) as described below. After seven days, intramedullary interstitial catheters were implanted into the left kidney and saline infused through the catheter for a period of 2 days. After this time, the infusion was switched over to QC-13 for an additional 3 days. Mice were then euthanized for determination of HO activity. Intramedullary interstitial catheters were modified as previously described [21] and implanted 1.5-2 mm into the left kidney. QC-13 was also administered by intraperitoneal (ip) injection (52 mg/kg) which is equivalent to 10 times the 25 μM concentration that was administered directly into the renal medulla. In this part of the study, mice were treated with CoPP as above. After 7 days, mice received single ip injections of QC-13 for two days and then were euthanized the next day and organs collected for determination of HO activity.

Drugs

QC-13, (2*R*,4*R*)-2-[2-(4-chlorophenyl)ethyl]-2-[(1*H*-imidazol-1-yl)methyl]-4-methyl-1,3-dioxolane hydrochloride, was synthesized as previously described [18]. Stock solutions of QC-13 were prepared in distilled water and diluted in saline for intrarenal medullay interstitial infusion (IRMI) and ip injections. HO-1 was induced by a single treatment with CoPP (50 mg/kg body weight, sc, Frontier Scientific, Logan, UT) as previously described [5].

Heme Oxygenase Assay

Heme oxygenase assay was performed on tissue lysates prepared as previously described [5]. The protein concentration was measured using a Bio-rad protein assay with BSA standards. Reactions were carried out in a 1.2 mls containing: 2 mM glucose-6-phosphate, 0.2 units glucose-6-phosphate dehydrogenase, 0.8 mM NADPH, 20 μM hemin, and 0.5 mg of lysate. The reactions were incubated for 1 hour at 37°C in the dark. The formed bilirubin was extracted with chloroform, and the change in optical density (ΔOD) at 464-530 nm was measured using an extinction coefficient of 40 mM/cm for bilirubin. HO activity was calculated as picomoles of bilirubin formed per hour per milligram of microsomal protein. For the IRMI studies, HO activity is expressed as percentage of non-infused cortex or medulla.

Western Blot

Western Blot for HO-1and HO-2 proteins were performed on lysates prepared from the renal cortex and medulla. Samples of 30 μg of protein were boiled in Laemmli sample buffer (Bio-Rad, Hercules, CA) for 5 min, electrophoresed on 12% SDS-polyacrylamide gels, and blotted onto nitrocellulose membrane. Membranes were blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE) for 2 hours at room temperature, then incubated with mouse anti-HO-1 antibody (StressGen, Vancouver, Canada, 1:2000) and rabbit anti-β-actin antibody (abcam, Cambridge, MA, 1:5,000) or rabbit anti-HO-2 polyclonal antibody (StressGen, Vancouver, Canada, 1:2000) and mouse anti-β actin antibody (abcam, Cambridge, MA, 1:5,000) overnight at 4°C. The membranes were then incubated with Alexa 680 goat anti-mouse IgG (Molecular Probes, Portland, OR, 1:5,000) and IRDye 800 goat anti-rabbit IgG (Rockland, Gilbertsville, PA, 1:5,000) or Alexa 680 donkey anti-rabbit IgG (Molecular Probes, Portland, OR, 1:5,000) or IRDye 800 donkey anti-mouse IgG (Rockland, Gilbertsville, PA, 1:5,000) for 1 hour at room temperature. The membranes were then visualized using an Odyssey infrared imager (Li-COR, Lincoln, NE). Experiments were performed in duplicated and densitometry analysis was performed using Odyssey software (LI-COR, Lincoln, NE). The level of HO protein is expressed as a ratio to the level of β-actin to normalize for protein loading.

Statistics

Mean values ± Standard Error (SE) are presented. Significant differences between mean values were determined using an unpaired T test or by ANOVA followed by a post hoc test (Tukey). A $p < 0.05$ was considered to be significant.

