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Regulation of Cyclooxygenase- and Cytochrome P450–Derived Eicosanoids by Heme Oxygenase in the Rat Kidney

Fady T. Botros, Michal Laniado-Schwartzman, Nader G. Abraham

Abstract—Heme oxygenase enzymes (HO-1 and HO-2) catalyze the conversion of heme to biliverdin, free iron, and carbon monoxide (CO). Heme and products derived from its metabolism potentially influence renal function and blood pressure by affecting the expression and/or activity of heme proteins, including cytochrome P450 (CYP4A) monooxygenases and cyclooxygenases (COX-1 and COX-2). We studied HO isoform expression and examined the effect of HO-1 induction by SnCl₂ on CYP4A and COX expression and activity in the rat kidney. HO-1 protein levels in kidney tissues from untreated rats were barely detectable, whereas HO-2 protein was expressed in all kidney structures examined and its levels were higher in the outer medulla followed by the inner medulla/papilla and cortex. HO-2 expression along the nephron followed its regional distribution, ie, the highest levels were detected in the medullary thick ascending limb (mTAL) and inner medullary collecting ducts followed by proximal tubules. SnCl₂ Treatment did not significantly affect HO-2 expression or distribution; however, it markedly increased HO-1 protein in the inner and outer medulla, specifically, in the inner medullary collecting ducts and mTAL. CYP4A expression and 20-hydroxyeicosatetraenoic acid (20-HETE) synthesis were the highest in the outer medulla followed by the cortex and inner medulla/papilla. SnCl₂ treatment reduced cortical and inner medullary CYP4A protein levels by 60% and 50% and inhibited 20-HETE synthesis by 90% and 60%, respectively. Despite a significant induction of HO-1 protein in the outer medulla, CYP4A expression and 20-HETE synthesis were hardly affected. SnCl₂ treatment did not affect COX-1 expression but markedly reduced cortical and medullary COX-2 protein levels. We conclude that HO isoform expression is segmented within the kidney and along the nephron and that treatment with an HO-1 inducer suppressed the levels of CYP4A and COX-2 proteins in a tissue-specific manner with concomitant effects on their activity. Such interactions may play an important role in the regulation of renal function. (*Hypertension*. 2002;39[part 2]:639-644.)

Key Words: cyclooxygenase ■ cytochrome P450 ■ heme oxygenase

Significant and mounting evidence indicates a close relationship among heme metabolism, kidney function, and blood pressure regulation. Heme functions as the prosthetic moiety for a number of heme-containing proteins with activities that are critical to vascular and renal function. These include soluble guanylate cyclase, nitric oxide synthase, and enzymes of the eicosanoid biosynthetic pathways, including cytochrome P450 monooxygenases, thromboxane and prostacyclin synthases, and the heme-dependent cyclooxygenases. Heme oxygenase (HO), as the key enzyme in heme degradation, not only controls cellular levels of heme available for the synthesis of heme proteins but also is responsible for the generation of the vasodepressor gas carbon monoxide (CO), which, by itself, can bind to the heme moiety of these heme proteins and cause either enzyme activation or inhibition.¹⁻³

The importance of HO thus resides in degrading heme and regulating the activity of heme proteins. To date, 3 isoforms,

which are the products of 3 distinct genes, have been identified. HO-2 is a constitutive isoform that is ubiquitously expressed in all tissues.^{4,5} The newly described HO-3 exhibits 90% homology to HO-2, and its mRNA has been detected in several tissues from the rat, including the kidney;⁶ however, it lacks significant catalytic activity and may function only as a heme regulatory protein through its capacity to bind heme.⁶ HO-1 is a 32-kDa heat shock protein that is inducible by numerous stimuli, including heavy metals, oxidative stress and injury, and cytokines.²

Renal HO activity arises primarily from 2 isoforms, HO-1 and HO-2. The functional impact of alteration in their expression and activity may be dependent on where in the kidney they are localized because both CYP450 and cyclooxygenase (COX) have been shown to localize to specific structures within the kidney. CYP450-dependent arachidonic acid metabolism and CYP4A expression demonstrated distinct localization along the nephron and within the renal

