

Spring 5-9-2010

The Effect of Heavy Metal Stress on Avian Proximal Tubule Urate Secretion

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Abstract

In both humans and birds, urate is an important antioxidant when maintained at normal plasma concentrations. Though human kidneys primarily reabsorb filtered urate, while those of birds perform mostly secretion, both maintain urate levels at $\sim 300\mu\text{M}$ (Dudas, Pelis, Braun, & Renfro, 2005; Gutman & Yu, 1972). The importance of controlling urate levels such that they are maintained within the homeostatic range was observed when the study of several major diseases revealed an association with hyperuricemia. These diseases include, but are not limited to: hypertension, cardiovascular disease, renal disease, acute renal failure, gout, diabetes, metabolic syndrome, and sleep-disordered breathing in obese children. Understanding the underlying mechanisms that control plasma urate concentration, therefore, bears much clinical significance.

The present study examined the effect of physiological stress, in the form of elevated zinc concentration, on the process of urate secretion. In this process urate is taken up into the proximal tubule cell by a tertiary active transport mechanism involving basolateral membrane organic anion transporter 1/3 (Oat1/3). In chicken renal proximal tubule epithelial cell monolayers (cPTCs), it has been demonstrated that urate exits primarily by apically located multi-drug resistance protein 4 (Mrp4) transport (Bataille, Goldmeyer, & Renfro, 2008). Currently, regulating factors remain uncertain. In other systems, cellular stress has been shown to affect transport properties by altering both stress protein levels and transporter expression or activity. We have shown that acute exposure of cPTCs to zinc had no effect on urate secretion. However, prolonged (6 hr, followed by 1.5 hr in otherwise normal culture medium) exposure resulted in zinc-induced cellular stress that inhibited active transepithelial urate secretion by cPTCs with no change in Mrp4 expression, glucose transport, or transepithelial resistance. Here, it is shown that zinc has no effect on urate transport by isolated brush border membrane vesicles; basolateral membrane vesicle analysis remains to be determined. The data suggest involvement

of a cellular stress adaptation that results in the observed inhibition of urate secretion. Previous studies have demonstrated that AMP-activated protein kinase (AMPK) serves as a critical metabolic regulator that is activated during cellular stress to promote survival. Activation of AMPK has been shown to conserve energy by shutting down non-essential ATP-utilizing processes and activating ATP-generating processes. Pharmacological activation of AMPK by AICAR produced decreased urate secretion by cPTCs similar to the effect seen with prolonged exposure to zinc, while the AMPK inhibitor Compound C prevented both AICAR and zinc inhibition of urate secretion, suggesting a stress induced mechanism of regulation. Supported by NSF. IACUC #A08-046.

Introduction

1.1 The importance of urate in physiological systems

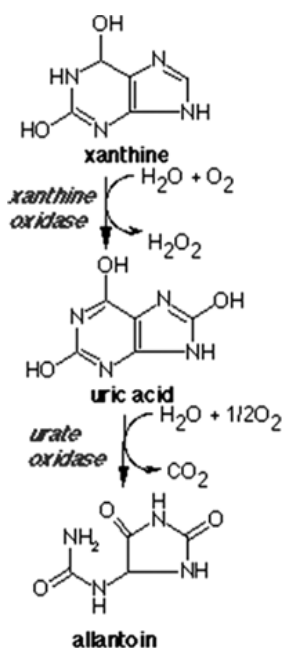


Figure 1: Uric acid generation and degradation, as provided by Ejaz et al. (2007)

Uric acid, the end product of purine metabolism in humans, is generated from xanthine by xanthine dehydrogenase, also known as xanthine oxidase (see **Figure 1**, as provided by (Ejaz

et al., 2007)). In most mammals, it is further degraded by the hepatic enzyme uricase (urate oxidase) to allantoin, but in humans a mutation in the uricase gene prevents this step.

The mutation in the uricase gene is thought by some to have provided evolutionary benefit to humans. In addition to maintaining elevated blood pressure during low-sodium diets and chelating transition metals to prevent their toxic accumulation, some argue that the resulting elevated serum levels of uric acid, which possesses high antioxidant capacity, could even have been partially responsible for the longer life-spans experienced by humans and great apes (Ames, Cathcart, Schwiers, & Hochstein, 1981); Ejaz et al., 2007);(Hediger et al., 2005);(Johnson et al., 2003). Clinically, it is the antioxidant capacity of uric acid that is of great interest. According to *Johnson et al. (2003)*, uric acid functions as “one of the most important antioxidants in plasma” (Johnson et al., 2003). Its soluble form, urate, can scavenge reactive oxygen species (ROSs), like superoxide anion, hydroxyl radical, and singlet oxygen, all of which damage lipids and DNA. Moreover, it may prevent the inactivation of superoxide dismutase (SOD3) by H_2O_2 . SOD3 is a protective enzyme that converts superoxide anion, O_2^- , to the slightly less damaging H_2O_2 ; in doing so, it prevents superoxide’s reaction with, and subsequent inactivation of, the vasodilator nitric oxide, NO (Johnson et al., 2003).

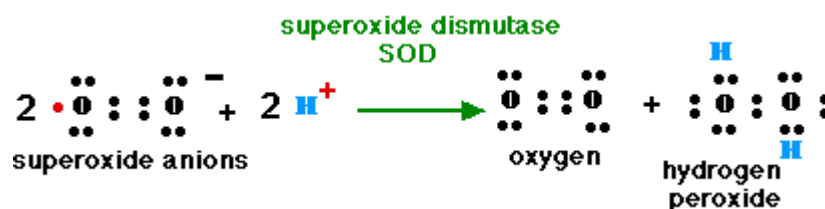


Figure 2: The conversion of superoxide anion to less damaging hydrogen peroxide, as catalyzed by superoxide dismutase

<http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/S/SOD.gif>

Ames et al. (1981) put in perspective the damage caused by ROSs; they suggest that the toxicity caused by oxygen radicals may be a major cause of prevalent diseases such as cancer and heart disease, and of even the aging process itself (Ames et al. 1981, pg. 6858). The antioxidant benefits of urate, therefore, could potentially be extended to include defense against these conditions too (Ames et al. 1981, 6858-6862).

To provide its antioxidant benefits in the normal, healthy individual, plasma urate must be homeostatically maintained between ~240 and 350 μ M (Hediger et al., 2005). The importance of such regulation becomes apparent from the study of a number of pathological states that are correlated with hyperuricemia. In addition to the more obvious connection to visceral gout, which is characterized by deposits of insoluble urate in the serous membranes of the heart, aorta, liver, and kidneys (Gutman & Yu, 1972), several other common pathological conditions, which are described in the following paragraphs, may be associated with the hyperuricemic individual (Hediger et al., 2005). Although the correlation exists in each case, whether or not uric acid is an independent risk factor for these diseases remains uncertain.

Recent literature has described a striking correlation between uric acid and cardiovascular risk when uric acid concentration is elevated above the normal range, which, according to *Feig et al. (2008)*, is > 6mg/dL (360 μ M/L) in women, and > 7mg/dL (420 μ M/L) in men¹ (Feig, Kang, & Johnson, 2008). As illustrated by Table 1 in **Figure 3** from (Johnson et al., 2003), uric acid concentration is also greater in groups of individuals with increased cardiovascular risk: postmenopausal women, African-Americans, hypertensive individuals, and patients with

¹ For comparison, most mammals have a urate concentration of < 2mg/dL due to the presence of uricase (Johnson et al., 2003).

metabolic syndrome, renal disease, and increased alcohol consumption (Feig et al., 2008; Johnson et al., 2003).

TABLE 1. Uric Acid Is Increased in Groups at Cardiovascular Risk

Group	Mechanism
Postmenopausal women and men	Estrogen is uricosuric ⁶
African Americans ⁷	Unknown
Renal disease	Decrease in GFR increases uric acid levels
Diuretics	Volume contraction promotes urate reabsorption
Obesity/insulin resistance	Insulin increases sodium reabsorption and is tightly linked to urate reabsorption ⁸
Hypertension ⁹	Urate reabsorption increased in setting of increased renal vascular resistance; ¹⁰ microvascular disease predisposes to tissue ischemia that leads to increased urate generation (from adenosine breakdown) and reduced excretion (due to lactate competing with urate transporter in the proximal tubule); ¹¹ some hyperuricemic hypertension may be due to alcohol ingestion ¹² or lead intoxication ¹³
Alcohol use	Increases urate generation, ¹⁴ decreases urate excretion ¹⁵
GFR indicates glomerular filtration rate.	

Figure 3: Groups with increased cardiovascular risk and the mechanism whereby this risk exists, as borrowed from *Johnson et al. (2003)*

Despite the correlation, it is possible that hyperuricemia is not actually a *predictor* of cardiovascular risk (Feig & Johnson, 2003; Feig et al., 2008; Johnson et al., 2003), but instead an *effect* of cardiovascular disease, which encompasses a number of pathological states, including hypertension (HTN), heart disease, arrhythmia, heart attack, and stroke. As *Feig et al. (2008)* point out, the elevation of serum uric acid in patients with cardiovascular disease may just be a result of common factors present in the disease. For example, they highlight that disease

components such as reduced glomerular filtration rate, hyperinsulinemia, renal vasoconstriction, and diuretic use, all reduce net renal excretion of uric acid. Moreover, alcohol use, tissue ischemia, and oxidative stress, can all increase uric acid generation (Feig et al., 2008). In other words, the role uric acid plays in cardiovascular disease involves a complex integration with many other factors and remains to be clearly established.

There appear to be some instances, however, where uric acid concentration assumes a true prognostic role. Specifically, high plasma uric acid, which is partly produced by ischemic heart tissue itself, has been shown to be a predictor of mortality in patients with congestive heart failure (CHF) (Sakai et al., 2006). Results from *Sakai et al. (2006)* indicated that in patients with CHF, uric acid concentration in the aortic arch and coronary sinus was elevated over that of uric acid concentration in the same locations of normal patients. Mechanistically, uric acid is elevated via activation of xanthine oxidase, a compound that causes oxidative damage in the myocardium. The increase in myocardial oxidative stress then further damages the heart tissue. A more recent study by *Alimonda et al. (2009)* extended this argument to acute heart failure (AHF) and was the first to demonstrate the “independent prognostic value” of uric acid for predicting increased long-term mortality of AHF patient (Alimonda et al., 2009). Here, xanthine oxidase was again identified as a significant ROS producer, with the resulting oxidative stress contributing to endothelial dysfunction.

One of the key factors associated with increased cardiovascular risk, which in many cases then contributes to the development of cardiovascular disease, is hypertension (HTN), and about 25%-50% of hypertensive individuals are hyperuricemic (Mazzali et al., 2001). Establishing a link between hyperuricemia and HTN would not only target a possible underlying cause of cardiovascular disease, but could perhaps reveal a therapeutic means by which to counteract its

progression. *Feig et al. (2008)* demonstrate such a link in a study of adolescents that suggests hyperuricemia may be the initial cause of primary HTN, or HTN that cannot be explained by any medical condition. In one particular study, elevated uric acid concentration ($> 5.5\text{mg/dL}$, or $> 330\mu\text{M/L}$) was observed in roughly 90% of adolescents with primary HTN; uric acid concentration was significantly lower in those with white-coat HTN or with secondary HTN, i.e., HTN that is secondary to some other medical condition, such as kidney disease (Feig & Johnson, 2003). Because primary HTN lacks an existing medical explanation, and uric acid concentration in the adolescent patients diagnosed with it is significantly elevated over that of those with secondary HTN, the hypothesis that uric acid concentration itself may be the causal link in primary HTN establishment seems quite plausible. Furthermore, with increasing age and duration of HTN, the correlation between uric acid and HTN was found to decline, suggesting that uric acid elevation may be more influential in the *initial* establishment of HTN as a young adult (Feig et al., 2008) rather than in its gradual development over time in some adults.