Results

Effect of Intrarenal Medullary Interstitial Infusion (IRMI) of QC-13 on HO Activity in CoPP Treated Mice

IRMI infusion of QC-13 at a concentration of 25 μM had no effect on HO activity in the medulla or cortex of control mice (Figure 1). This result is likely due to the low levels of HO-1 protein present in the kidney under basal conditions. In order to determine the

effectiveness of QC-13 in inhibiting HO-1, mice were pre-treated with CoPP which is a strong inducer of HO-1 protein *in vivo* [5]. CoPP treatment resulted in a 3 fold increase in HO activity in both the renal cortex and medulla as compared to control levels (data not shown). The dose response curves of QC-13 on HO activity in the renal cortex and medulla of CoPP treated mice are presented in Figure 2. IRMI of QC-13 at a concentration of 2.5 or 25 μM had no effect on renal cortical HO activity. HO activity averaged 106 ± 17 and $111 \pm 8\%$ of that observed in the non-infused CoPP treated kidney (Figure 2A). IRMI infusion of QC-13 at a concentration of 250 μM resulted in a significant decrease in renal cortical HO activity as compared to the non-infused CoPP treated kidney averaging $59 \pm 9\%$ (Figure 2A).

IRMI of QC-13 at a concentration of 2.5 μM had no effect on renal medullary HO activity in the infused kidney of CoPP treated mice averaging $105 \pm 11\%$ (Figure 2B). However, IRMI of QC-13 at concentrations of 25 and 250 μM resulted in significant decreases in medullary HO activity in the infused kidney of CoPP treated mice averaging 46 ± 14 and $38 \pm 7\%$ of that observed in the non-infused CoPP treated kidney (Figure 2B).

Effect of Intraperitoneal Injection QC-13 on HO Activity in CoPP Treated Mice

In order to determine the effect of systemic administration of QC-13 on renal HO activity, CoPP treated mice were treated with two ip injections (52 mg/kg) over a 48 hour period. Systemic administration of QC-13 at this concentration resulted in a significant decrease in both renal cortical and medullary HO activity in CoPP treated mice as compared to non-QC-13 treated mice (Table 1). However, systemic administration of QC-13 at this concentration did not result in a significant decrease in HO activity in heart or liver of CoPP treated mice (Table 1).

IRMI Infusion of QC-13 Does Not Induce HO-1 Protein

In order to determine if IRMI infusion of QC-13 was associated with induction of HO-1 or HO-2 protein, Western blot was performed on lysates from the renal cortex and medulla of control mice in which QC-13 was infused via intrarenal medullary interstitial catheters as well as control, IRMI QC-13, CoPP, and CoPP + IRMI QC-13 mice. CoPP treatment resulted in a 10-fold increase in the level of HO-1 protein in the renal medulla (Figure 3). IRMI infusion of QC-13 did not induce HO-1 protein either in control or CoPP treated mice (Figure 3). Next, we determined the effect of IRMI infusion of QC-13 at several concentrations (2.5, 25, and 250 μM) on the levels of HO-1 and 2 protein in the cortex and medulla of the infused kidney. IRMI infusion of QC-13 at all concentrations tested had no effect on the levels of HO-1 or 2 protein in the renal cortex of infused mice (Figure 4). Likewise, IRMI infusion of QC-13 at all concentrations tested had no effect on the levels of HO-1 or 2 protein in the renal medulla of infused mice (Figure 5). These results indicate that unlike porphryrin-based HO inhibitors imidazole-dioxolane inhibitors do not induce HO-1 protein *in vivo*.