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vasculature in the rat kidney.^{7,8} COX activity is localized mainly to the outer and inner medullary parts of the nephron. Moreover, the biological activities of CYP450- and COX-derived eicosanoids are diverse, and the functional outcomes (effect on renal function and blood pressure) of such activities depend on the site of their synthesis in the kidney. For example, 20-hydroxyeicosatetraenoic acid (20-HETE), formed in the thick ascending loop of Henle (TALH), inhibits K-channels and ion transport mechanisms;^{9,10} inhibition of tubular synthesis of 20-HETE is associated with an increase in blood pressure, whereas induction leads to a reduction in blood pressure.^{11,12} In the renal vasculature, 20-HETE is a potent vasoconstrictor, and inhibition of its synthesis results in blood pressure reduction.^{13,14} PGE₂ has been shown to affect transport mechanisms and vascular tone, indicating the possibility that the impact of its biological activity may depend on the site of its synthesis.¹⁵ Likewise is the effect of HO-derived CO; it activates K-channels in the TALH¹⁶ and causes vasodilation in renal vessels.¹⁷ On the basis of these findings, it can be postulated that induction of HO in the TALH may lead to inhibition of eicosanoids that have the ability to inhibit ion transport as well as production of CO, which activates K-channels, leading to a contrasting outcome with regard to renal function.

The present study was designed, first, to characterize HO-1 and HO-2 expression in different regions of the kidney and, second, to examine the effect of HO-1 induction, by the selective renal inducer SnCl₂, on the levels and activity of the heme proteins CYP450 and cyclooxygenases.

Methods

Animal Treatment

Seven-week-old male Sprague-Dawley rats (Charles River, Wilmington, MA) were housed for 4 to 5 days before the beginning of the study. SnCl₂ (5 mg/100 g body wt per day) was administered subcutaneously. Control rats received an injection of saline. Control and treated rats were euthanized after 48 hours. When kidneys were processed for isolation of microvessels and tubules, both kidneys were used.^{18,19}

Tissue Preparation

Kidneys were immediately perfused with ice-cold HEPES solution, sliced, and dissected to separate the cortex, outer medulla, inner medulla, and papilla. Microsomes were prepared as previously described.^{18,19} Protein concentration was determined according to the method of Bradford (BioRad). Proximal tubules were isolated by Percoll gradient separation.²⁰ This method yields approximately 6 to 8 mg of microsomal protein from 1 rat (2 kidneys). medullary thick ascending limb (mTAL) and inner medullary collecting ducts (IMCD) segments were isolated from the inner strip of the outer medulla and the inner medulla, respectively, using an enzymatic digestion followed by a sieving technique as described by Ito et al.⁸

Arachidonic Acid Metabolism

Microsomal 20-HETE synthesis was measured as previously described.¹⁹ The rate of PGE₂ synthesis was detected by incubation of 100 μg of microsomes with 30 μmol/L arachidonic acid and NADPH (1 mmol/L) in 100 mmol/L potassium phosphate buffer (pH 7.4) containing 10 mmol/L MgCl₂ (final volume 0.6 mL) at 37°C for 15 minutes. Metabolites were extracted, and ELISA assay (NEOGEN Corporation, Lexington, KY) was used to measure PGE₂ levels.