Development of the first experimental model that monitored the progression from *mild* hyperuricemia,² as is documented in cardiovascular disease patients, to development of HTN lends credence to the hyperuricemia- HTN link. When *Mazzali et al. (2001)* treated male rats with the uricase inhibitor oxonic acid, which prevents the degradation of uric acid to allantoin and so results in hyperuricemia, HTN developed after 3 weeks (Mazzali et al., 2001). At least part of the mechanism whereby uric acid may contribute to the development of HTN may be due to endothelial dysfunction. Antioxidants, like uric acid, contribute to endothelial dysfunction by reaction with, and subsequent removal of, the vasodilator nitric oxide (NO); *Mazzali et al. (2001)* point out that the hyperuricemic rats also had increased juxtaglomerular renin and decreased

² *Mazzali et al., 2001* considered mild hyperuricemia as a 1.5- to 2-fold increase in serum uric acid.

macula densa neuronal NO synthase (Mazzali et al., 2001). A second consideration may be the salt sensitivity exhibited by hyperuricemic rats, which is a consequence of preglomerular vascular disease (Johnson, Herrera-Acosta, Schreiner, & Rodriguez-Iturbe, 2002). Development of preglomerular vascular disease is a key event in the progression to HTN in that it is followed by: renal ischemia, production of leukocytes, generation of oxidants, and the overall shift towards vasoconstriction. With vasoconstriction, sodium excretion is reduced, and the corresponding salt retention results in elevated blood pressure, or HTN (Johnson et al., 2002; Johnson et al., 2003). However, to clearly illustrate that the causal link to HTN was hyperuricemia, in their study *Mazzali et al. (2001)* treated their rats with allopurinol, a xanthine oxidase inhibitor, or benzbromarone, a uricosuric agent, both of which lower uric acid concentration. The two showed direct correlation: a decrease in uric acid level resulted in a corresponding decrease in blood pressure.

Perhaps an even more interesting, and more clinically enlightening, connection between hyperuricemia, HTN, and the diuretics commonly used to treat HTN is highlighted in the review provided by *Nakagawa et al. (2008)* in which they begin to characterize the links between hyperuricemia, metabolic syndrome, and renal disease. Here, they reveal that diuretics may actually play a role in elevating serum uric acid concentration (Nakagawa, Cirillo, Sato, Gersch, Sautin, Roncal, Mu, Sanchez-Lozada, & Johnson, 2008b). Specifically, diuretics increase urine output, and it is the corresponding decrease in blood volume that contributes to a reduced blood pressure. The decrease in blood volume, however, then acts as a stimulus for 1.) sodium reabsorption at the proximal tubule, and 2.) the renin-angiotensin-aldosterone system (RAAS), both of which counteract the volume depletion and, consequently, increase blood pressure. Moreover, the authors point out that uric acid reabsorption appears to be “coupled with sodium

reabsorption,” (Nakagawa, Cirillo, Sato, Gersch, Sautin, Roncal, Mu, Sanchez-Lozada, & Johnson, 2008a), which contributes to uric acid elevation. Though further studies are required to more fully characterize this finding, these preliminary indications suggest that, clinically, diuretic use might contribute to serum uric acid elevation, perhaps increasing the risk of many of the other diseases that have been found to be associated with hyperuricemia.

Another condition observed to be associated with hyperuricemia is metabolic syndrome, also known as insulin resistance syndrome, a condition characterized by the simultaneous presence of several risk factors for cardiovascular disease: truncal obesity, hypertriglyceridemia, elevated blood pressure, insulin resistance and its associated hyperinsulinemia, and hyperuricemia (Cirillo et al., 2006; Nakagawa et al., 2006). It is also strongly associated with the development of further complications, such as diabetes, HTN, and cardiovascular disease itself (Cirillo et al., 2006). *Nakagawa et al. (2006)* found that uric acid and the development of metabolic syndrome are linked via an increase in the intake of fructose, which comprises about 50% of table sugar and a significant percentage of high-fructose corn syrup. After fructose is ingested, it is phosphorylated by fructokinase to fructose-1-phosphate, a process that generates ADP, and ultimately, uric acid, after this ADP is metabolized (Hallfrisch, 1990; Nakagawa et al., 2006). Uric acid inhibits NO, the vasodilator critical for normal insulin action since it increases blood flow, and therefore glucose uptake, into skeletal muscle. Uric acid elevation therefore causes a dampening of insulin’s effects at the level of the skeletal muscle (Nakagawa et al., 2006). In short, uric acid’s role in contributing to the development of metabolic syndrome features, especially insulin resistance, appears to be quite complex and not fully understood.

The 2006 Nakagawa study (Nakagawa et al., 2006) clearly establishes that the role of fructose intake in metabolic syndrome development is rooted in uric acid. Here, rats were fed

either a fructose or a control diet, and half of the fructose-fed rats were treated with the uric acid-lowering compound allopurinol. While the fructose-fed rats as a group exhibited features of metabolic syndrome, including elevated serum uric acid concentration, hypertriglyceridemia, and elevated blood pressure, those simultaneously treated with allopurinol did not experience uric acid elevation or hypertriglyceridemia, and they exhibited increased blood pressure to a lesser degree. In addition, fasting insulin levels were significantly lower in the allopurinol group as opposed to the fructose-fed rats whose diets did not include allopurinol (Nakagawa et al., 2006). The uricosuric agent benzbromarone also prevented metabolic syndrome features, providing further evidence that it is via the lowering of uric acid, and not of other oxidants of the xanthine oxidase pathway, that lessened the degree of metabolic syndrome (Nakagawa et al., 2006). With such a causal role in the development of metabolic syndrome, a syndrome that represents a “transition state” to many other diseases, it may be possible to pursue a therapy that targets uric acid elevation in order to prevent, or at least lessen the severity of, metabolic syndrome and the cascade of other diseases that can result.

Finally, the condition associated with hyperuricemia that is quite indicative of how broad a role it plays in the health spectrum is the correlation between hyperuricemia and sleep-disordered breathing (SDB) in overweight and obese children and adolescents. The first study on the subject was published in 2007 by *Verhulst et al.*, where they concluded that the severity of SDB was independently correlated with increased serum uric acid concentration. Because the urinary uric acid/creatinine ratio is actually higher in young children, but declines to adult levels as childhood progresses, *Verhulst et al. (2007)* point out that they needed to control for gender and developmental stage in their study (Verhulst et al., 2007). Here, increased serum uric acid level was interpreted as a marker of oxidative stress and tissue hypoxia, which occurs in the more

severe cases of sleep apnea (Verhulst et al., 2007). It remains to be proven whether serum uric acid may also be the link between the observed association between sleep apnea and cardiovascular morbidity (Verhulst et al., 2007).

1.2 The importance of urate secretion in maintaining urate plasma concentration and the current model by which it occurs

One of the earliest reviews providing an analysis of uric acid handling in humans and other physiological systems was the 1972 study by Gutman and Yu entitled “Renal Mechanisms for Regulation of Uric Acid Excretion, With Special Reference to Normal and Gouty Man.” At the time, tubular secretion of urate had already been established as the major mechanism of uric acid excretion in the uricotelic bird (Mayrs, 1924), while urate filtration, followed by reabsorption of about 98% of the filtered load³ (Gutman & Yu, 1972) and excretion of the non-reabsorbed fraction, were the generally accepted components of the mammalian process. For humans, secretion had been shown to be the more variable part of the process, the step that could be altered appropriately in order to maintain homeostatic plasma urate concentration (Steele & Rieselbach, 1967). Moreover, it is the more influential step in that 70% of the excreted urate is due to tubular secretion (Dudas, P.L. 2005). In one of the experiments performed by *Steele and Rieselbach, (1967)*, human subjects were fed RNA to raise their plasma urate concentration. 98% of the filtered load was still reabsorbed, only at an enhanced rate. In other words, the increase in urate excretion was the result of an accelerated rate of tubular urate secretion, and not the result of an excess of filtered urate that had escaped reabsorption (Gutman & Yu, 1972). Simple, yet elegant, these early experiments were critical in establishing that the secretory system provides the more robust reaction to changes in urate level, whether an increase resulting in an increased

³ Filtered urate in the human kidney is reabsorbed by the transporter URAT1 (Hediger, Johnson, Miyazaki, & Endou, 2005);(Enomoto et al., 2002)).

rate of tubular secretion as this experiment demonstrates, or a decrease resulting in a decreased rate of tubular secretion, in order to maintain plasma urate concentration within the homeostatic range (Steele & Rieselbach, 1967).

Although most mammals have the enzyme uricase, which converts urate to the more soluble allantoin, and therefore do not demonstrate urate excretion as their major mechanism of urate elimination, humans are an exception. *Gutman et al. (1972)* highlight that regulation of overall plasma urate concentration, as well as the concentrations of other plasma components, is chiefly dependent upon elimination in the urine, which in turn is regulated by secretion, as just described. They clearly remark that in some pathological states the more “primitive” process of secretion is what adjusts the extent of elimination of a particular substance in the urine, and in doing so, is what is more influential in maintaining homeostatic plasma levels (Gutman & Yu, 1972). Herein lies the major reason for which the current experiment is applicable to uric acid regulation. Since plasma urate concentration is regulated in large part by tubular urate secretion, studying the factors that affect urate secretion is essentially an examination of the part of the process that has marked implications for overall urate level, and thus overall health. The bird provides an ideal experimental model in that it does not demonstrate urate reabsorption, the overriding process in humans and other mammals. By using the avian model system, therefore, we are isolating the secretion process for further study (Dudas et al., 2005);(Bataille et al., 2008; Gutman & Yu, 1972).

The generally accepted model of net transepithelial urate transport in the bird (see **Figure 4A**) is one characterized by tertiary active transport, which, as with many tertiary active transport systems in physiology, depends on the basolaterally-located Na^+ , K^+ -ATPase for establishment and maintenance of the electrochemical gradients that provide the energy for all the following

steps in the process. The avian system, which does not exhibit urate reabsorption, is somewhat simpler than its mammalian counterpart and will be described here. Briefly, in chick proximal tubule epithelial cells (cPTCs), low internal Na^+ and the resulting steep electrochemical gradient energizes co-transport of Na^+ with α -ketoglutarate ($\alpha\text{-KG}^{2-}$) via a Na^+ -dicarboxylate co-transporter (Shimada, Moewes, & Burckhardt, 1987), also located on the basolateral membrane (BLM). Although there exists an outwardly-directed gradient for $\alpha\text{-KG}^{2-}$ due to its continual generation during cellular metabolism, the Na^+ -dicarboxylate co-transporter uses the Na^+ gradient to transport $\alpha\text{-KG}^{2-}$ against its concentration gradient. A third BLM transporter functions as an organic anion/dicarboxylate exchanger, outwardly transporting $\alpha\text{-KG}^{2-}$ in exchange for inward transfer of urate. This third transporter was first proposed to be organic anion transporter 3 (Oat3), the Oat with greatest expression in the kidney, by (Sweet et al., 2003) when uptake experiments strongly suggested organic anion/dicarboxylate exchange coupled to the Na^+ gradient via the Na^+ -dicarboxylate co-transporter (Sweet et al., 2003). Although all of the mechanisms that then facilitate urate uptake across the brush-border membrane (BBM) into the lumen of the proximal tubule have not yet been completely characterized, recent evidence has strongly implicated multidrug resistance protein 4 (Mrp4) in this role. (Bataille et al., 2008).

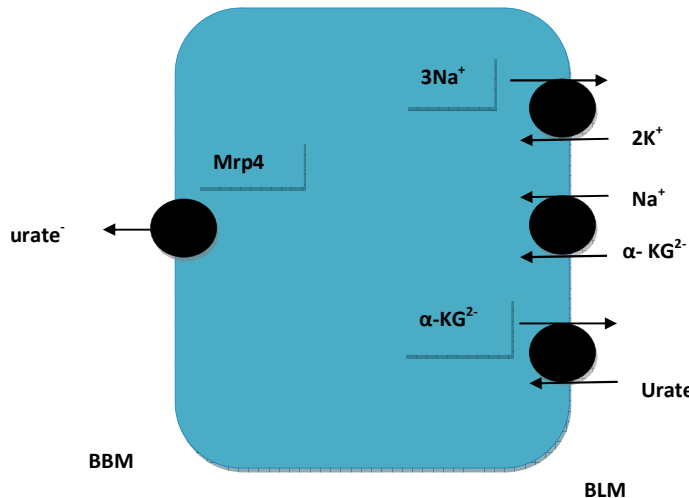


Figure 4A: Cartoon of proposed urate secretion mechanism in the avian proximal tubule epithelial cell. Note that Mrp4 represents the major secretory route. BLM= basolateral membrane; BBM = brush border membrane

Urate secretion into the proximal tubule cell lumen in mammals (see **Figure 4B**) demonstrates a higher level of complexity due its 98% reabsorption rate. Nonetheless, the same basic tertiary active transport principles apply: establishment of low internal Na^+ gradient by Na^+ , K^+ -ATPase, followed by Na^+ -dicarboxylate co-transport, and Oat3-mediated inward transfer of urate. In humans, URAT1 appears to perform the majority of urate reabsorption (Enomoto et al., 2002). The following diagram and figure legend, taken from (Terkeltaub, 2010), reveals the complexity of the urate secretion-reabsorption system in humans.

