Acute Isolated Increased Renal Venous Pressure and Kidney Functions in an Experimental Rat Model

by

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Abstract

Increased renal venous pressure is a recognized risk factor for kidney dysfunction. It is not rare in clinical settings with venous congestion, such as heart failure, and is associated with worsening kidney function and increased mortality. However, the underling mechanisms are not well understood. In addition, advanced kidney dysfunction might result in sodium and fluid retention, which exacerbates the congestive state. Sodium handling at high renal venous pressure has not been well addressed. In this thesis, we first review principal mechanisms governing renal hemodynamics and sodium handling. Then we investigate how these mechanisms would relate to increased renal venous pressure. Pathophysiologically, both intravascular and extravascular factors can lead to an increased renal venous pressure. Limited data could be found regarding a comprehensive understanding of how increased renal venous pressure contributes to kidney dysfunction in disease states. Thus, it is not well understood how increased renal venous pressure contributes to renal dysfunction. It is therefore that we embarked on a number of studies to define the role of increased renal venous pressure on renal function.

Increased renal venous pressure forms a physical factor that directly decreases renal blood flow. However, neurohumoral factors are likely involved in mediating effects of increased renal venous pressure on renal blood flow, glomerular filtration rate and sodium handling. To separate the role of renal venous pressure from the role of a congestive state as a whole, we adapted a model of isolated increased renal venous pressure in the rat. The acute isolated increase in renal venous pressure results in decreases in RBF, renal vascular conductance, glomerular filtration rate and heart rate.

Two very powerful modulators are the renin-angiotensin system and renal sympathetic nerve activity. The roles of these two mechanisms in the renal response to increased renal venous pressure are not clearly delineated. Moreover, it has been suggested that the intrinsic mechanism intended to maintain renal blood flow and glomerular filtration rate stable upon fluctuations in renal perfusion pressure, renal autoregulation, is compromised by increased renal venous pressure, but this has hardly been studied in this context. Our data indicate that other than renal sympathetic nerve activity, renin-angiotensin system and autoregulation, have primary roles in vasoconstrictive response to renal venous pressure elevation. Furthermore, the supression of renal sympathetic nerve activity and inappropriate activation of the renin-angiotensin system contribute to the impact on heart rate and mean arterial pressure. Increased renal venous pressure also modulates the sodium handling. The inappropriate activation of renin-angiotensin system is also responsible for the sodium retention in renal venous pressure elevation. Increased renal venous pressure also tend to incease the tubular pressure, which might affect the tubular sodium tansport.

In conclusion, increased renal venous pressure impairs kidney function, which involves potential modulators such as the renal sympathetic nerve activity, the renin-angiotensin system and others. This report describes an initial exploration of this area, with emphasis on the renin-angiotensin system, renal nerves and renal autoregulation.

Preface

The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethic Board, project title 'Renal venous pressure, renal function and experimental heart failure'. NO: RES0019970, Date: Oct 20, 2014.

Experiments in Chapter 2 and 3 were done with the assistance of Dr. Shereen Hamza. Data in chapter 2 and 3 have been published as 'Xiaohua Huang, Shereen Hamza, Wenqing Zhuang, William A Cupples, Branko Braam. Sodium intake but not renal nerves attenuates renal venous pressure-induced changes in renal hemodynamics in rats.' *Am J Physiol Renal Physiol. 2018 Sep 1;315(3): F644-F652. doi: 10.1152/ajprenal.00099.2018. Epub 2018 Jun 6.* I was responsible in data collection and contributed to manuscript edits. Dr. Shereen Hamza contributed equally in data collection and manuscript edits. Wenqing Zhuang contributed to analytical methodology development. Drs. William A Cupples and Branko Braam were the supervisory author and were involved with concept formation and manuscript revision.

Data analysis in chapter 4 and 5 are my original work, as well as the literature review in chapter 1. Chapter 4 will be submitted for publication.