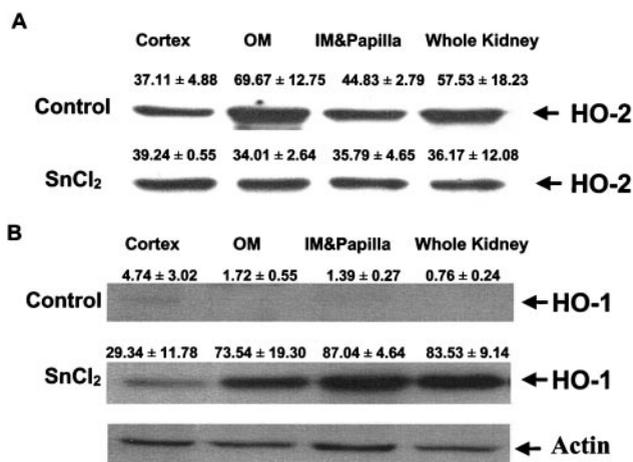


Figure 1. Effect of SnCl₂ on HO-1 and HO-2 protein levels in different regions of the rat kidney. Western blot and densitometry analyses of HO-2 (A) and HO-1 (B) protein levels in renal microsomes (10 μg) from control rats and rats treated with SnCl₂. The values above each lane represent the mean ± SE of densitometry analyses in arbitrary units of 4 immunoblots from 4 separate experiments.

Western Blot Analysis

Western blot analysis of proteins in different kidney preparations was performed as previously described.¹⁹ The antibodies used were the following: rabbit anti-rat HO-1 and HO-2 polyclonal antibodies (1:1000; Stressgen Biotechnologies Corp, Victoria, BC, Canada), rabbit anti-mouse COX-1 and COX-2 polyclonal antibodies (1:250; Cayman Chemical, Ann Arbor, MI), and goat anti-rat CYP4A1 polyclonal antibodies (1:250; GENTEST Corporation, Woburn, MA).

Statistical Analysis

Results are presented as mean ± SE for the number (n) of replicate determinations. Statistical significance of differences between the experimental groups was determined by using *t* test with *P* < 0.05 considered significant.

Results

HO Isoform Expression in Rat Kidney in Response to SnCl₂

We examined the effect of SnCl₂ on HO-1 and HO-2 protein levels. Western blots of cortical, outer medullary, inner medullary/papillary, and whole kidney microsomal preparations from untreated rats indicated significant levels of HO-2 protein with relatively high levels in the outer medulla followed by inner medulla/papilla and cortex (Figure 1A). HO-2 protein levels did not significantly change in microsomes from SnCl₂-treated rats. In contrast, HO-1 protein was barely detected in tissues from untreated rats but was markedly increased after SnCl₂ treatment (Figure 1B). The increase of HO-1 protein levels was most prominent in the outer medulla and inner medulla/papilla with estimated 40- and 60-fold increases, respectively, whereas the cortex showed only a 6-fold increase (Figure 1B).

Localization of HO Protein Along the Nephron

Western blot analysis was used to study the expression of HO isoforms in bulk preparations of proximal tubules (PT), mTAL, and IMCD. In untreated rats, HO-1 protein was hardly detected in any of the 3 nephron segments (Figure 2A),

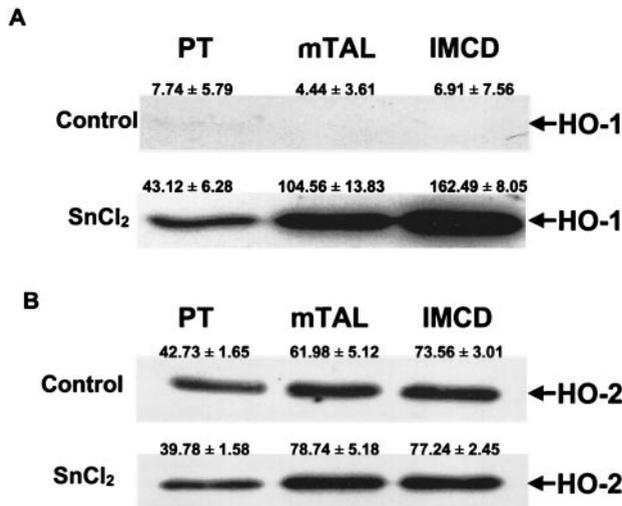


Figure 2. Effect of SnCl₂ on HO-1 (A) and HO-2 (B) protein levels in isolated PT, mTAL, and IMCD. Homogenates (15 μg) were subjected to electrophoresis and immunoblotting as described in Methods. The values above each lane represent the mean ± SE of densitometry analyses in arbitrary units of 3 immunoblots from 3 separate experiments.

whereas in SnCl₂-treated rats, HO-1 protein levels markedly increased with the highest increase seen in the IMCD followed by mTAL and PT (Figure 2A). HO-2 protein levels demonstrated a similar pattern of expression as HO-1, but, unlike HO-1, HO-2 was expressed in untreated rats and the levels of its protein remained unchanged after treatment with SnCl₂ (Figure 2B).