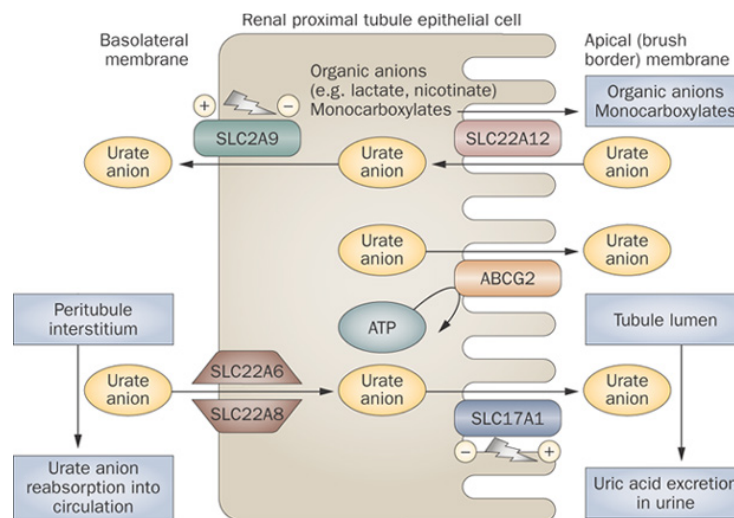
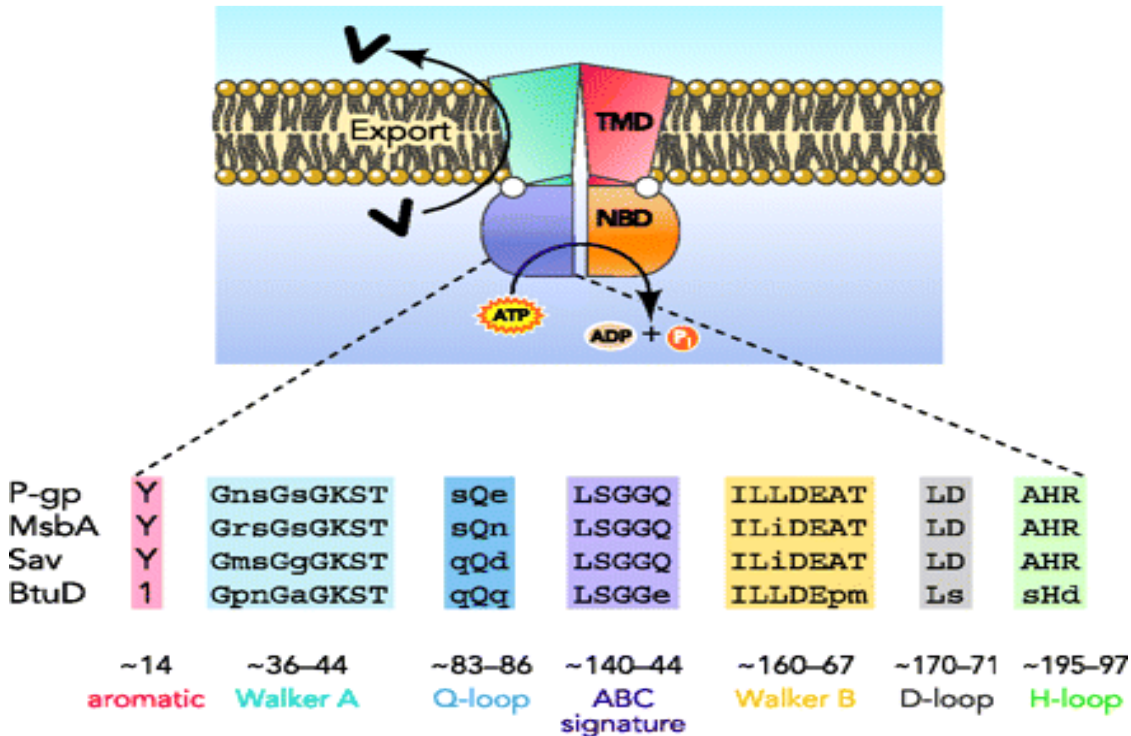


Figure 4B: The complexity of the urate reabsorption-secretion mechanism in the mammalian proximal tubule, as provided by (Terkeltaub, 2010).

Figure 3: The principal transporters in the current model of bidirectional urate anion movement in proximal tubule epithelial cells are shown. The balance between urate reabsorption and secretion is critically linked to net uric acid elimination in urine. This schematic shows the major transporters that are linked with serum urate levels and susceptibility to gout in genetic studies; other transporters at the apical and basolateral membrane (ABCC4 and SLC22A11, not shown) are also implicated in urate disposition. Urate reabsorption at the apical membrane is critically regulated by SLC22A12-mediated exchange of urate for intracellular organic anions (for example, lactate, nicotinate) and monocarboxylates (for example, pyrazinamide metabolites). The purine nucleoside transporter ABCG2 and the voltage-driven transporter SLC17A1 mediate urate secretion at the apical membrane. The hexose transport facilitator SLC2A9 carries out voltage-dependent urate anion transport, which at the basolateral membrane leads to urate reabsorption into the circulation. Some evidence also indicates a role for SLC2A9 at the basolateral membrane in reabsorption of urate anion in proximal tubule cells. Importantly, the uricosuric drugs probenecid and benzbromarone inhibit both SLC22A12 and SLC2A9. Abbreviations: ABC, ATP-binding cassette family member; SLC, solute carrier family member **[NOTE: the SLC22A12 gene encodes URAT1]**.

1.3 ABC transporters

To better understand the role Mrp4 plays in urate transport, it is useful to first describe the class of transporters to which it belongs. Mrp4 is an ATP-binding cassette (ABC) transporter, or a transporter that relies on ATP binding and hydrolysis for the conformational change that moves a ligand across a particular membrane. ABC transporters can be found in all species (Higgins & Linton, 2004; Linton & Higgins, 2007; Linton, 2007), and in humans alone, there exist 48 different types (Linton, 2007). In general, ABC transporters possess four core domains in their overall structure, as depicted in **Figure 5**, borrowed from (Linton, 2007).



four core domains of an ABC transporter, as depicted by *Linton et al. (2007)*

The two transmembrane domains (TMDs) are structurally described as “multiple membrane-spanning α -helices...[that] form the pathway through which substrate crosses the membrane” (Linton & Higgins, 2007). Unique in their sequence identities, they are the sites of attachment for specific ligands that are to be exported. Below the TMDs, the nucleotide binding domains (NBDs), which share similar motifs among all ABC transporters, dimerize to create an ATP-binding “pocket” that then binds and hydrolyzes 2 ATP molecules to drive the translocation of that ligand across the membrane (Higgins & Linton, 2004; Linton, 2007);(Linton & Higgins, 2007);(Lu, Westbrooks, Davidson, & Chen, 2005). The NBDs, since they perform a similar role in all ABC transporters, possess very similar sequence motifs.

ABC transporters perform their export function in a series of specific steps, termed the “ATP-switch model” by (Linton, 2007), as illustrated in their diagram, reproduced in **Figure 6**

below. Both of the upper domains that are embedded in the lipid bilayer represent the TMDs, while the two lower domains that bind ATP represent NBDs. The ATP-switch model to be described was proposed based on structural data for the drug transporter P-glycoprotein, but, according to *Linton et al. (2007)*, with “minor modifications,” it can explain the behavior of many other ABC transporters (Linton, 2007).

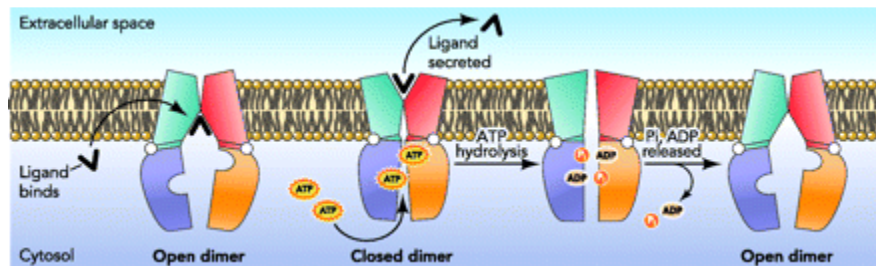


Figure 6: The ATP “switch” model based on P-glycoprotein data, as proposed by *Linton et al.*

The first step in the ATP-switch model begins with the ABC transporter positioned in what is termed the “open dimer” formation (see above). When in this conformation, the NBDs possess a low affinity for ATP, but the TMDs express high affinity for the cytoplasmic ligand, which, upon binding, causes a conformational change in the NBDs. It has been suggested that ligand binding itself directly increases the affinity of the NBDs for ATP via specific NBD motifs, and the subsequent binding of 2 ATPs to the NBDs catalyzes the formation of the “closed dimer” configuration (Higgins & Linton, 2004; Linton & Higgins, 2007; Linton, 2007). These two sequential steps result in the large-scale conformational change in the TMDs that translocates the ligand across the membrane; at the same time, the affinity for the ligand decreases, and the ligand unbinds. In the third step, ATP hydrolysis destabilizes the closed dimer formation. For P-glycoprotein, it is necessary to hydrolyze both of the ATPs, albeit non-

simultaneously, but in other ABC transporters, it may be necessary to hydrolyze only a single ATP (Linton, 2007; Lu et al., 2005);(Higgins & Linton, 2004). Though the actual trigger of ATP hydrolysis is unknown, it is believed to be an automatic effect of the closed NBD dimer formation; for example, *Lu et al. (2005)* demonstrate a model of maltose transport that implicates the closing of the NBDs in ATP hydrolysis initiation (Lu et al., 2005). In the final step of the process, first inorganic phosphate, and then ADP, is released from the NBDs. After phosphate release, high affinity of the TMDs for the specific ligand is restored, which resets the system. Even though this switch mechanism is highly conserved among ABC transporters, of which Mrp4 is a part, the main differences among ABC transporters include: the type of ligand transported, in which direction the ligand is transported, and whether or not both ATP-binding pockets have equal ability to bind and hydrolyze ATP (Higgins & Linton, 2004).

1.4 Mrp4 and its role in urate regulation

Although nine Mrps have thus far been identified, Mrp4 appears to have the ability to transport one of the broadest ranges of substrates, especially cellular signaling molecules like cAMP and cGMP, as well as antiviral medications, antibiotics, cardiovascular drugs, and cytotoxic agents (Russel, Koenderink, & Masereeuw, 2008). In addition, unlike other Mrps, Mrp4 is present in a variety of locations in addition to the apical membrane of renal proximal tubule cells: prostate tubuloacinar cells, hepatocytes, choroid plexus epithelium, brain capillary endothelium, testis, ovary, adrenal gland, and several blood cells and neurons (Russel et al., 2008). Since urate is a substrate of Mrp4, it is believed that the presence of Mrp4 in each of its tissue locations helps to locally regulate urate concentration (Russel et al., 2008). Moreover, as *Russel et al. (2008)* point out, Mrp4's specificity for signaling molecules and tissue localization is suggestive of the ability to perhaps target the Mrp4 in a defined location for therapeutic

approaches such as: inhibition of nociception and inflammation, prevention of myocardial infarction and stroke, or improvement of brain penetration of antiviral nucleosides (Russel et al., 2008)).