Discussion

The present study is the first to test the efficacy of the imidazole-dioxolane inhibitor, QC-13, in the *in vivo* inhibition of HO-1 in the kidney by either direct infusion into the renal medulla or by systemic administration. Our results demonstrate that when QC-13 was infused directly into the renal medulla of CoPP treated mice at a concentration of 25 μM specific inhibition of medullary HO activity was observed. This concentration of QC-13 was based on the findings of a previous *in vitro* study which demonstrated that this concentration of QC-13 resulted in maximal inhibition of HO activity in cultured proximal tubule cells treated with hemin [20]. While direct medullary infusion of QC-13 at this concentration was

able to inhibit HO activity in CoPP pretreated mice, no significant inhibition of HO activity was observed when infused into the renal medulla of control mice. One reason for this observation may be that under basal conditions HO-1 is expressed at very low levels in the kidney and the majority of HO activity present may be derived from HO-2 [5,22]. Previous studies in cultured renal proximal tubule cells have demonstrated that QC-13 is a specific inhibitor of HO-1 but not HO-2 while another imidazole-dioxolane inhibitor, QC-1, can inhibit both isoforms of HO [20].

IRMI of QC-13 at a lower concentration (2.5 μM) did not result in inhibition of HO activity in the renal medulla and cortex of CoPP treated mice. However, IRMI infusion of QC-13 at a 100 fold higher concentration (250 μM) resulted in significant inhibition of both renal medullary and cortical HO activity in CoPP treated mice. Classically, compounds that are infused into the renal medulla are selectively accumulated because of the efficient countercurrent exchanger in the vasa recta circulation [23]. When compounds are infused at very high concentrations as with 250 μM QC–13, it is possible that excess accumulation of the compound increases the amount that gets reabsorbed by the peritubular capillaries resulting in increased levels of the compounds which are then able to diffuse into the cortex. Thus, it is important to always test activity in both regions of the kidney when administering drugs into the renal medullary interstitium to ensure the degree of inhibition in each region.

IRMI infusion at the 25 μM concentration resulted in selective inhibition of renal medullary but not cortical HO activity. However, we found that systemic administration of QC-13 at 10 at times this concentration inhibited both renal cortical and medullary HO activity without having any effect on HO activity in the heart and liver. The reason for this observation is not known but may be due to the increased accumulation of QC-13 in the kidney upon being filtered from the blood. A portion of the QC-13 could then be reabsorbed by the tubule cells mainly in the cortex and to a lesser extent in the renal medulla to inhibit HO activity. QC-13 administered via IP injection at this dose may not have reached a high enough plasma concentration effectively inhibit HO activity in these organs. While we did observe a significant decrease in renal cortical and medullary HO activity with this dose of QC-13, the degree of inhibition was much greater (56%) in the renal cortex as compared to the medulla (20%). Thus, it may be possible to selectively inhibit medullary versus cortical HO activity with a lower dose of QC-13 in order to determine the role of HO in these different regions in various renal pathologies. For example, both renal ischemia and acute renal injury due to radiocontrast agents are believed to cause preferential damage to the renal medulla especially thick ascending loop of Henle cells [24,25] while nephrotoxins such as cisplatin are believed to target proximal tubule cells in the cortex [26,27]. Several studies have suggested that induction of HO-1 may play a protective role in the response of the kidney to all of these pathological injuries [28-31]. Differential delivery methods as well as infusion of different concentrations of the imidazole-dioxolane inhibitor, QC-13, could be used as a strategy to target either medullary or cortical HO-1 to further investigate the protective role of HO-1 induction in each of these conditions.

The most widely used class of HO inhibitors are metalloporphyrin based compounds such as stannous mesoporphyrin, Zinc Deuteroporphyrin IX 2,4 Bis Etylene Glycol (ZnDPBG), and chromium mesoporphyrin (CrMP) [32-34]. While these compounds can block HO activity, they are also potent inducers due to the metal containing groups [16,17]. The induction of HO-1 by these compounds could lead to decreased efficiency of blockade *in vivo* especially in situations in which these compounds are administered chronically. Imidazole-dioxolane inhibitors such as QC-13 offer a distinct advantage over metalloporphyrin based HO inhibitors in that they do not induce HO-1 *in vivo* which is less likely to complicate interpretation of data resulting from their chronic use. They are also water soluble and do not undergo photosensitization which also makes them easier to chronically infuse *in vivo*.