Effect of HO-1 Induction by SnCl₂ on CYP4A Expression and 20-HETE Synthesis

In untreated rats, CYP4A protein levels were the highest in the outer medulla followed by the cortex and inner medulla/papilla. Induction of HO-1 by SnCl₂ markedly reduced cortical and inner medullary levels but did not significantly alter outer medullary CYP4A expression (Figure 3A). Because CYP4A proteins are the primary enzymes that catalyze the ω-hydroxylation of arachidonic acid to 20-HETE, we measured 20-HETE synthesis in renal tissues from control and SnCl₂-treated rats. 20-HETE synthesis was the highest in the outer medulla followed by the cortex and inner medulla/papilla, viz, 165.3 ± 16.7, 137.4 ± 8.4, and 57.2 ± 1.5 pmol 20-HETE/mg per min, respectively. Induction of HO-1 by SnCl₂ inhibited cortical 20-HETE synthesis by 80% and inner medullary synthesis by 60%, whereas outer medullary 20-HETE synthesis was decreased by only 25% (Figure 3B).

Effect of HO-1 Induction by SnCl₂ on COX Protein Expression

COX proteins also demonstrated a distinct renal regional distribution. Immunoreactive COX-1 protein was detected primarily in the inner medulla/papilla, whereas COX-2 immunoreactive protein was detected in all renal regions examined, with the highest levels in the cortex followed by outer and inner medulla. SnCl₂ treatment markedly reduced the levels of COX-2 protein without significantly affecting those of COX-1 (Figures 4A and 4B). COX activity was measured

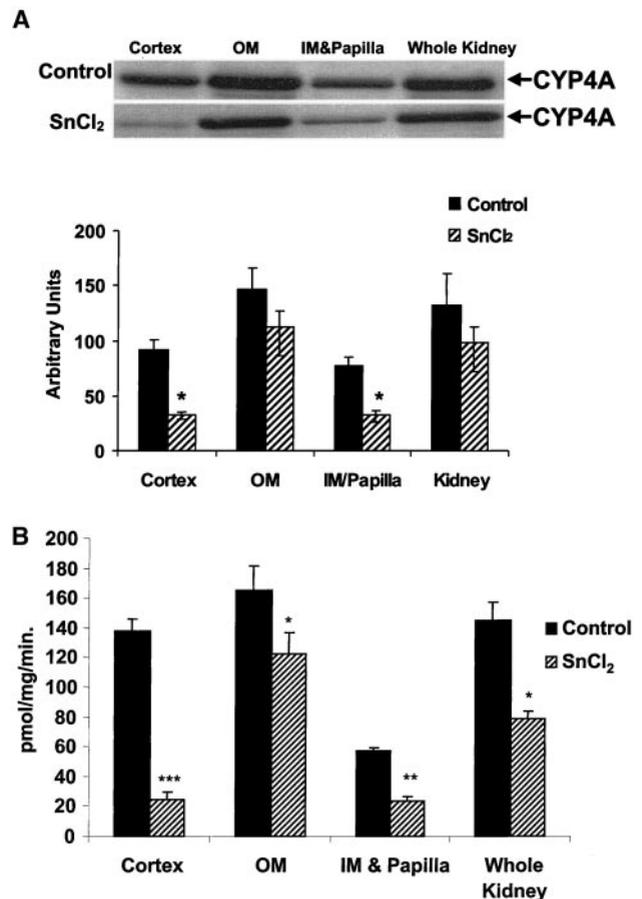


Figure 3. Effect of SnCl₂ on CYP4A protein levels and 20-HETE formation in different regions of the rat kidney. A, Western blot and densitometry analyses of CYP4A protein levels in renal microsomes from control rats and rats treated with SnCl₂ (5 mg/100 g body wt). Results are mean ± SE (n=4); *P<0.05 as compared with control. B, Arachidonic acid metabolism to 20-HETE. Results are the mean ± SE (n=5); *P<0.05; **P<0.005; ***P<0.0005 compared with control.