Dedication

To my late father…

To my beloved mother, sister and brother

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Contents

List of Tables

List of Figures

Abbreviations

ACEi	angiotensin-converting-enzyme inhibitor
AVP	Vasopressin
ANG II	angiotensin II
BSA	bovine serum albumin
BW	body weight
CHF	congestive heart failure
CKD	chronic kidney disease
CVP	central venous pressure
ENaC	epithelial sodium channel
FE_{Na}	fractional excretion of sodium
GFR	glomerular filtration pressure
HCT	Hematocrit
HR	heart rate
HS	high salt
K_f	filtration constant

L-NAME	L-NG-nitroarginine methyl ester
MD	macula densa
MR	myogenic response
NCC	Na-Cl co-transporter
NHE3	Na+/H+ exchanger
NKCC2	Na-K-Cl co-transporter
NO	nitric oxide
NOS	nitric oxide synthase
NS	normal salt
P_B	hydrostatic pressure in the Bowman's capsule
P_C	capillary hydrostatic pressure
P_G	glomerular capillary hydrostatic pressure
P_i	interstitial hydrostatic pressure
RBF	renal blood flow
RPP	renal perfusion pressure
RSNA	renal sympathetic nerve activity

RVC	renal vascular conductance
RVP	renal venous pressure
RVR	renal vascular resistance
SGK-1	serum- and glucocorticoid-inducible kinase-1
SNS	sympathetic nervous system
TC	time control
TGF	tubuloglomerular feedback
$U_{Na}V$	urinary sodium excretion
σ	reflection coefficient
π_B	oncotic pressure in the Bowman's capsule
π_C	capillary oncotic pressure
π_G	glomerular capillary oncotic pressure
π_i	interstitial oncotic pressure

CHAPTER 1:

What is the fuss about renal venous pressure?

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1.1 Introduction

Physical obstruction of renal venous outflow [1], increased intra-abdominal pressure [2] and venous congestion such as found in chronic kidney disease (CKD) [3] or congestive heart failure (CHF) [4] all can lead to increased renal venous pressure (RVP). Impaired kidney function is commonly found in these situations. While pre-renal factors are frequently considered in renal dysfunction, the influence of increased 'renal afterload' [5, 6] is less often considered. However, the importance of RVP is now getting growing attention.

Increased RVP can be due to extravascular factors such as mechanical obstruction and increased ambient pressure, or an intravascular factor such as congestion and renal vein thrombosis. Inadequate renal venous outflow is the underlying cause for diminished renal function in renal vein compression and renal vein thrombosis [7, 8]. Increased intra-abdominal pressure causes renal dysfunction in both experimental and clinical studies [2, 9]. It has been shown in swine that the compression of the renal vein, but not the kidney itself, reduced GFR [2]. Congestive state in acute decompensated heart failure (ADHF) is often accompanied with increased venous pressure [5]. Retrospective studies have shown a clear correlation between increased central venous pressure (CVP) and reduced GFR in ADHF [5]. Impaired renal function per se increases mortality risk in ADHF [10]. Similarly, chronic increase of CVP is associated with impaired kidney function in patients with cardiovascular disease [11].

In summary, all these diseases, despite the different etiologies, often result in impaired renal function, which is probably induced by an increased RVP. The focus of this review is to discuss how increased RVP impacts renal function and to explore potential mechanisms including physical and neurohormonal factors, in both physiology and pathophysiology states.

1.2 Overview of the major parameters to affect kidney function

1.2.1 Hemodynamics

This section introduces different parameters modulating kidney function that will be affected by increased RVP. Increased RVP reduces the pressure gradient between arterial pressure and venous pressure, thus decreases renal perfusion pressure (RPP) (Eq.1).

$$RPP = MAP - RVP \qquad (Eq.1)$$

The changes in RPP might induce subsequent changes in RBF, which also depend on the changes in renal vascular conductance (RVC) (Eq.2).

$$RBF = RPP \times RVC \qquad (Eq.2)$$

RVC is an important parameter in the microcirculation and renal hemodynamics. Both RVC and renal vascular resistance (RVR) have been used to indicate the changes in vascular tone. RVC is the reciprocal of RVR (Eq.3). Decreases in RVC indicate more resistance for blood to flow through the vessels, in other words, vasoconstriction. In comparison to RVR, RVC change is considered better in reflecting regional vascular responses in the pressure changes [12, 13] since RVC varies linearly with RBF. Kidneys have continuous adjustment of RVC to maintain the stabilization of RBF. The changes of RVC are mainly from afferent and efferent arterioles. However, it is important to note that most of the intrinsic adaptations of RVC occur predominantly at the pre-glomerular segments (afferent arterioles) [14].