In conclusion, the present study demonstrates that the imidazole-dioxolane inhibitor, QC-13, is an effective inhibitor of renal HO-1 *in* vivo. HO-1 activity in the renal medulla or cortex can be selectively blocked with QC-13 depending upon how the compound is delivered to the kidney. Systemic administration of QC-13 preferentially inhibits renal cortical HO-1 while direct infusion into the renal medulla at a concentration of 25 μM selectively inhibits medullary HO-1 activity. Blockade of HO-1 with QC-13 is not associated with induction of endogenous HO-1 as observed with classical metalloporphyrin based HO inhibitors. Imidazole-dioxolane HO inhibitors have the potential to be excellent pharmacological tools to investigate the role of renal HO in a number of different physiological and pathological processes in the kidney.

Acknowledgments

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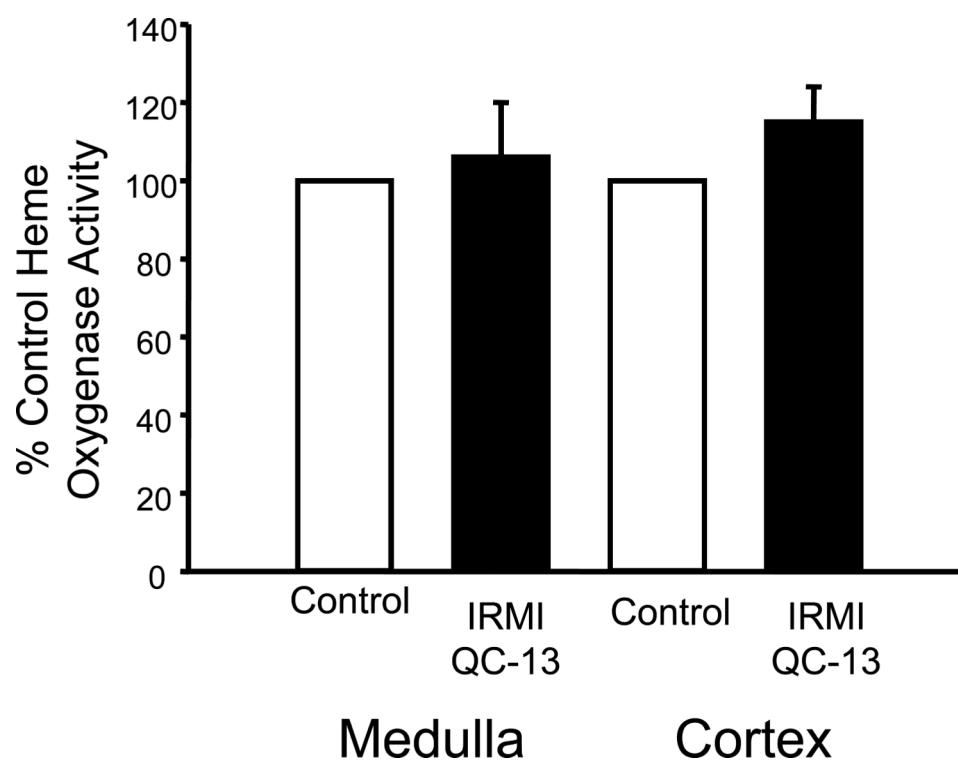
Figure 1.
Effect of intrarenal medullary interstitial infusion of QC-13 (25 μM) on renal medullary and cortical heme oxygenase activity in control mice. QC-13 was infused for two dyas and HO assays were performed as described in the *Methods*. HO activity is expressed as the percent of activity observed in the control non-infused kidney, n=7.
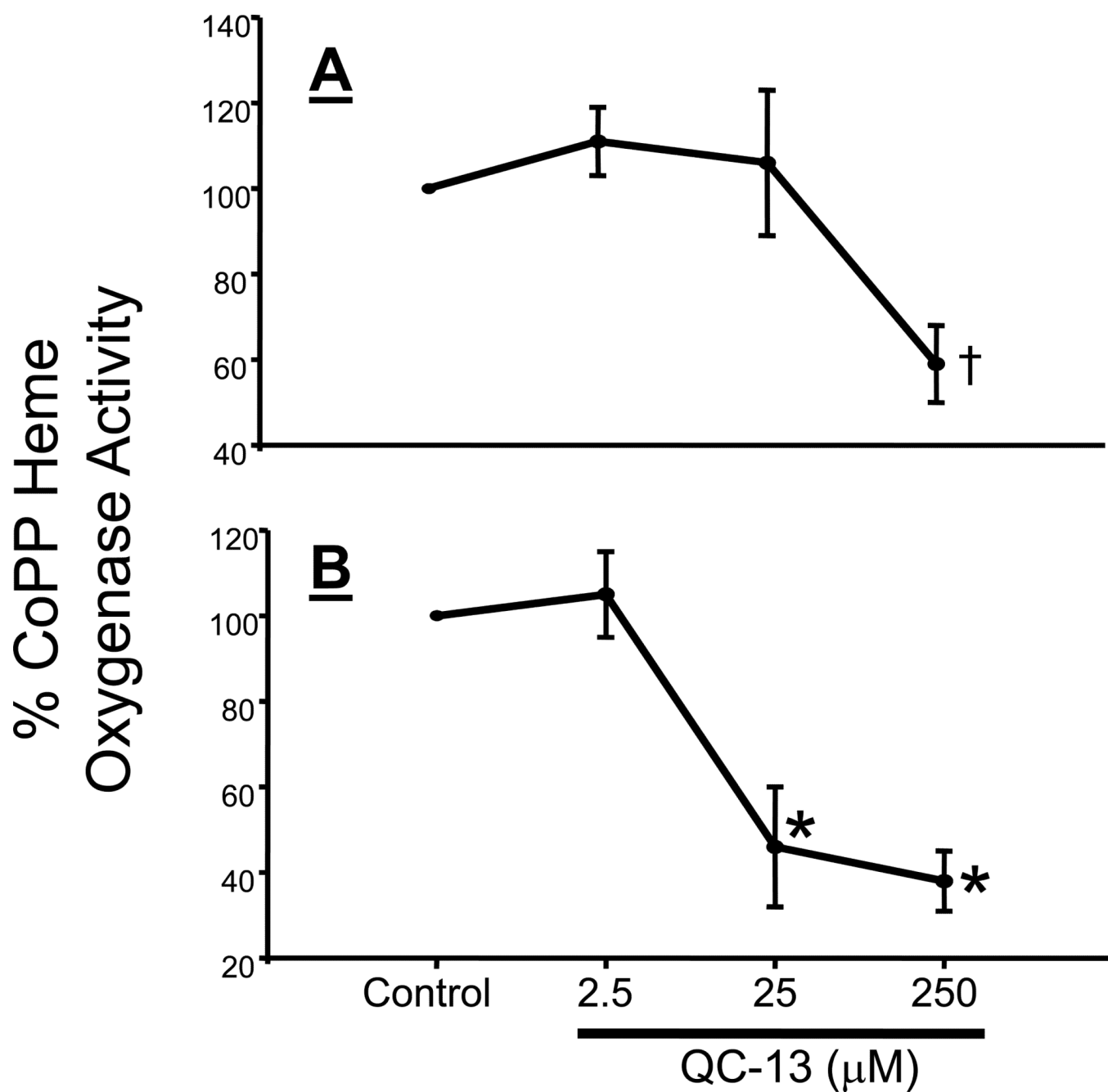