as the conversion of arachidonic acid to PGE₂. The results depicted in Figure 4C indicated that COX activity followed the levels of COX proteins. The inner medulla/papilla, where the highest levels of COX-1 protein were detected, showed the highest activity followed by the cortex, in which the highest expression of COX-2 was located. Treatment with SnCl₂ had little effect on PGE₂ production by the various renal tissues. As seen in Figure 4C, PGE₂ production in microsomes from the cortex and outer and inner medulla/papilla of rats treated with SnCl₂ was not significantly different from that in microsomes from untreated rats.

Discussion

The present study was designed to explore the effect of differential expression of HO-1 and HO-2, in various kidney structures, on hemoproteins, specifically CYP450 and COX. The results of this study clearly show that HO isoform expression is segmented within the kidney and along the nephron and that treatment with an HO-1 inducer suppressed the levels of CYP4A and COX-2 proteins in a tissue-specific manner with concomitant effects on their activity.

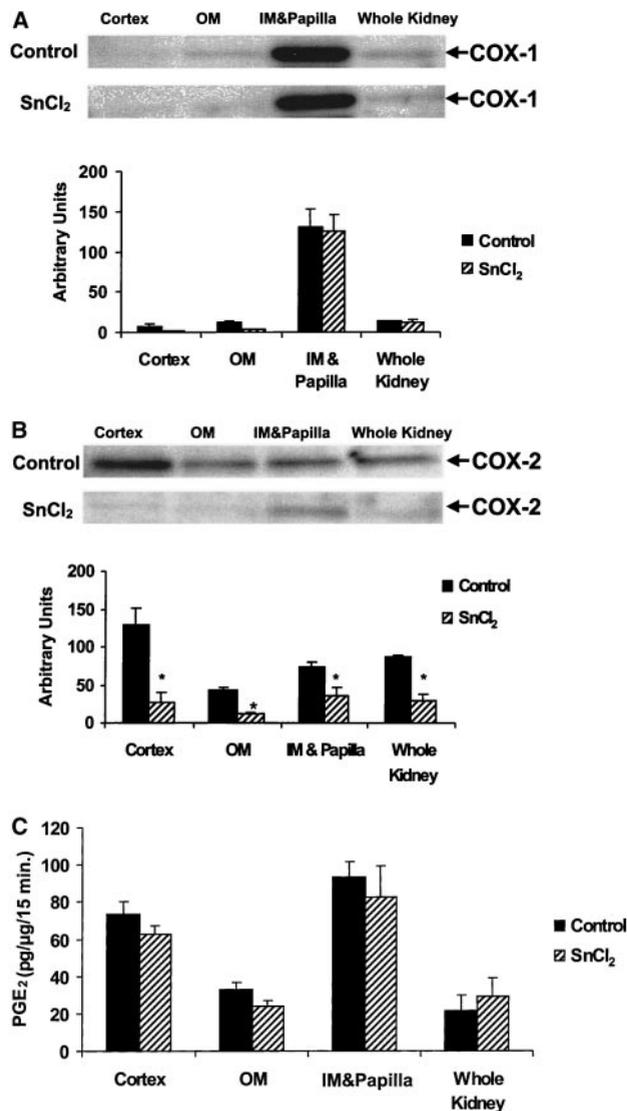


Figure 4. Effect of SnCl₂ on COX protein expression and PGE₂ formation in different regions of the rat kidney. A, Western blot and densitometry analyses of COX-1 protein levels in renal microsomes from control rats and rats treated with SnCl₂ (5 mg/100 g body wt). Results are mean ± SE (n=4); *P<0.05 as compared with control. B, Western blot and densitometry analyses of COX-2 protein levels in renal microsomes from control rats and rats treated with SnCl₂ (5 mg/100 g body wt). Results are mean ± SE (n=4); *P<0.05 as compared with control. C, PGE₂ formation in renal tissues. Microsomes (100 μg) were incubated with arachidonic acid (30 μmol/L) for 15 minutes. PGE₂ was quantified by ELISA as described in Methods. The results are the mean ± SE (n=5).