Mrp4's involvement in urate transport and regulation is complex, and current research is only beginning to characterize its role. One of the first studies to directly implicate its involvement in avian urate secretion was published in October 2008 by *Bataille et al.* Here, avian renal proximal tubule epithelium urate secretion was examined using ^{14}C -urate flux experiments on chicken proximal tubule epithelial cell (cPTC) monolayers mounted in Ussing chambers. When Mrp4 expression was knocked down, transepithelial urate secretion under short-circuited conditions was decreased to 35% of the control value; the transepithelial resistance and sodium-dependent glucose transport were not affected (Bataille et al., 2008). Thus, for the first time, this study not only demonstrated Mrp4's localization at the BBM of cPTCs but also implicated the dependence of urate secretion upon the extent of Mrp4 expression in this location (Bataille R2031).

1.5 Stress and Transporter Expression

As plasma urate concentration is critical to overall health, and it has been demonstrated that, at least in the avian proximal tubule cell, the ABC transporter Mrp4 is critical to urate secretion (Bataille et al., 2008), one of the overarching themes in this study is the investigation into how stress affects avian urate excretion, and, presumably, Mrp4 expression and/or function. There is a rapidly expanding body of evidence that regulation under stress occurs for both uptake and efflux transporters. While much of it focuses on transporters other than Mrp4, some has begun to tackle the complexity behind Mrp4 regulation in different tissue types and, subsequently, to reveal the other critical roles that it performs.

One study that clearly demonstrates that stress can have an effect on transporter expression is the first to document liver and kidney Mrp gene regulation after hepatic ischemia reperfusion (IR) (Tanaka, Chen, Maher, & Klaassen, 2008). Hepatic IR is identified by *Tanaka et al. (2008)* as “a common event in hepatic resectional surgery and liver transplantation” (Tanaka et al., 2008), a description that makes the results of interest to humans who undergo these surgical procedures. In the study, different groups of rats underwent either 60 minute partial hepatic ischemia via clamping of the hepatic artery and portal vein or a sham surgery without clamping. Results indicated that after partial hepatic ischemia, multidrug resistance protein 2 (Mrp2) mRNA and protein decreased in the liver, but increased in the kidney; Mrp4 expression transiently increased in the kidney. Whereas the downregulation of liver Mrp2, which is possibly the result of reactive oxygen species (ROSs) and cytokine secretion by Kupffer cells, may contribute to development of cholestasis that sometimes occurs after hepatic IR, the upregulation of renal Mrp2 and Mrp4 may provide protection against the accumulation of toxic substrates (Tanaka et al., 2008).

Another example regarding regulation of transporter expression based on stress comprises a set of studies involving the induction of Mrp4 expression after acetaminophen (APAP) hepatotoxicity (Aleksunes et al., 2005; Aleksunes, Augustine, Scheffer, Cherrington, & Manautou, 2008); (Campion et al., 2008); (Campion et al., 2009). With ingestion of supra-therapeutic doses of APAP, the bioactivation of APAP to N-acetyl-p-benzoquinone imine (NAPQI) greatly contributes to centrilobular hepatic damage, which leads ultimately to hepatic cell death (Campion et al., 2008). Notably, for Mrp4 induction to occur following APAP toxicity, Kupffer cells (macrophages present in the liver) must be functioning normally; in mice that were pre-treated with clondronate liposomes, or liposomes that provoke apoptosis of

Kupffer cells, Mrp4 upregulation was inhibited (Campion et al., 2008). Oppositely, in a more recent study of the effects of allyl alcohol, a periportal hepatotoxicant, on transporter expression, it was found that Mrp4 expression increased whether or not Kupffer cells demonstrated normal function, with a greater degree of Mrp4 expression occurring in the latter case. As in the APAP toxicity studies though, Mrp4 expression was localized to centrilobular- and not periportal-hepatocytes (Campion et al., 2009). Although the details of the response in each case differ, both the APAP and allyl alcohol stressors illustrate the dynamic nature of transporter expression in the stress response. This study takes on particular importance in light of the fact that, as of 2005, APAP poisoning accounted for about 42% of acute liver failure cases in the United States, (Campion et al., 2008; Larson et al., 2005) . Further study to fully characterize the body's steps towards recovery- as well as to use this information to develop other effective therapeutic interventions- is well warranted.

Such studies have provided clues to the other roles of Mrp4 and to its regulation of expression. For instance, the balance between regulation of an efflux transporter such as Mrp4 and uptake transporters in tissues like the liver and kidney determines how effective the body is at preventing dangerous accumulation of toxic chemicals and/or byproducts of cellular injury. Moreover, in some cases, insight into the mechanisms whereby Mrp4 regulation occurs is revealed. For example, it is believed that Kupffer cell products, perhaps tumor necrosis factor α (TNF- α) or interleukin-1 β (IL-1 β), may be the critical elements for upregulation of Mrp4 expression after APAP damage (Campion et al., 2008). Essentially, studies such as these provide evidence that regulation of transporter expression is dynamic, yet precisely balanced, given the situation.

The set of stress experiments most influential in the development of the present study regarding urate secretion by Mrp4 under physiologically-stressful zinc concentrations include those performed by (Terlouw et al., 2002) and (Notenboom et al., 2005), both of which use the model of fluorescent methotrexate (FL-MTX) transport by Mrp2 in isolated killifish (*Fundulus heteroclitus*) proximal tubules. For true integration of these experiments, it is necessary to first consider the proposed role of endothelin (ET) and nitric oxide (NO) in the reduction of Mrp2-mediated transport of this drug. Briefly, data have suggested that acute exposure (30 minutes) to nephrotoxics, such as the aminoglycoside antibiotic gentamicin, and the heavy metal salts HgCl_2 and CdCl_2 , reduce Mrp2-mediated FL-MTX transport through a Ca^{2+} -mediated increase in ET release (Notenboom, Miller, Smits, Russel, & Masereeuw, 2002). ET then binds to the ET_B receptor, which stimulates NO production by NO synthase (NOS), and activation of cGMP (Notenboom et al., 2002; Notenboom et al., 2005; Terlouw, Masereeuw, Russel, & Miller, 2001; Terlouw et al., 2002). cGMP then acts as the major second messenger in mediating the stress effect, or reduction of Mrp2 FL-MTX transport.

Of even greater interest are the effects observed in the studies of (Terlouw et al., 2002) and (Notenboom et al., 2005), which examine both short- and long-term exposure to the proximal-tubule-targeting heavy metal salts and gentamicin, respectively. In both studies, data from acute exposure to the nephrotoxicant demonstrate the previously-observed reduction of FL-MTX transport by Mrp2 through the proposed ET, NOS, and cGMP signaling pathway. However, long-term exposure (6-24 hrs) to the nephrotoxicant resulted in a significant *increase* in both Mrp2-mediated FL-MTX transport and Mrp2 expression, although both of these factors did not increase in parallel most likely because of the involvement of other response mechanisms (Notenboom et al., 2005). Hypotheses of the specific effects of the second-messenger cGMP

include increased Mrp2 synthesis, increased rate of Mrp2 insertion into the luminal membrane, or decreased rate of endocytosis of Mrp2 from the luminal membrane (Notenboom et al., 2005), all of which would contribute to the observed increase in Mrp2-mediated FL-MTX transport. This “multiphasic” response functions as a “preconditioning mechanism” (Notenboom et al., 2005), whereby an acute exposure to a small amount of nephrotoxicant protects against a more prolonged exposure that occurs later on.

1.6 Zinc as a physiological stressor

Based upon the studies involving Mrp2-mediated FL-MTX transport in the face of nephrotoxicant stress, a study regarding the effect of Mrp4-mediated cPTC urate secretion when challenged with physiological stress began to take shape. Similar questions were asked: How does urate secretion by Mrp4 in the avian system respond to physiological stress? Does the response differ depending on the length of exposure to the stress? What is the mechanism whereby the stress response, if observed, occurs? Before describing the present study in detail, it is necessary to briefly explain the rationale for the use of the heavy metal zinc as a model for physiological stress. Considered an “essential trace element,” zinc has a total body concentration between 2 and 3g, and in individual tissues, its concentration ranges only between 10 and 200µg/g (Folin, Contiero, & Vaselli, 1994). *Mertz, 1981* broadly defines an essential trace element not based upon its concentration in the body, but upon the effects of its deficiency. He classifies an element as essential if a “deficient intake” of it causes impairment of a certain function, and if this impairment is only prevented or relieved by supplementation of homeostatic levels of the element (Mertz, 1981). A “deficient intake” can be measured according to the daily recommended intake, which varies for each trace element and is documented in the following table from (Mertz, 1981).

Table 1. Recommended safe and adequate dietary intakes for adults. [From (1)]

Element	Intake (mg/day)
Iron (males)	10
Iron (females)	18
Zinc	15
Manganese	2.5 to 5.0
Fluorine	1.5 to 4.0
Copper	2.0 to 3.0
Molybdenum	0.15 to 0.5
Chromium	0.05 to 0.2
Selenium	0.05 to 0.2
Iodine	0.15

Figure 7: Daily Recommended Intake of Several Trace Elements, from Mertz, 1981

Zinc was officially added to the list of essential trace elements in 1961 after observation of Iranian and Egyptian populations that were suffering from suspected zinc deficiency (Prasad, 2008). Such populations exhibited growth retardation, hypogonadism in males, hepatosplenomegaly, rough and dry skin, geophagia, severe iron deficiency anemia, and severe immune dysfunctions that often resulted in death by 25 years of age (Prasad, 2008). It is now known that there exist many zinc-regulated genes of the small intestine, thymus, and monocytes, most of which underlie signal transduction pathways contributing to global stress responses (Cousins et al., 2003; Prasad, 2008). With this knowledge, it is easier to understand why such a broad range of health effects are observed with its deficiency. Notably, however, early work by Bertrand (*Eighth international congress of applied chemistry, washington and new york, september 4 to 13, 1912* ..1912; 1913) demonstrated mathematically that *any* essential nutrient has the potential to become “toxic” if homeostatic interruptions that either decrease or increase concentration from the optimal range occur. **Figure 8** is a representation of such a mathematical depiction found in (Mertz, 1981), illustrating that toxicity, and death, can occur both above and below the homeostatic range, or plateau of the graph.

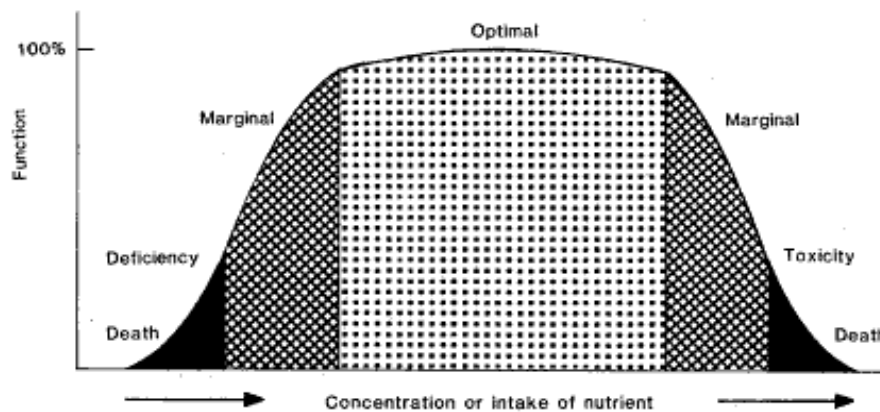


Fig. 2. Dependence of biological function on tissue concentration on intake of a nutrient.

1333

Figure 8: Mathematical Representation of homeostatic vs. nonhomeostatic concentrations of nutrients, from Mertz, 1981

Mertz comments that although there is a homeostatic range specific to each essential trace element, Bertrand's model is likely pertinent to all essential nutrients (Mertz, 1981).