Figure 2.
Dose response curves of intrarenal medullary interstitial infusion of QC-13 (2.5, 25, 250 μM) on A) renal cortical and B) renal medullary heme oxygenase activity in CoPP treated mice. Mice were administered a single dose of CoPP seven days prior to QC-13 infusion. QC-13 was then infused for two days and HO assays were performed as described in the *Methods*. HO activity is expressed as the percent of activity observed in the non-infused CoPP treated kidney, n=6. † =statistically different at *P* <0.004. *= statistically different at *P*<0.001.
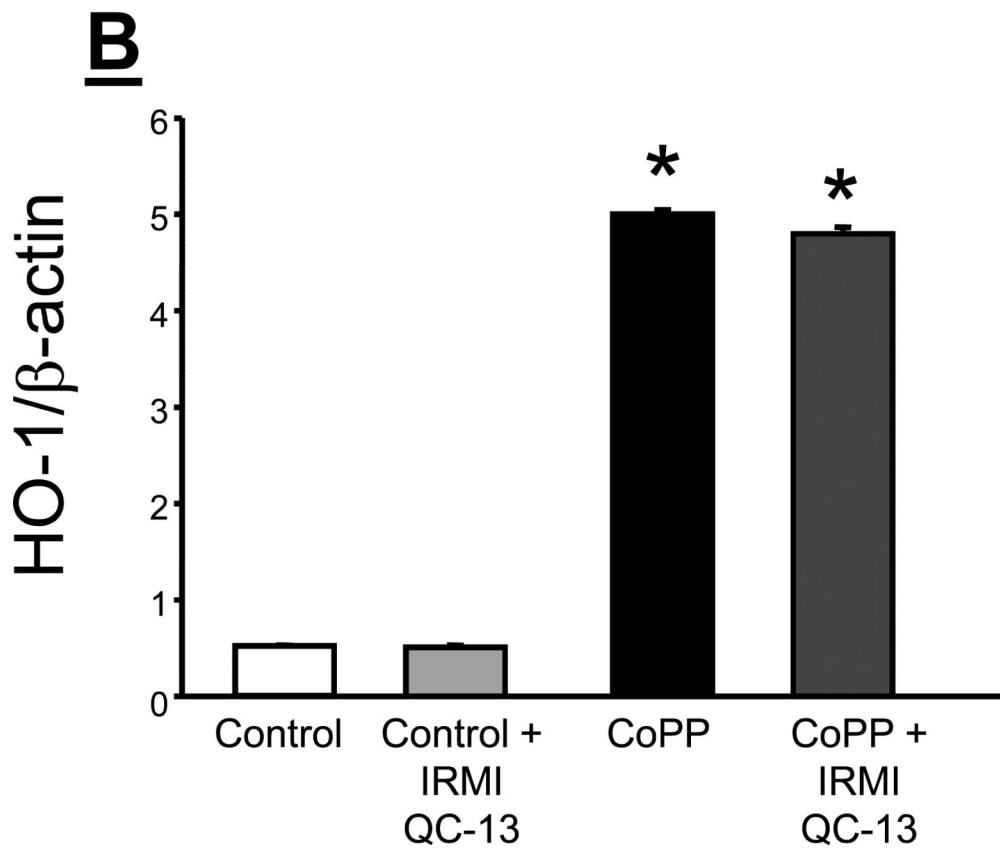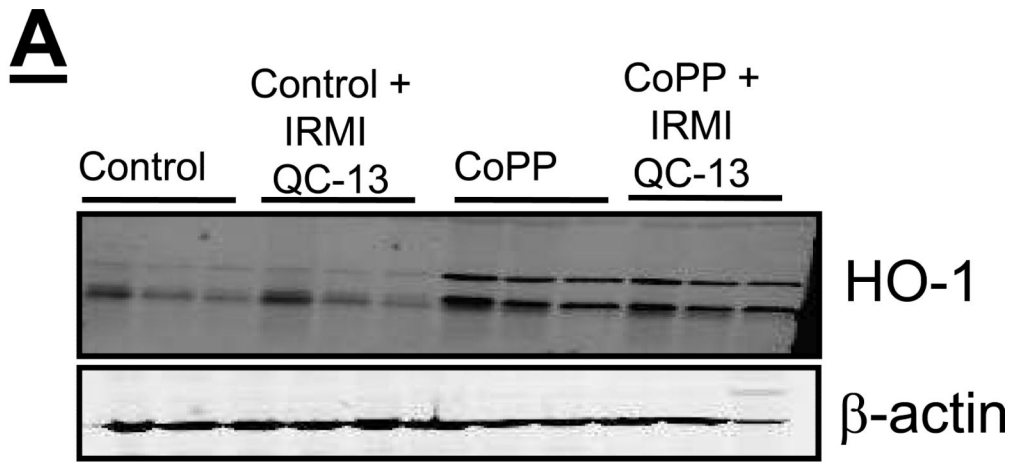
A