$$RVC = \frac{1}{RVR} \qquad (Eq.3)$$

GFR is not only an important parameter for the evaluation of kidney function, but also the first essential step of urine formation. According to the Starling equation (Eq.4), the hydrostatic pressure gradient is considered the primary driving force for glomerular filtration under physiological situation. The

question remains unclear here is how increased RVP impacts hydrostatic pressure in glomerulus and renal tubules due to secondary changes caused by neurohormal factors.

$$GFR = K_f \times [(P_G - P_B) - \sigma(\pi_G - \pi_B)] \qquad (Eq. 4)$$

(K_f: filtration constant, P_G: glomerular capillary hydrostatic pressure, P_B: hydrostatic pressure in the Bowman's capsule, σ: reflection coefficient; π_G: glomerular capillary oncotic pressure; π_B: oncotic pressure in the Bowman's capsule.)

1.2.2 Autoregulation

The kidney has its intrinsic mechanism to stabilize RBF and GFR in response to the pressure fluctuations. Renal autoregulation protects glomerular structure and maintains stable kidney function. Both quick (myogenic response, MR) and relatively slow (tubuloglomerular feedback, TGF) mechanisms are involved in RBF and GFR autoregulation [15]. MR refers to the autonomous response of vessel tone to changes in renal perfusion pressure. An acute increase in renal perfusion pressure elicits a rapid constriction of the afferent arteriole that is triggered by stretch of smooth muscular cells [16]. The underlying mechanisms include vascular smooth muscle cells depolarization, activation of voltage-gated L-type Ca^{+2} channels and Ca^{+2} entry triggering a rapid vasoconstriction [17, 18].

TGF is a negative feedback mechanism, which senses the changes in NaCl concentration at the macula densa (MD) and elicits adjustments in glomerular arteriolar resistance (Figure 1.1). Increased perfusion pressure induces the increase of glomerular capillary pressure and glomerular filtration. This results in an increase in tubular flow, which taken in isolation means increased NaCl arriving at MD. MD, as a chemosensor, detects the changes in NaCl concentration mostly via apical NaCl transport mechanisms, which is mainly through NKCC2 [19, 20], and elicits constriction of the afferent arterioles. The exact mechanism of the vasoconstriction elicited by TGF is not clear, yet the development of gene manipulation technique has shed insight. It is suggested that transcellular NaCl transport induces the

generation of adenosine, which plays an essential role on afferent arteriolar constriction [21]. The constriction of afferent arterioles therefore decreases the glomerular capillary pressure, restores the blood flow and decreases the single nephron GFR.