As the current study utilizes a greater-than-normal, and not a deficient, concentration of zinc as a physiological stressor, it is of interest to mention several human pathological states that have been associated with elevated zinc concentration. Examples include elevation of zinc (and iron) levels in the globus pallidus pars externa of patients with spinocerebellar ataxia as opposed to controls (Popescu, Robinson, Chapman, & Nichol, 2009), elevated zinc levels in the erythrocytes of patients with multiple sclerosis as compared with controls (Ho, Catalanotto, Lisak, & Dore-Duffy, 1986), and elevation of zinc levels in postmortem neocortical samples of brains from patients who had suffered from Alzheimer Disease (AD) (Religa et al., 2006). In the AD study, *Religa et al. (2006)* specifically state some effects of excessive zinc concentration: inhibition of mitochondrial respiration, abnormal microtubule assembly, inhibition of proteasome activity, and, possibly, effects upon the metabolism of zinc metalloproteinases (Religa et al., 2006). Thus, elevation of zinc appears to cause respiratory, networking, proteolytic, and metabolic stress upon the cell. From a comparative perspective, the ability to

effectively deal with zinc supplementation varies among species. As an example, *Ansari et al.* (1976) revealed that rats exhibit much more effective homeostatic control mechanisms in the face of zinc supplementation than calves (Ansari et al., 1976). Such variation should be kept in mind when analyzing data from zinc supplementation performed on tissues from different animals.

1.7 The present study

The present study examines the effect of ZnSO_4 on the uptake of ^{14}C -urate into brush border membrane vesicles (BBMV) that were isolated from the kidneys of the domestic chicken, *Gallus gallus*. The BBM of the renal proximal tubule epithelium is the location of Mrp4, the dominant transporter involved in urate secretion into the proximal tubule lumen in the avian system (Bataille et al., 2008). As such, ^{14}C -urate uptake into these vesicles is thought to occur primarily via this transporter, and results were presumably indicative of its function. Previous work with flux experiments in Ussing chambers by *Bataille et al.* indicated inhibition of urate secretion after pretreatment of cPTC monolayers with prolonged exposure (6 hrs, followed by 1.5 hr recovery time) to ZnCl_2 (see **Results**). The next logical step, therefore, was to determine whether zinc was directly interacting with Mrp4 at the BBM and thereby preventing urate secretion. Results from this study revealed that ^{14}C -urate uptake was not significantly different between the vesicles incubated in control vs. ZnSO_4 – containing buffers. The direction of future research is focused on what is thus suggestive of a more complex cellular stress adaptation to zinc supplementation. AMP- activated protein kinase (AMPK), which responds to stress by shutting down non-vital ATP-utilizing processes, seems a likely player.

1.8 AMPK and a new direction

AMPK is the downstream component of a kinase cascade that responds to the cellular AMP: ATP ratio, or overall energy status of the cell. Highly conserved among almost all species (Hallows, 2005; B. B. Kahn, Alquier, Carling, & Hardie, 2005), AMPK is a heterotrimeric complex, consisting of a catalytic α subunit and two regulatory (β and γ) subunits. Its sensitivity is due to the existence of several mechanisms whereby AMP can activate the complex, including AMP's allosteric activation, phosphorylation at the major activation site Thr-172, and inhibition of dephosphorylation at Thr-172 (Hallows, 2005; B. B. Kahn et al., 2005). Globally, AMPK activation results in the shift from ATP-utilizing processes to ATP-generating and glucose-uptake pathways in order to restore the energy status of the cell (Hallows, 2005; B. B. Kahn et al., 2005). The following figure and legend are borrowed from (B. B. Kahn et al., 2005), where they illustrate the global effects of AMPK activation:

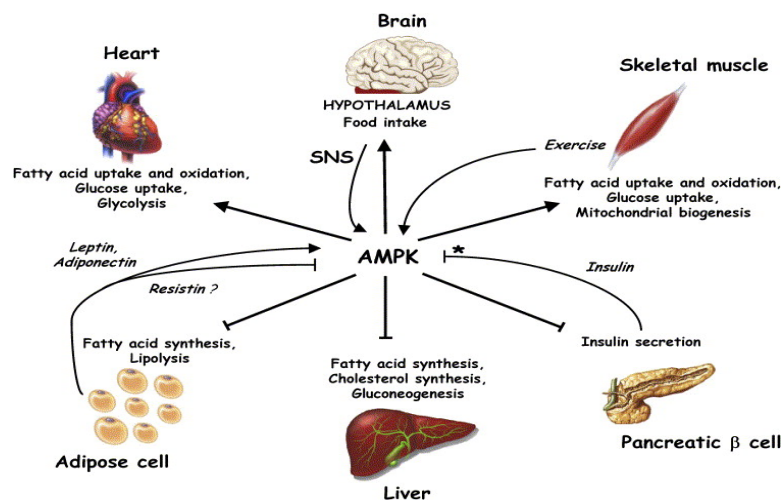


Figure 9: Role of AMP-activated protein kinase (AMPK) in the control of whole-body energy homeostasis, from Kahn et al. (2005)

Activation of AMPK in many tissues switches off ATP-consuming processes while switching on catabolic processes that generate ATP. Some key metabolic effects are shown. The adipocyte-derived hormones leptin and adiponectin, as well as exercise, activate AMPK in skeletal muscle, stimulating fatty acid oxidation. Leptin's activation of AMPK in skeletal muscle involves the hypothalamic-sympathetic nervous system (SNS) axis. Adiponectin also activates AMPK in liver, increasing fatty acid oxidation and reducing gluconeogenesis, and in adipocytes, where the downstream biologic pathway has not been studied. Resistin inhibits AMPK in liver. AMPK inhibits insulin secretion from pancreatic β cells.

Currently, it is becoming ever more apparent that AMPK is indeed quite instrumental in total body metabolism. For example, in humans there is evidence regarding AMPK's role in diabetes; the diabetic drugs metformin and rosiglitazone both exhibit their metabolic effects partially through AMPK (Misra & Chakrabarti, 2007). In addition, it appears as though caffeine mediates its effects on glucose regulation and lipid metabolism in skeletal muscle via AMPK α isoforms (Egawa et al., 2009). In short, AMPK is involved in an expanding assemblage of metabolic effects, a phenomenon that raises the possibility of someday crafting drugs that target its activity to help combat certain metabolic imbalances.

It is possible that AMPK may even play a role in the inhibition of Mrp4-mediated urate secretion in the face of a physiologically-stressful concentration of zinc, as observed by *Bataille et al.* Recent data has shown that pharmacological activation of AMPK by 5-aminoimidazole-4-carboxamide- β -D-ribofuranoside (AICAR) decreases urate secretion by cPTCs to an extent similar to that seen with prolonged exposure to ZnCl₂. Moreover, the AMPK inhibitor Compound C prevents both the AICAR-induced and zinc-induced inhibition of urate secretion (see **Results**). Since vesicle work suggests that zinc is not directly exerting its effects at the level of the BBM, zinc may indeed trigger a more global cellular stress adaptation involving AMPK, and ongoing study aims to better characterize its role.

1 Materials and Methods

2.1 Solutions and chemicals

The following solutions and/or compounds used in the homogenization medium and incubation buffers were purchased from Sigma Chemicals [St. Louis, MO]: D- Mannitol, HEPES, probenecid, potassium gluconate, urate, valinomycin, ouabain and oligomycin (for use

in the Na^+ , K^+ -ATPase assays), and Compound C (for AMPK-investigating Ussing chamber experiments). AICAR was purchased from BioMol International [Plymouth Meeting, PA]. Percoll for use in the basolateral membrane vesicle preparations was from GE Healthcare. ^{14}C -urate for both the Ussing chamber and vesicle uptake experiments was purchased from American Radiolabeled Chemicals, Inc. [St. Louis, MO]. All other chemicals were purchased from Fischer Scientific [Pittsburgh, PA].

2.2 Brush-border membrane vesicle (BBMV) preparation

Vesicle studies have become a useful model for studying the transport of substances across membranes. In addition to being used to characterize transport in the avian kidney, specifically in the domestic chicken (Kuo & Austic, 1987);(Bataille, Goldmeyer, and Renfro 2008) and turkey (Grassl 2002, C1155-62), the technique has been successful in studies of other species, including humans (Roch-Ramel, Werner, & Guisan, 1994), pigs (Krick, Wolff, & Burckhardt, 2000), dogs (Kinsella, Holohan, Pessah, & Ross, 1979) and rats (A. M. Kahn & Weinman, 1985);(Shimada et al., 1987). The steps in BBMV preparation, enzyme analysis, and flux experimentation in this study are based upon those described in *Grassl (2002)* and *Kuo et al. (1987)*, but have modifications. They are described in the following paragraphs.

All steps of BBMV preparation were performed on ice. BBMV preparations began when kidneys from 5-7 day old chicks (*Gallus gallus*) in a 1:30 dilution with homogenization medium (50mM Mannitol, 1 Tris/HEPES, pH 7.4) were homogenized using a Polytron homogenizer at speed 8 for three 20 second intervals, separated by two intervening 1 minute rest periods. The membrane fragments produced began to spontaneously form spherical vesicles of right-side-out orientation. Isolation of the BBM fragments was achieved by a series of steps. First, addition of 30 mM MgCl_2 to the homogenate followed by slow stirring for 20 minutes on ice was used to

separate out the BLM fragments. Removal of the BLM and other unwanted fragments was then accomplished by differential centrifugation in vesicle buffer (200 mM Mannitol, 20 Tris/HEPES, pH 7.4) in a Sorvall centrifuge with an SS34 rotor. Briefly, the vesicle mixture was spun at 5000 rpm for 10 minutes to draw down the BLM fragments into a pellet. Then subsequent spins with the resulting supernatant were performed at 10,000 rpm for 10 minutes and 17,000 rpm for 30 minutes; during these spins, the mitochondria and then the BBM were spun out, respectively. The resulting pellet was refluxed through a 23 gauge needle to re-suspend BBM vesicles in ~500 μ L of vesicle buffer. A second 30-minute spin at 17,000 rpm displaced the Mannitol from inside the intravesicular space, replacing it with vesicle buffer. The final supernatant was discarded, and the BBM pellet was re-suspended in 500 μ L of filter-sterilized vesicle buffer before being again refluxed through a 23 gauge needle to re-suspend the vesicles. The vesicles were stored in liquid nitrogen pending flux experimentation.

Though the protocol described here is straightforward, vesicle preparation of any kind is quite difficult for many reasons. For example, contamination with even a minute amount of detergent in the glassware inhibits proper vesicle formation, which is revealed by the lack of the characteristic overshoot (described in section 2.4) during flux experimentation. BBMV preparation in this study was no exception: many preparations were performed to no avail. Moreover, the ^{14}C -urate compound that is used is relatively unstable, and it degrades within a time frame of about 3 weeks. Therefore, care needed to be taken in order to plan experiments accordingly.

2.3 Assaying for degree of BBMV purity

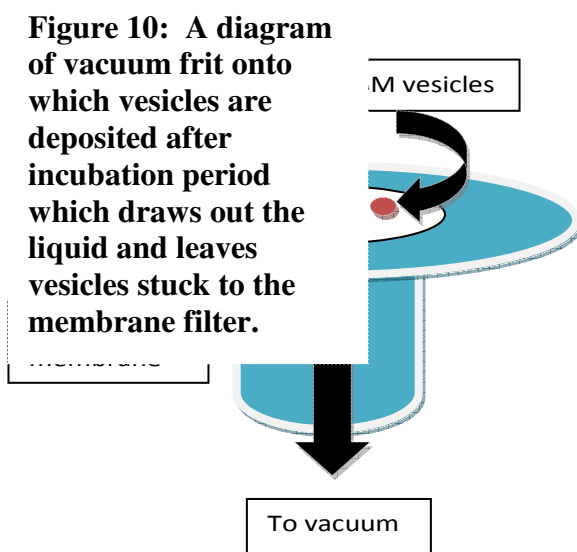
Enzyme assays were performed on lyophilized homogenate and BBM samples to ascertain the degree of BBM enrichment in the vesicle preparations. Three principal assays were

used: the BioRad assay [BioRad Laboratories Inc., Hercules, CA] to determine the amount of protein present, an alkaline phosphatase assay [phosphatase substrate from Sigma Aldrich, St. Louis, MO], and a $\text{Na}^+ \text{K}^+$ -ATPase assay [ouabain and oligomycin from Sigma Aldrich, St. Louis MO; MgCl_2 from Fischer]. Alkaline phosphatase is a BBM marker enzyme, so its enrichment in the BBMV preps needed to be significantly greater than that in the homogenate. On the other hand, the $\text{Na}^+ \text{K}^+$ -ATPase assay is used to check for contamination by BLM, the primary location of $\text{Na}^+ \text{K}^+$ -ATPase, and as such, values for BBMV preps needed to be quite low. All assays were measured spectrophotometrically, quantified through absorbance readings at specific wavelengths (595 λ for BioRad, 405 λ for alkaline phosphatase, and 700 λ for $\text{Na}^+ \text{K}^+$ -ATPase).