B

Figure 3.
Western blot of renal medullary lysates from control, control + intrarenal medullary interstitial infusion (IRMI) QC-13 (25 μM), CoPP, CoPP + IRMI QC-13 treated mice. A) Representative blot of HO-1 and β-actin from 30 μg of tissue lysate. B) Quantitation of the level of HO-1 protein. The level of HO-1 protein is expressed as a ratio to the level of β-actin, n=3. *=statistically different at $p<0.05$ by ANOVA followed by a post hoc test (Tukey).
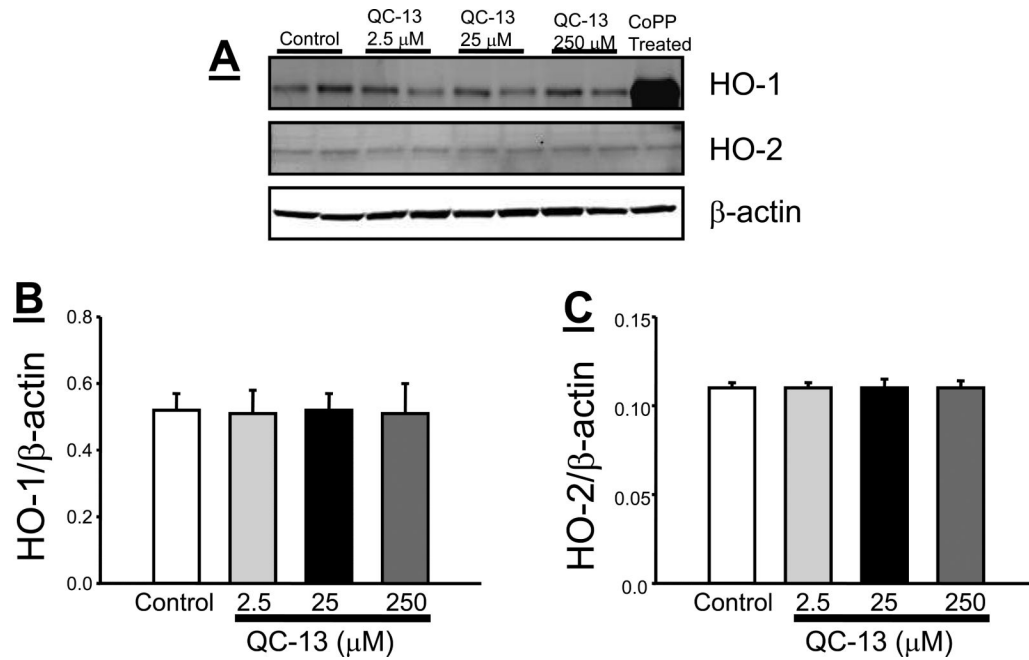
Figure 4.
Western blot of renal cortical HO-1 and HO-1 proteins from QC-13 infused mice. A)
Representative blots of HO-1, HO-2, and β-actin from the renal cortex of QC-13 treated
mice. QC-13 was infused via intrarenal medullary interstitial catheters for two days after
which time kidneys were collected for protein measurements. B) Quantitation of the level of
HO-1 protein. The level of HO-1 protein is expressed as a ratio to the level of β-actin, n=4.
C) Quantitation of the level of HO-2 protein. The level of HO-2 protein is expressed as a