HO isoforms exhibit distinct regional and segmental expression. The expression of the constitutive HO-2 was shown to localize to all regions of the kidney with relatively high levels in the outer medulla followed by the inner medulla/papilla and cortex. HO-2 expression in isolated nephron segments showed a corresponding pattern with the IMCD and mTAL having a relatively high abundance, whereas the HO-2 level of expression in PT was approximately 30 to 40% lower. As demonstrated previously,²¹ HO-1 protein was hardly detectable in kidneys from control untreated rats. However, administration of SnCl₂, a strong inducer of renal

HO-1 expression, markedly increased HO-1 protein levels, and this increase was region and segment specific. That HO-2 expression is apparent under normal conditions and is not affected by SnCl₂ treatment reinforce its status as a constitutive isoform and further suggests that under normal conditions, HO-2 is the major isoform that contributes to the regulation of cellular heme levels and the production of CO. The ability of HO-1 to be rapidly induced within the kidney and the localized expression of this inducible protein, as well as that of the constitutive HO-2, may have important direct and indirect implications with regard to renal vascular and tubular function.

The direct implications of HO activity with respect to renal function relate primarily to the production of CO. Numerous studies have documented the vasodilatory actions of CO in isolated renal arterioles²² and have implicated its production, via HO isoforms, in the regulation of renal medullary blood flow.²³ Recent reports indicating that CO generated by HO-dependent heme catabolism stimulates the apical 70-pS K-channel of the mTAL¹⁶ further underscores the potential importance of HO to the regulation of kidney function. HO may also have an indirect impact on renal function through its activity. Heme is essential to the function of hemoproteins, whereas CO binds to heme moiety, causing inhibition or activation of hemoprotein function. In this capacity, HO expression and activity may regulate hemoproteins, the function of which is important for the regulation of kidney hemodynamics and excretory functions.

Of particular interest are the eicosanoid biosynthetic enzymes COX and CYP450 monooxygenases. Both COX and CYP450 monooxygenases metabolize arachidonic acid to active metabolites that play an important role in the regulation of kidney function. The COX-derived prostanoids PGE₂, PGI₂, and TXA₂ have been implicated in the regulation of vascular tone¹⁵ and in the control of renin release.²⁴ Likewise, the CYP450-derived eicosanoids 20-HETE and epoxyeicosatetraenoic acid have been shown to affect renal vascular tone and tubular epithelial ion transport mechanism; their production has been implicated in the regulation of renal blood flow, tubuloglomerular feedback, and control of blood pressure.^{25,26} We and others have shown that induction of HO-1 by SnCl₂ or heme in spontaneous hypertensive rats (SHR) depleted the levels of CYP450 in the kidney and that this depletion is associated with inhibition of 20-HETE synthesis and a decrease in blood pressure.^{18,27,28} A recent study by Haider et al²⁹ demonstrated that in cells overexpressing HO-1, COX isoform expression and activity are impaired.