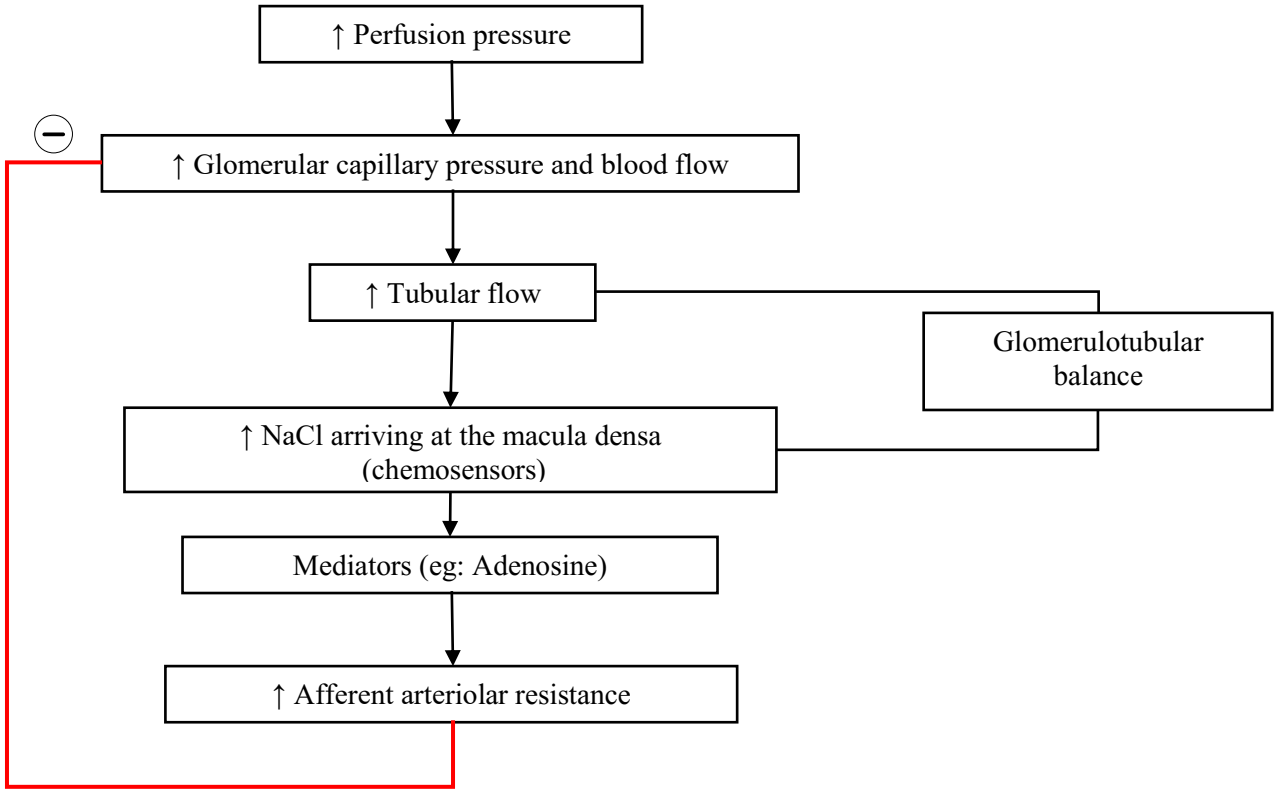

Figure 1.1 TGF in response to increased perfusion pressure.

Other than TGF, glomerulotubular balance also contributes to the stabilization of distal delivery. Glomerulotubular balance refers to a constant fractional proximal tubular reabsorption in response to the delivery fluctuation [22]. This means that more tubular reabsorption occurs when there is increased tubular flow. In case of increased filtration, plasma leaving the glomeruli has a higher oncotic pressure, which favors the tubular reabsorption. The increased filtration of glucose and amino acids due to an increased GFR also results in increased sodium reabsorption coupled with glucose and amino acid in the early proximal tubule [23, 24]. Finally, increased GFR would increase delivery to distal segments and subsequently increase transport in distal segments [22]. In sum, kidneys have intrinsic mechanisms

to maintain stable RBF and GFR despite fluctuation of RPP. The question is whether autoregulation might be impaired by RVP elevation.

1.2.3 Sodium excretion

Maintaining balance between sodium intake and excretion is key to body fluid homeostasis. The kidney plays a critical role in maintaining sodium and water balance. Regulation of tubular sodium reabsorption is important in determining the urinary sodium excretion. The sodium is transported across the tubular epithelial wall into the renal interstitial fluid and then across the peritubular capillary membrane back into the blood. Different segments of the nephron have different capacity in sodium reabsorption. Experiments in rabbits and rats have shown about 65% of the sodium is reabsorbed from the proximal tubular lumen together with potassium, chloride, bicarbonate, glucose and amino acids [25, 26]. About 25% of sodium reabsorption takes place in the loop of Henle, specifically in the thick ascending loop of Henle, mainly by the Na-K-Cl co-transporter (NKCC2) [27], which is the target of loop diuretics, as well as Na-H exchanger (NHE3) [28, 29]. About 5% -10% of sodium reabsorption occurs in the early distal tubule through the Na-Cl co-transporter (NCC), inhibited by thiazide diuretics [30], and in the late distal tubule, where both NCC and amiloride-sensitive epithelial sodium channels (ENaCs) are involved in sodium reabsorption [31]. A small portion of the sodium (<5%) is reabsorbed from medullary collecting ducts [32]. Na^+-K^+-ATPase at the basolateral cell membrane is essential to the active transport throughout the nephron [33].