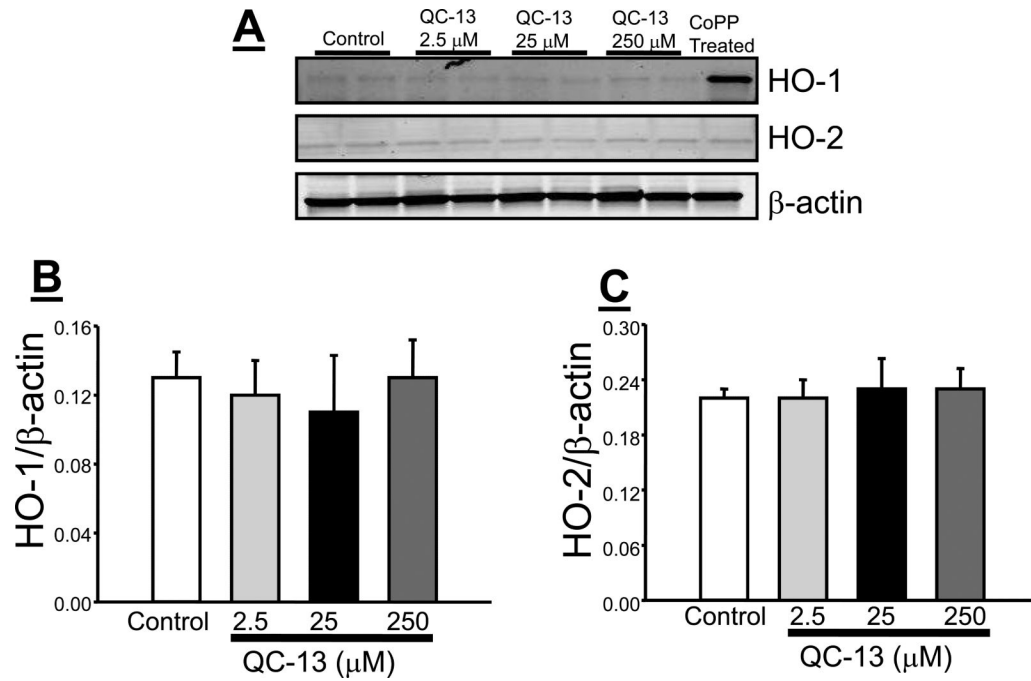ratio to the level of β-actin, n=4.

Figure 5.
Western blot of renal medullary HO-1 and HO-1 proteins from QC-13 infused mice. A) Representative blots of HO-1, HO-2, and β-actin from the renal medulla of QC-13 treated mice. QC-13 was infused via intrarenal medullary interstitial catheters for two days after which time kidneys were collected for protein measurements. B) Quantitation of the level of HO-1 protein. The level of HO-1 protein is expressed as a ratio to the level of β-actin, n=4. C) Quantitation of the level of HO-2 protein. The level of HO-2 protein is expressed as a ratio to the level of β-actin, n=4.

Table 1

Effect of intraperitoneal injection of QC-13 (52/mg/kg) on HO activity in CoPP Treated Mice.

Organ	Heme Oxygenase Activity	
	CoPP	CoPP + QC-13
Heart	35+ 15	24+ 7
Liver	250 + 64	195 + 26
Renal Cortex	219 + 38	98 + 27*
Renal Medulla	51 + 6	41 + 2*

Heme oxygenase activity given as pmoles bilirubin per hr per milligram of protein.

*
p<0.05 as compared to CoPP treated mice. n=6 per group.