2.4 Flux Experimentation

Vesicle flux experiments were performed in triplicate and consisted of vesicle incubation in control (*100 mM K^+ gluconate, 10 mM HEPES, 1M KOH to pH 7.4*) and experimental (*100 μM ZnSO_4 , 100 mM K^+ gluconate, 10 mM HEPES, 1M KOH to pH 7.4*) buffers. Equimolar concentrations of ^{14}C -urate (*1.7 μL per mL incubation buffer*) and cold urate were added to the incubation buffers immediately prior to incubation start. 20 mg/mL of valinomycin, a K^+ ionophore, was added to the vesicle preparation to facilitate inward K^+ movement; the resulting inside-positive electrical gradient that stimulated rapid ^{14}C -urate influx, or “overshoot,” was characteristic of a viable BBMV preparation. This overshoot occurred normally within 15 seconds of incubation (see **Figures 13 and 14**). Incubation (*100 μL of incubation buffer added to 10 μL vesicles*) was performed for 15s and 60 minute (equilibration) time-points before ice cold stop solution (*100 mM KCl, 2 mM probenecid, 30mM NaOH, HEPES to pH 7.6*) was introduced. The entire solution was then transferred to a membrane filter on a vacuum frit that

drew out the liquid, leaving the vesicles stuck to the filter membrane (see **Figure 10** below). After rinsing the membrane 5 times with stop solution to wash away excess ^{14}C -urate, the amount of radioactivity left on the filter (the amount of urate taken up into the vesicles) was determined by a liquid scintillation counter. Finally, bath samples ($2 \times 10\mu\text{L}$) of each incubation buffer were taken to determine their initial ^{14}C -urate concentration. Also, triplets of filter blank flux experiments for each incubation buffer were performed; these included only the incubation buffer and stop solution to reveal any nonspecific binding of ^{14}C -urate to the filter membrane.



2.5 Integration of Intact Tissue Studies

Despite the usefulness of the vesicle technique in studies such as this one, it is important to consider the limitations of using isolated vesicle preparations to draw conclusions about a process that functions optimally in whole tissue. *Pritchard, 1987* adequately voices this concern, urging that vesicle data are best interpreted along with data from the tissue itself (Pritchard,

1987). In this particular case, the vesicle data were not simply being considered in isolation; in fact, they were initiated to further characterize a phenomenon observed in intact tissue.

Integration of the vesicle and intact tissue data is what truly renders the vesicle data more valuable. More specifically, the results from zinc stress and AICAR experiments (see **Results**) on intact tubule monolayers in Ussing chambers (see **Figure 11**) demonstrated inhibition of urate secretion upon prolonged exposure to a stressful concentration of zinc. Ussing chamber study technique has been refined by the Renfro lab (Bataille et al., 2008); (Dudas & Renfro, 2001; Dudas et al., 2005). Briefly, unidirectional fluxes were measured by adding ^{14}C -urate to one side of each monolayer at time 0, and samples were taken from the unlabeled side of each monolayer every 30 minutes for 1.5 hours. Net transepithelial flux was determined by the difference between the secretory (peritubular to lumen) and reabsorptive (lumen to peritubular) unidirectional fluxes (Bataille et al., 2008). Notably, flux measurements were taken during short circuited conditions with no electrical or chemical gradients present. Moreover, glucose current was measured at the beginning and end of experiments to assess normal tissue function.

Figure 11:

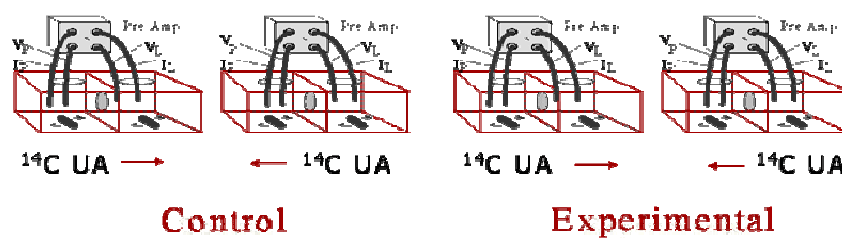


Figure 11: Diagram of Ussing Chamber setup. cPTCs are mounted in the center before both halves are sealed together and voltage and current electrodes are placed into the fluid on both sides of the tissue. V = voltage; I = current; P = peritubular; L = lumen

2.6 BLMV preparation

In considering the data observed so far, namely the inhibition of Mrp4-mediated urate secretion in the presence of zinc stress and AICAR in addition to the apparent lack of direct inhibition by zinc at the BBM, the next logical step in this study was examination of whether or not zinc was directly interacting with the BLM and thereby inhibiting urate secretion. The preparation of basolateral membrane vesicles (BLMV) for uptake experiments similar to those performed with BBMV stems from that described by (Sacktor, Rosenbloom, Liang, & Cheng, 1981) and (Grassl, 2002), though with significant modifications. As in BBMV preparation, all steps of the BLMV preparation were performed on ice. First, 8-10g of kidneys from 5-7 day old chicks were minced with a dissecting scissors in a small amount of homogenization medium (*250 mM sucrose, 10 mM Tris*HCl, pH 7.4*). After mincing, more homogenization medium was added, the tissue was allowed to settle, and the blood was decanted. This procedure was repeated about 4 times in order to remove as much blood as possible, since blood contains alkaline phosphatase, an enzyme that has greater enrichment in the BBMV preparations. After decanting, a 1:6 (w/v) dilution with the homogenization medium was made with the minced kidneys, and 20 strokes with a glass-teflon homogenizer were performed. Importantly, the low temperature provided by the ice significantly slows the rate at which the released lysosomal enzymes can degrade the tissue. After this first round of homogenization, the tissue was transferred into a graduated cylinder and homogenized further by a Polytron at speed 8 for three 20-second periods, separated by two 1-minute intervening rest periods. During this stage, the fragmented membrane begins to form spherical vesicles.

After homogenization, BLM isolation proved to be a more involved process than BBM isolation. Spins in the SS34 rotor in a Sorvall centrifuge were performed at 3500 rpm for 15 minutes, followed by 13,000 rpm for 20 minutes with the resulting supernatant. After this spin,

three layers were evident in the centrifuge tube: a blood red supernatant, a light beige “fluffy” layer, and a dark greenish-yellow pellet at the very bottom of the centrifuge tube. The light fluffy layer, which contains the crude membrane extract, was extracted with a Pasteur pipet and transferred to a graduated cylinder placed on ice. Homogenization medium was added to bring the volume to 32.0 mL.

18 strokes with the glass-teflon homogenizer comprised the third stage of homogenization, and it was followed by the addition of 3 mL Percoll. Percoll addition is the critical element of the BLMV preparation because, upon its addition to a mixture, it separates the components of the mixture such that each migrates to the layer where the Percoll particles are of equal density. After Percoll addition, 2 additional strokes with the glass-teflon homogenizer were performed, and the mixture was spun at 17,000 rpm for 60 minutes in the SS34 of the Sorvall centrifuge to generate the Percoll density gradient. After this spin, the top milliliters 1- 6 were removed and discarded, whereas milliliters 7 -13 were removed and transferred into an empty ultracentrifuge tube (variability exists with regard to the position of majority of the BLM within the Percoll-generated density gradient; this approach differs from milliliters 9-15, as described by (Sacktor et al., 1981)).

The first step in the final stage of BLMV preparation was the removal of the Percoll from the BLM isolate: the BLMV is spun in homogenization medium at 28,500 rpm in the SW41Ti rotor in the Beckman ultracentrifuge for 60 minutes. At the end of this spin, the Percoll exists as a “glass-like” pellet at the bottom of the tube, above which sits the BLMV pellet. The supernatant was removed with a Pasteur pipet and discarded; the BLMV was removed and transferred to another ultracentrifuge tube containing vesicle buffer (*200 mM Mannitol, 20 Tris/HEPES, pH 7.4*). A second spin 60 minute spin at 28,500 rpm in the SW41Ti of the Beckman ultracentrifuge

replaced the homogenization medium with vesicle buffer. Finally, the resulting BLM pellet was re-suspended in 500 μ L of filter sterilized vesicle buffer and refluxed through a 23 gauge needle in order to facilitate vesicle formation.

Despite the lengthy process of BLMV preparation and the numerous attempted trials, none of the enzyme analyses clearly revealed that BLM isolation had occurred. Vesicle volume calculation indicated little vesicle formation in most cases, suggesting a problem in the prep itself. Work is ongoing in the attempt to expose where in the preparation the error lies.

3. Results

3.1 Results of Ussing Chamber zinc stress experiments (Bataille et al., 2008)

Recent work by *Bataille et al.* in our laboratory has revealed that, with prolonged exposure (6 hrs followed by a 1.5 hr recovery period in untreated culture medium) to 250 μ M ZnCl_2 pretreatment, cPTCs demonstrated significant inhibition of net urate flux (**Figure 12A**). Inhibition was not observed in cPTCs treated acutely (only during the 1.5 hr experiment) with the same concentration of ZnCl_2 (**Figure 12B**). These data prompted the set of vesicle experiments in order to determine whether or not zinc was directly inhibiting the BBM transporters, especially Mrp4, and thereby directly inhibiting urate uptake. Moreover, these data suggest that the cellular stress adaptation to zinc takes time to develop, as cPTCs under acute zinc exposure did not exhibit inhibition of net urate flux.

Figure 12:

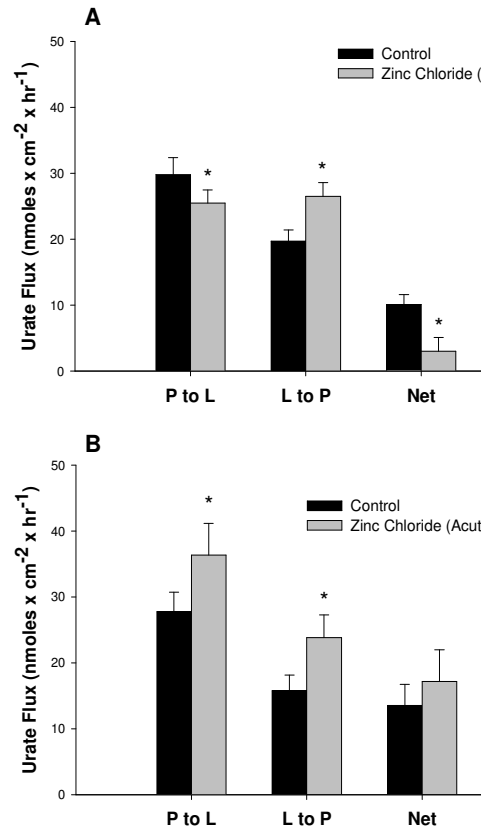


Figure 12: Transepithelial urate transport by chicken proximal tubule cell monolayers \pm extended (A) and acute (B) zinc exposure. Unidirectional flux data measured in Ussing chambers is shown (P to L, peritubular-to-lumen side; L to P, lumen-to-peritubular side) and net urate flux is determined by the difference between the two. (A) Fluxes were measured in untreated control monolayers and those that have experienced a 6 hour exposure to 250 μ M ZnCl₂, followed by a 1.5 hour recovery period in untreated culture medium. (B) Fluxes measured in untreated controls and those acutely exposed to 250 μ M ZnCl₂ for the 1.5 hour duration of the experiment. Values shown are means \pm SEM determined at 1.5 hours. * Indicates significantly different from paired controls at $P < 0.05$.