Sodium reabsorption in the proximal tubule is modulated by angiotensin II (ANG II) [34], adenosine [35] and the sympathetic nervous system (SNS) [36, 37], among others. In the loop of Henle, main modulators are ANG II, SNS and prostaglandins [38-40]. In the distal and cortical collecting ducts, reabsorption is modulated by aldosterone [41] and atrial natriuretic peptide [42]. Water permeability in collecting ducts is modulated by vasopressin [43].Different from the transport across the tubular epithelial membrane, sodium uptake across the peritubular capillary wall from the interstitium into the

peritubular capillaries, is purely driven by the Starling forces, as referred to as the 'physical factors' (Eq. 5).

$$Reabsorption = K_f \times [(P_c - P_i) - \sigma(\pi_c - \pi_i)] \qquad (Eq. 5)$$

(K_f: filtration constant, P_c: capillary hydrostatic pressure, P_i: interstitial hydrostatic pressure, σ: reflection coefficient; π_c: capillary oncotic pressure; π_i: interstitial oncotic pressure.)

The increase of RVP increases the renal interstitial pressure, which has been shown to decrease sodium reabsorption in volume depletion, but to increase sodium reabsorption in volume expansion animals [44]. Normally, the active transport across tubular epithelium instead of the physical factors across the peritubular capillaries is considered the principal determinant in tubular sodium reabsorption [45]. However, the increase in RVP might become important in modulating the active tubular transport[45]. This indicates that the physical factors affected by increased RVP might not be directly responsible for the changes in sodium reabsorption, yet they can induce changes in active transport in tubular epithelium. The question here is, where, what and how epithelial sodium transporters are modified by increased RVP.

1.2.4 Neuro-hormonal factors

Multiple neuro-hormonal factors are involved in the regulation of kidney functions. The following section aims to describe three of the most important modulators, a brief introduction of their pathways and their impacts on regulation of renal hemodynamics as well as tubular function.

Renal sympathetic nerve activity (RSNA)

RSNA plays an important role in regulating kidney function. Anatomically, kidney vasculature, tubules and juxtaglomerular cells are well innervated. Norepinephrine released from the renal sympathetic nerve terminals stimulates predominantly α_1-adrenoceptors on the renal vasculature. Other cofactors such as ATP [46] and neuropeptide Y [47] also act on the renal vasculature to cause

vasoconstriction. Regarding neural control in kidney function, it has been shown that mild activation of RSNA has little impact on RBF and GFR under physiological status [47, 48]. A good example is that after kidney transplantation, recipients were able to modulate fluid and sodium hemostasis similarly as donors [49]. However, the possibility of re-innervation of the transplanted kidney could not be ruled out [50].

Abundant evidence supports neural modulation of kidney function. One is that the transection of the renal nerves increases RBF and urinary flow rate [48]. Conversely, an increase of efferent RSNA, either by direct or reflex stimulation of renal sympathetic nerves, induces immediate reduction in RBF and RVC [51]. This indicates the direct neural control of renal hemodynamics.

Neurotransmitters released by the renal sympathetic nerve induce immediate vasoconstriction. It has been shown in rats that moderate electrical stimulation (3Hz) of the renal nerves causes about 40% vasoconstriction in afferent and 30% vasoconstriction in efferent arterioles [52]. The predominant preglomerular vasoconstriction results in decreased the glomerular hydrostatic capillary pressure and decreased GFR [53]. RSNA also directly alters tubular sodium reabsorption. In experimental animal models, renal α-adrenoceptor blockade or renal denervation increases urinary sodium excretion in the absence of hemodynamic changes [54, 55]. A low frequency of RSNA stimulation was shown to increase kidney renin release and decrease sodium excretion despite having no impact on the RBF or GFR [56]. Taken together, stimulation of RSNA results in the regulation of renal hemodynamic changes, sodium handling and renin release. However, it is unclear how it is involved when RVP is increased.