The present study suggests that the functional outcomes of the interactions between HO activity and these hemoproteins greatly depend on the specific localized expression of each component. For example, the expression of CYP4A proteins that catalyze the formation of 20-HETE is greatest in the outer medulla. The primary nephron structures in the outer medulla are the mTAL, where 20-HETE has been shown to inhibit the activity of the Na-K-2Cl cotransporter⁹ and K-channels.¹⁰ Moreover, inhibition of outer medullary 20-HETE synthesis increased blood pressure in salt-sensitive hypertensive rats.¹² It is interesting that in our study, outer medullary CYP4A protein expression was not affected by

SnCl₂, whereas 20-HETE synthesis was reduced by only 25%. This apparent discrepancy may be explained by the fact that the detected CYP4A immunoreactive band represents at least 4 proteins, CYP4A1, 4A2, 4A3, and 4A8, thus masking changes in a specific CYP4A that may contribute the most to 20-HETE synthesis in this part of the kidney.³⁰ Moreover, CYP4A isoforms may differ in their susceptibility to CO and/or cellular heme levels. Thus, outer medullary tissues may contain a CYP4A protein whose activity is less sensitive to either inhibition by CO and/or reduction in heme levels brought about by HO-1 induction than that present in cortical tissues. Hence, inasmuch as the actions of 20-HETE in the mTAL are conducive of an antihypertensive effect, the relatively small change in 20-HETE synthesis after HO-1 induction may contribute to the lowering blood pressure effect seen after administration of SnCl₂ in SHR.²⁷ However, despite relatively lower levels of HO-1 in the cortex, its induction, albeit smaller in magnitude, had a profound effect on cortical CYP4A protein levels and 20-HETE production. Preglomerular arteries are the primary site for 20-HETE synthesis and action. 20-HETE of vascular origin has been implicated in the regulation of myogenic tone,^{31,32} and inhibition of its production lowers blood pressure in normotensive and hypertensive rats.^{14,33} The marked reduction of cortical CYP4A protein levels and 20-HETE synthesis may contribute to the decrease of blood pressure seen in SHR treated with SnCl₂ to induce renal HO-1 expression.²⁷

In our study, COX-1 protein expression was most prominent in the inner medulla, whereas COX-2 protein was detected in all regions with the highest levels in the cortex. These patterns of localization concur with others' reports. In the rat kidney, COX-1 is constitutively expressed and has been localized to arteries and arterioles, glomeruli, and collecting ducts.³⁴ Although COX-2 is considered to be the inducible form, it is constitutively expressed in the TALH and in the region of the macula densa, primarily a cortical structure,^{35,36} where it is involved in the stimulation of renin release from the juxtaglomerular cells.²⁴ It is interesting that despite that COX-1 and COX-2 share similar catalytic characteristics, HO-1 induction via SnCl₂ administration only affected COX-2 protein expression, leading to a marked decrease in its levels in the cortex as well as in the inner and outer medulla. However, the decrease in COX-2 protein levels was not accompanied by a significant decrease in PGE₂ synthesis. One explanation is that PGE₂ synthesis in these tissues is primarily driven by COX-1. However, COX activity, measured as PGE₂ production in microsomal protein fortified with arachidonic acid, did not correlate with COX-1 protein levels. A recent study in our laboratory indicated that heme level, rather than CO per se, is the primary factor in regulating COX activity in cultured endothelial cells.²⁹ Thus, one may argue that heme levels were not reduced to levels that may affect COX activity. Additional preliminary studies revealed a significant 15% reduction in PGI₂ production in the inner medulla of rats treated with SnCl₂ (Abraham et al, unpublished data). Because PGI₂ production entails the engagement of 2 heme proteins for its synthesis, COX and PGI₂ synthase, it is possible that this HO-1 induction regimen is sufficient to affect PGI₂ in a tissue with the highest capacity

to produce it. It should be noted that PGI₂ synthase, like TxA₂ synthase, is a CYP450 protein and thereby may be more susceptible to levels of cellular heme as well as CO. To this end, a study by our laboratory demonstrated a reduction in TxA₂ synthesis in the kidney of hypertensive rats treated with SnCl₂.³⁷

In summary, HO isoform expression is segmented within the kidney and along the nephron, and treatment with an inducer of HO-1 expression suppressed the levels of CYP4A and COX-2 proteins in a tissue-specific manner with concomitant effect on their activity. Such interactions could present an important mechanism for the regulation of kidney function.

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