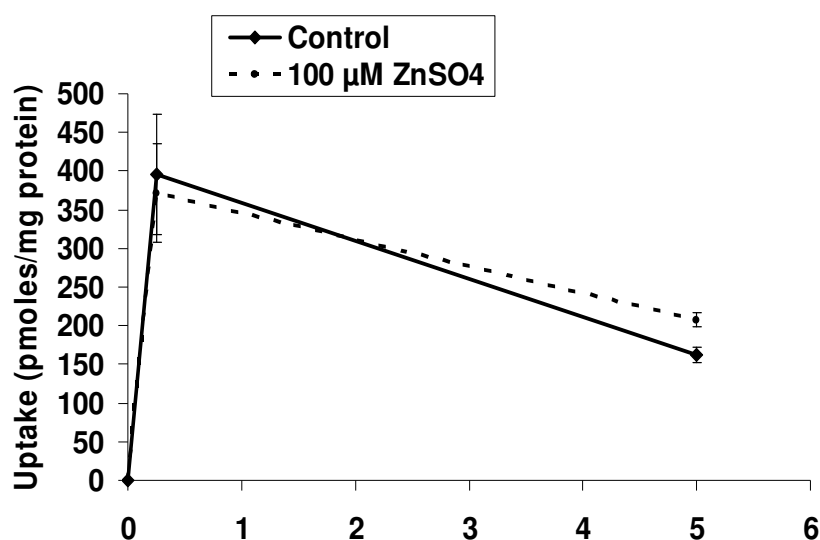
3.2 Results of BBMV Experiments

Data for vesicle flux experiments follow in **Figures 13** and **14**. The valinomycin treatment of all vesicles created a very rapid uptake of 14 C-urate (an overshoot) that occurred within the first 15 seconds after 14 C-urate-containing incubation buffer addition. The effect of 100 μ M ZnSO₄ was explored both extravesicularly (**Figure 13A, 13B**), in which the 100 μ M

ZnSO₄ was added to the buffer just prior to flux experimentation, and intravesicularly (**Figure 14A, 14B**), in which experimental vesicles were pre-incubated with the 100 μ M ZnSO₄ for 30 minutes before flux experimentation, allowing zinc entry into the vesicles. In each case, ZnSO₄ did not alter what was observed for control vesicles: rapid ¹⁴C-urate uptake occurred at almost exactly the same time and to the same extent as it had for control vesicles incubated in zinc-free buffers. Though a difference in maximum uptake between extravesicular (400 pmoles/mg protein) and intravesicular (800 pmoles/mg protein) experiments occurs, which is likely due to differences in the stage of degradation of the very unstable ¹⁴C-urate at the particular time of experimentation, only relative changes between the uptake in control and zinc-containing buffers were the critical part of data interpretation here.

Figure 13:

A.



B.

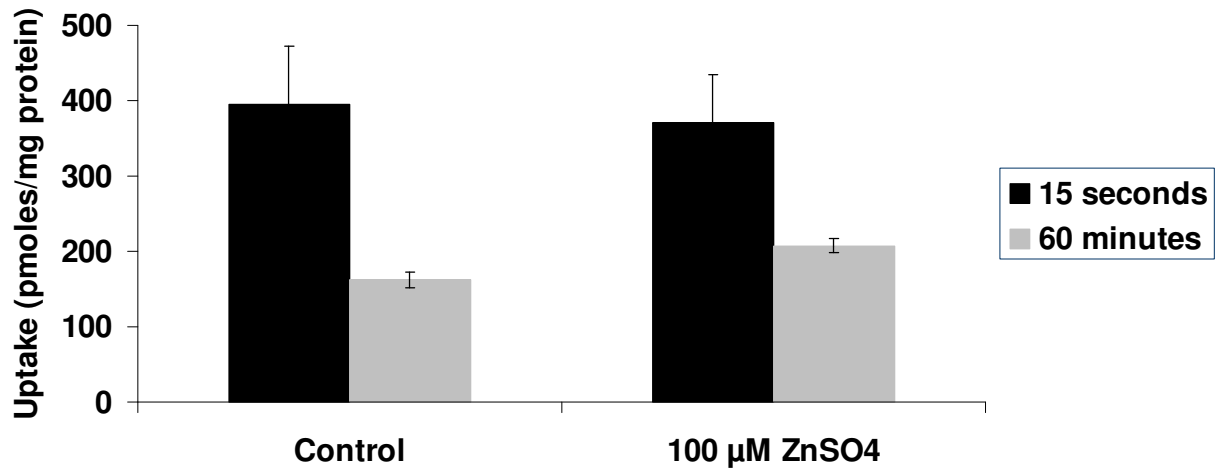
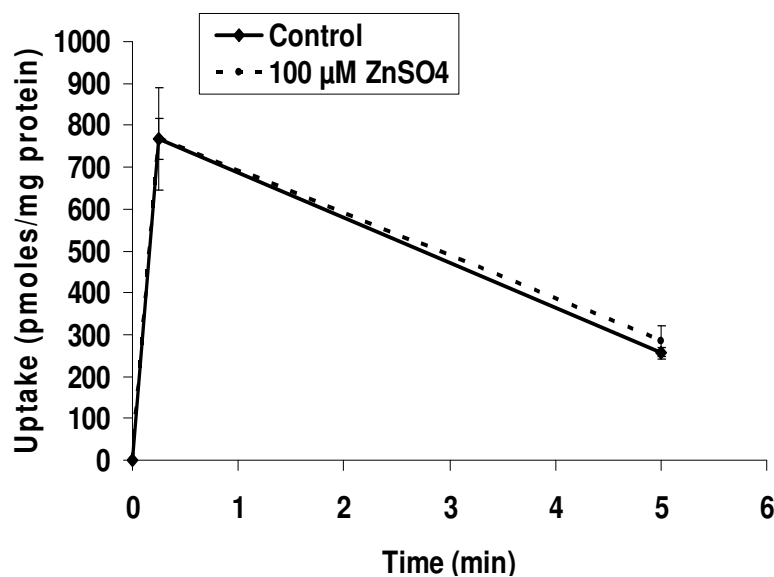


Figure 13: Net urate flux in isolated brush border membranes in the presence and absence of extravesicular 100μM ZnSO₄. A. A plot of urate uptake in pmoles/mg protein in the presence and absence of 100μM ZnSO₄ in the incubation buffer illustrating the characteristic overshoot at the 15 second point. B. Bars are summary data from 4 flux experiments. Time points for all experiments were 15 seconds and an equilibrium time of 60 minutes. Valinomycin, a K⁺ ionophore, was added to all vesicles (20 mg/mL), in order to provide an inside-positive electrical gradient that facilitated the rapid inward movement of the negatively charged ¹⁴C-urate. Control incubation buffer, experimental incubation buffer, and stop solutions are listed in the Materials and Methods section. (Enzyme enrichments as means ± SEM: Alk. Phos 10.8 ± 2.7, Na⁺,K⁺-ATPase 1.5 ± 0.9, and Mg-ATPase 3.2 ± 2.0)

Figure 14:

A.



B.

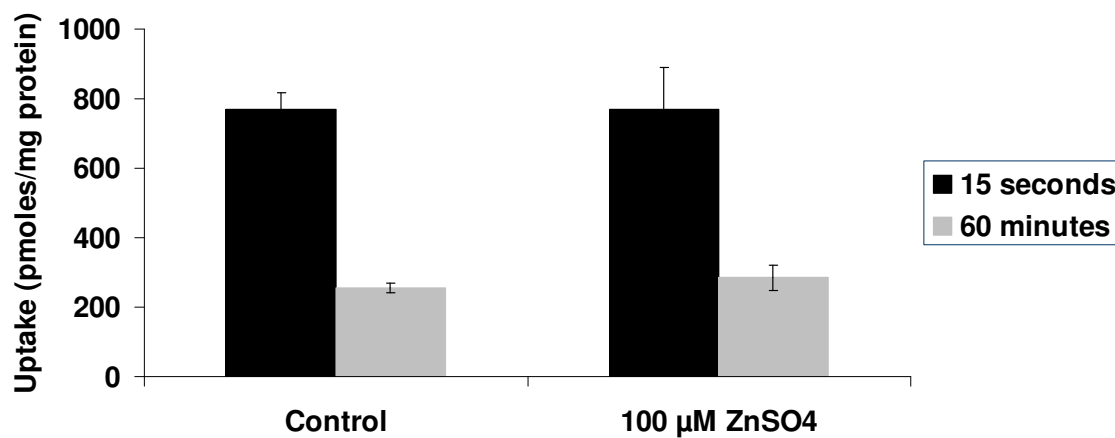


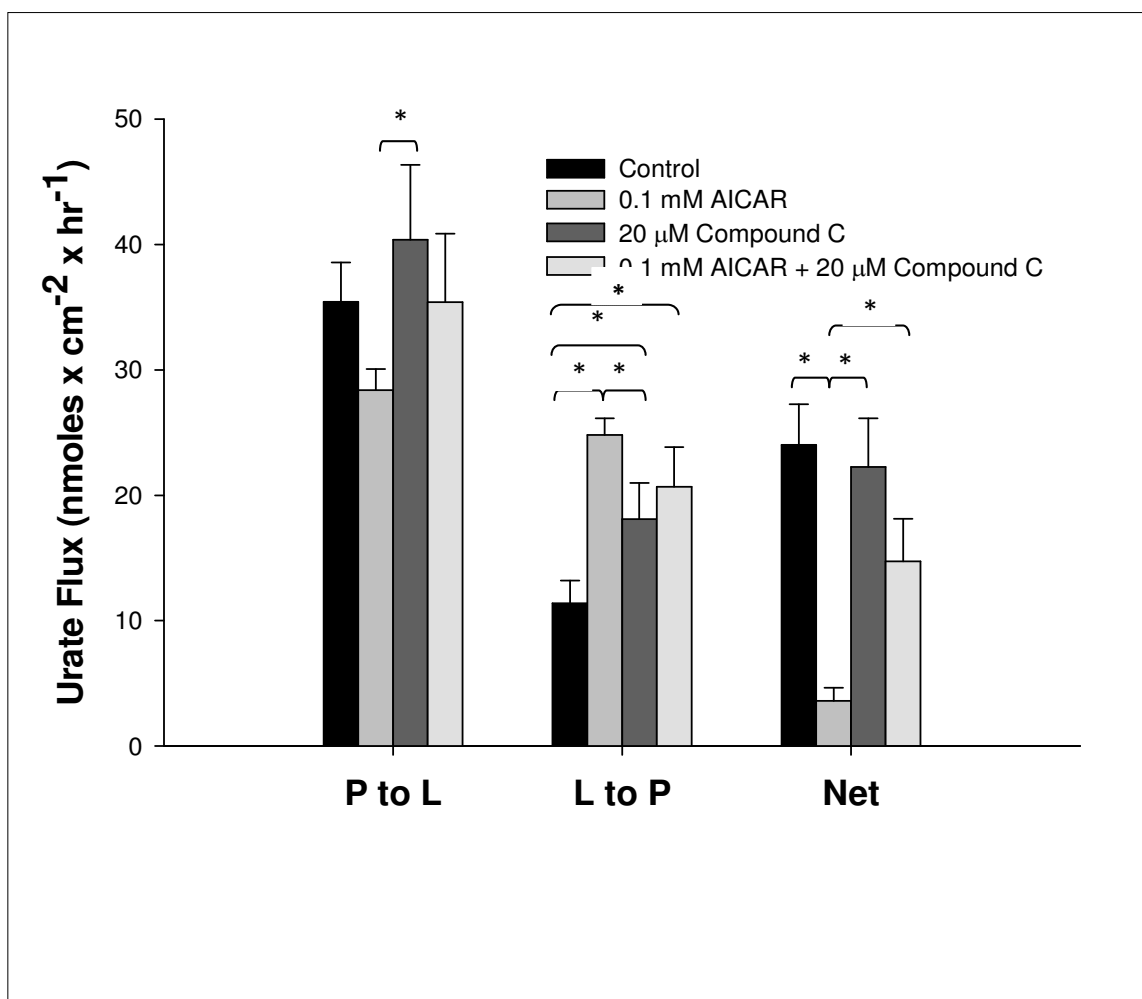
Figure 14: Net urate flux in isolated brush border membranes in the presence and absence of intravesicular 100 μM ZnSO_4 . A. A plot of urate uptake in pmoles/mg protein in the presence and absence of 100 μM ZnSO_4 . In those treated with the ZnSO_4 , the vesicles were preincubated for 30 minutes before flux experiments. Time

points remained both 15 seconds and 60 minutes, and as before, there is the characteristic overshoot in presence of valinomycin (20mg/mL). B. Bars are summary data from 4 flux experiments. (Enzyme enrichments as means \pm SEM: Alk. Phos 15.23 ± 0.95 , Na^+ , K^+ -ATPase 2.89 ± 0.25 , and Mg-ATPase 3.35 ± 0.68).