Renin-angiotensin system (RAS)

The RAS directly modulates renal hemodynamics. As a powerful vasoconstrictor, ANG II constricts both the afferent and efferent arterioles via binding to ANG II type 1 receptors, reduces RBF. Direct

infusion of ANG II into the renal artery causes a dose-dependent vasoconstriction in both afferent arterioles and efferent arterioles. However, the efferent arterioles resistance increased more due to the smaller resting luminal diameters [57]. The differential vasoconstriction in afferent and efferent arterioles changes glomerular capillary hydrostatic pressure, thereby modulating GFR. Furthermore, there are studies showing that ANG II causes contraction of mesangial cells *in vitro* [58, 59]. However, the observation of mesangial cells contraction in response to ANG II has not been shown *in vivo*. That said, ANG II might affect the glomerular permeability coefficient [60]. Overall, the RAS regulates the RBF and GFR by vasoconstrictor effects and modulating glomerular capillary hydrostatic pressure and can affect the glomerular ultrafiltration barrier.

ANG II can act on tubules by affecting many different pathways. Other than influence on physical factors, ANG II can increase expression of the protein subunits of the Na^+-K^+-ATPase complex and increase Na^+-K^+-ATPase activity at the basolateral membrane leading to increase sodium transport across the basolateral proximal tubular cell membrane [61]. ANG II perfusion also induces an increase protein expression of NHE3 in the proximal tubule [62], NKCC2 in the cortical thick ascending limp of the loop of Henle [63] and ENaC in the collecting duct [64]. Furthermore, ANG II stimulates the secretion of aldosterone. Similar to ANG II, aldosterone enhances sodium reabsorption primarily in late distal tubules and collecting ducts. There are studies showing that aldosterone stimulation induces increase of serum and glucocorticoid regulated kinase 1 (SGK1) [65], which plays a key role in regulation of sodium transport in both proximal and distal tubule as well as the cortical collecting duct [66, 67], via stimulating the ENaC and NHE3 activity [67]. Taken together, the RAS results in vasoconstriction and retention of water and sodium. On the other hand, it contributes to stabilizing RBF and GFR. The question here is whether and how ANG II is induced by increased RVP.

Nitric oxide (NO)

Endothelial-derived nitric oxide (NO) is important in vasodilation of the kidney. The kidney has an abundant amount of NO synthase (NOS) which is essential in NO bioavailability. There are three isoforms of NOS present in the kidney: endothelial NOS (eNOS) found in the endothelium, inducible NOS (iNOS) which presents in the interstitial cells and expresses in response to stimuli like oxidative stress [68] and inflammation, and neuronal NOS (nNOS), expressed in MD cells and modulating TGF [69]. NO released by endothelial eNOS increases the production of cGMP by guanylate cyclase and causes relaxation of vascular smooth muscle cells [70-73]. NO is important in regulation of renal hemodynamics. Conversely, administration of L-NAME, which is a non-selective NOS inhibitor, decreases GFR, RBF and RVC [74, 75]. It is suggested in *in vitro* study that the NO contribution to renal hemodynamics is more profound in the afferent than the efferent arteriole [76]. Inhibiting NO synthesis causes a 40% reduction in diameters of the isolated afferent arteriole from rabbits but not the efferent arteriole [76]. However, NOS inhibitors have been shown to increase filtration fraction in *in vivo* study in rats [77]. This suggests that the efferent arteriole is more affected than the afferent arteriole [78], therefore the glomerular capillary hydrostatic pressure and thus filtration fraction increase. Furthermore, NO can inhibit tubular sodium reabsorption by inhibition of sodium transporters [79] and Na^+-K^+-ATPase [80]. The overall effect of NO is to promote natriuresis and diuresis [81]. NO has been shown to blunt RBF autoregulation in dogs [82] and rats [77, 83, 84]. Both nonselective [77, 85] and nNOS selective inhibition [86] have been shown to enhance the TGF responses. Lastly, NO has been shown to induce renin release in response to decreased perfusion pressure in conscious dogs [87]. Activation of ANG II type 1 receptors results in production of NO [88]. Despite all this information, it is unclear how NO modulates the kidney function when RVP is elevated. Moreover, the interaction between NO and ANG II makes it more complex to interpret the role of NO.