3.3 AMPK Results

Since the BBMV studies did not reveal any specific interaction of zinc with the BBM, further experimentation was performed in order to better characterize what appears to be a more integrated cellular stress response possibly involving the metabolic regulator AMPK. **Figure 15** reveals that AMPK activation by AICAR resulted in a significant reduction in net urate flux, a reduction that was mostly prevented by simultaneous treatment of the AMPK inhibitor Compound C. Moreover, as **Figure 16** points out, the net inhibition of urate secretion that had been previously observed with prolonged exposure to ZnCl_2 was almost completely prevented by the simultaneous treatment of Compound C. As such, the data suggest that the zinc-induced inhibition of urate secretion may have occurred through AMPK, and this hypothesis is the focus of further study.

Figure 15:



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transport in cPTCs. Net transepithelial transport of urate was significantly reduced in cPTCs treated with 0.1 mM AICAR. 20 μM Compound C prevented the effect of AICAR and restored net urate transport to that of the paired controls. All treatments were added to the physiological saline bath, and fluxes were measured 90 minutes after treatments were begun. Values are means ± SEM. Asterisks indicate significant differences among treatments at $P < 0.05$.

Figure 16:

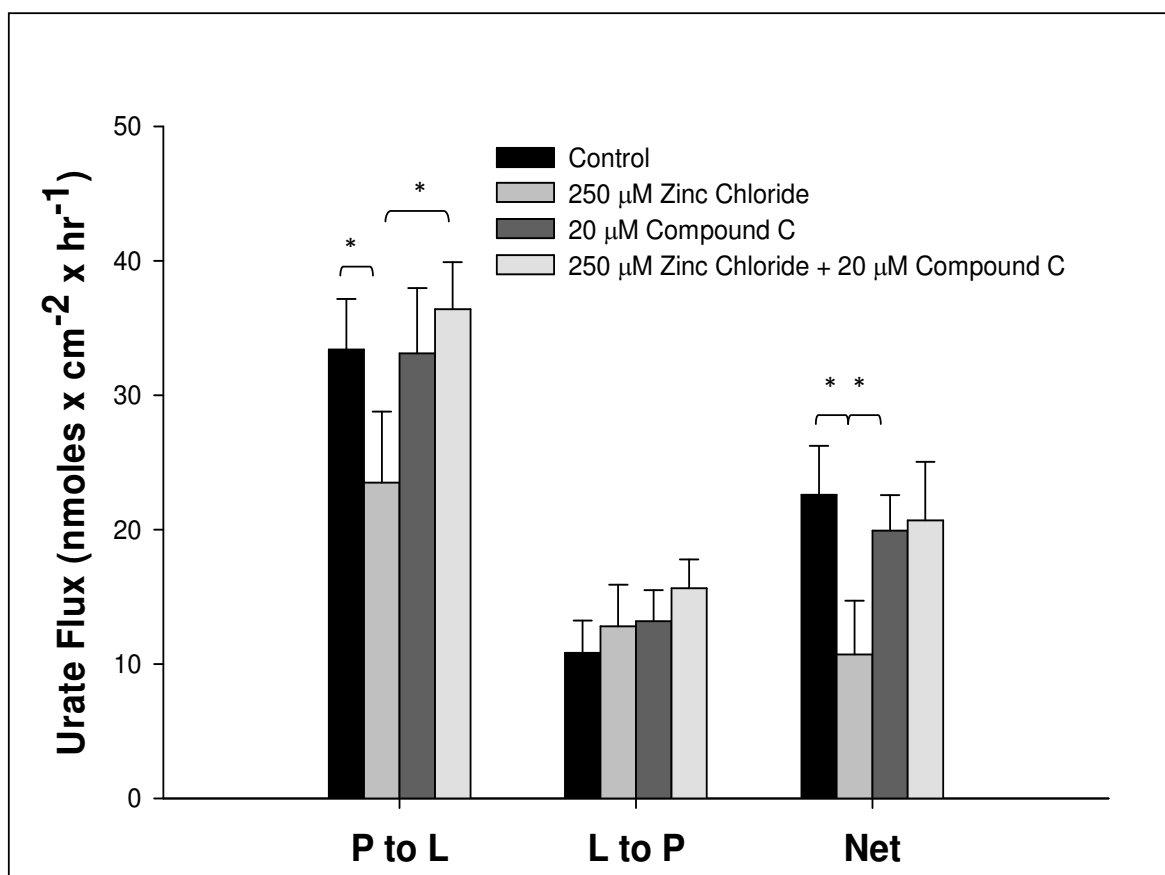


Figure 16: Effect of zinc chloride and Compound C on unidirectional and net urate active transepithelial transport in cPTCs. Net transepithelial transport of urate was significantly reduced in cPTCs treated with 250 μ M ZnCl_2 . Treatment with 20 μ M Compound C prevented the effect of the ZnCl_2 treatment on net urate transport, restoring it to that of the paired controls. All treatments were added to the cPTCs in otherwise normal culture medium, incubated for 6 hours, and allowed to recover for 1.5 hours in normal culture medium prior to flux measurements. Values are means \pm SEM. Asterisks indicate significant differences among treatments at $P < 0.05$.

Recent literature pertaining to AMPK has demonstrated that an AMPK activator similar to AICAR, A-769662, also inhibits the Na^+ , K^+ -ATPase (Benziane et al., 2009). Its similarity to AICAR prompted the question of whether or not the observed inhibition of Mrp4-mediated urate secretion upon AICAR administration (**Figure 17**) was fundamentally due to the inhibition of

this critical enzyme. Na^+ , K^+ -ATPase enzyme assays were performed on several BLMV preparations in the presence of: AICAR, ouabain, and the combination of ouabain and oligomycin. The data suggest that AICAR is not promoting this inhibition.

Figure 17:

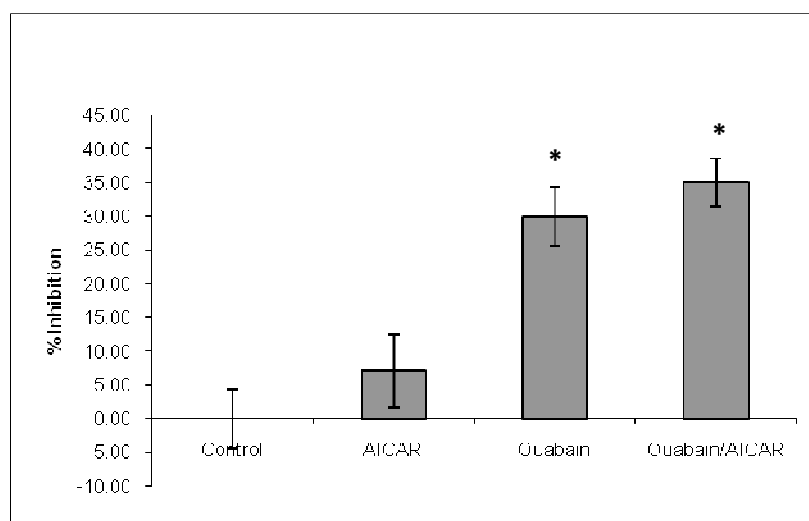


Figure 17: The effect of AICAR on Na^+ , K^+ -ATPase activity. BLMV samples were pre-incubated in Na^+ , K^+ -ATPase buffer (125 mM Tris, 1.8 mM EDTA, 150 mM NaCl, 14 mM KCl, pH 7.7) containing AICAR (1mM) and/or ouabain (1.36 mM) for 10 minutes at 37°C. Reaction was initiated by addition of Mg^{2+} -ATPase (45 mM Mg^{2+} + 30mM ATP) and allowed to occur for 15 minutes at 37 °C. Reaction was stopped with ammonium molybdate-based stop solution (3.6 mM ferrous sulfate in 8.09 mM ammonium molybdate solution). Na^+ , K^+ -ATPase activity was determined by colorimetric analysis in a spectrophotometer at 700 nm. Bars are summary data for 4 separate BLMV preparations using 1:100 dilutions of lyophilized BLMV samples. Values shown are means \pm SEM. * Indicates significantly different from controls at $P < 0.05$.

4. Conclusions

The questions posed by this particular study were greatly influenced by the work of authors such as Notenboom and Terlouw that revealed inhibition of Mrp2-mediated FL-MTX uptake in isolated proximal tubules of killifish when acutely challenged with nephrotoxics that included gentamicin and heavy metal salts (Notenboom et al., 2005; Terlouw et al., 2002). When long-term exposure to a low concentration of these nephrotoxics resulted in a beneficial “preconditioning” effect that protected against the reduction of FL-MTX transport upon later exposure to the same nephrotoxicant, (Notenboom et al., 2005; Terlouw et al., 2002), it further complicated the stress response, bolstering the need for further explanation in this and other Mrps. Previous work in our lab had demonstrated a prominent role of Mrp4 in urate secretion in the avian proximal tubule (Bataille et al., 2008), and a series of zinc-stress Ussing chamber experiments, followed by BBMV and (ongoing) BLMV uptake experiments, and AMPK Ussing chamber experiments, progressed in order to characterize the stress response of this system. Thus, the vesicle work that is the specific focus of this thesis was a small, yet necessary, contributing factor in this ongoing analysis.

Despite the inherent difficulty in preparing isolated BBM and BLM vesicles, lack of BLM vesicle data as of the writing of this thesis, and the inherent instability of ^{14}C -urate, the major vehicle for the analysis of Mrp4-mediated urate secretion in the avian system, there are several key aspects of urate secretion in response to a stressful zinc concentration suggested by the data presented. First, preliminary data in Ussing chamber transepithelial transport studies have shown that there is no significant difference between the net urate flux by chicken proximal tubule cell monolayers treated with acute exposure to zinc in the form of $250\ \mu\text{M}\ \text{ZnCl}_2$ compared to the control. In addition, it shows a statistically significant difference between the net urate flux measured in monolayers that have had a more extensive 6 hour exposure to $250\ \mu\text{M}$

ZnCl₂, followed by a 1.5 hour recovery period in untreated culture medium, as opposed to controls. Importantly, in both acute and long-term conditions, the zinc treatment had no significant effects on the electrical resistance or glucose current exhibited by the tissues; these measurements are indicative of good overall tissue health.

In response to the Ussing chamber urate flux experiments, isolated BBMVs experiments were undertaken to reveal any specific interaction and inhibition at the BBM. Results here indicated no significant difference between ¹⁴C-urate uptake for vesicles with an inward-positive electrical gradient that have been acutely exposed to a physiologically stressful extravesicular or intravesicular 100 μM concentration of ZnSO₄ as compared to controls. In other words, it does not appear as though zinc is directly interacting with Mrp4, or any of the other BBM transporters, as its mechanism for inhibition of urate secretion. BLMV experiments and analysis is pending, and if these data reveal no significant difference in control vs. Zn-containing incubation buffers as well, it is likely that a more complex cellular response is initiated in the face of zinc supplementation. Preliminary evidence based upon AICAR and Compound C points towards involvement of the metabolic sensor AMPK, and it is the major focus of future experiments.

Acknowledgements

Many thanks are warmly extended to Dr. Larry Renfro, Amy M. Bataille, and Sonda Parker. This manuscript could not have been completed without your unwavering support and guidance. Thank you to Dr. Joseph Crivello and Dr. José Manautou who were also on my advisory committee. Finally, thank you to the University of Connecticut University Scholar Program and Honors Program. This work was supported by the National Science Foundation.

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