1.3 Physiology:

1.3.1 Increased RVP and Hemodynamics

It has been over a hundred years since the first study about hemodynamic impact of increased RVP was published [89]. Yet evidence is still conflicting. This is possibly due to different experimental settings, different species used, different levels of the RVP elevation and the complexity of the problem itself. As a result, the impact of increased RVP on renal hemodynamics is still poorly understood. This section is a short literature review on how RVP could affect renal function.

Back in 1949, Dr. Selkurt showed that RBF decreased upon RVP elevation by using a bubble flow meter connected between the carotid and renal artery [90]. However, this method would cause appreciable loss of perfusion pressure, which might reduce the RBF by itself. By using the electromagnetic flow probe around the renal artery, Kastner PR *et al* reported that in dogs, RBF was not reduced until RVP was increased to 50 mmHg [91]. The inconsistency of the RBF response to increased RVP can be partly due to technique limitation in some early studies. Increased RVP decreases the RPP which is responsible for decreased RBF since the arteriovenous pressure gradient allows the generation of flow (Eq.2).

There is also inconsistency regarding the RVC changes in response to increased RVP. Abildgaard showed that RVC remained unchanged when RVP was elevated to 30 mmHg but decreased when RVP was increased to 60 mmHg in dogs [92]. Others reported an increased RVC upon increased RVP (21-75 mmHg) in both intact and isolated perfused kidneys in dogs [93]. They tried to explain that the increase of total RVC came from decreased resistance of the venous segment where intrarenal pressure acted as the 'pressure buffer' to alleviate the impact of increased RVP [93]. However, since the renal vascular resistance is mainly determined by the resistance of afferent and efferent arterioles, the contribution of venous segment is unimportant. One of the possible reasons for the inconsistency of RVC changes might be the activation of autoregulation. As an autoregulation action, the decreased

vascular resistance could happen at pre-glomerular sites to maintain stable GFR [14]. This is supported by a study, in which arterial pressure was increased to the same degree as RVP in the oil perfused kidney. In this study, the RVC remained unchanged [94]. It has also been suggested that factors other than physical forces modulate the changes in RVC [95]. Early in 1956, Dr. Haddy *et al* suggested that the increase of RVP induced vasoconstriction partially via a nervous reflex, 'venous-arterial reflex' and some unrecognized factors [96]. All of these observations suggest factors other than physical forces could be responsible for the hemodynamic changes.

It is similarly complicated how increased RVP could affect GFR. Increased RVP per se would not decrease glomerular capillary hydrostatic pressure. Increased RVP was shown to be positively correlated to increased renal interstitial pressure [44, 94]. In 1983, James R Dilley *et al* showed that increased RVP to 22 mmHg in rats increased glomerular capillary and Bowman's space pressure similarly [97], therefore it did not change the hydrostatic pressure gradient in the glomerulus. There is also study showing the increased RVP impaired GFR due to decreased ultrafiltration coefficient [97]. However, the ultrafiltration coefficient measurement might not be reliable at low RBF because some capillaries are no longer perfused [98, 99]. Furthermore, neurohormonal factors activated by increased RVP could modulate the renal hemodynamics as well, which will be explained in later sections. In conclusion, increased RVP modulates renal hemodynamic changes via alterations in ultrafiltration driving forces. In addition, subsequent neurohomornal factors contribute to the hemodynamic adaptations. The inconsistency in kidney response to the increased RVP might be due to the complex interaction of multiple mediators.

1.3.2 Increased RVP and sodium reabsorption

Data are conflicting in terms of how increased RVP impacts sodium excretion. Published data have shown both that increased RVP caused sodium retention [100] and conversely induced sodium

excretion [101]. In the following section, possible mechanisms, though incompletely studied, will be discussed.

To influence tubular sodium reabsorption, modulation must take place either at the trans-tubular or trans-capillary site. Sodium transporters are considered the main determinants for sodium transport across tubules while physical (Starling) forces determine sodium and fluid fluxes between the capillary lumen and interstitium. So far, there is no study about how increased RVP can modulate sodium transporters. At the trans-capillary site, increased RVP correspondingly increases peritubular capillary pressure [102]. However, it is unlikely an important determinant since interstitial hydrostatic pressure also increases. The impact on glomerular filtration of increased RVP should not be overlooked. Although no specific level of RVP was indicated in the report, high RVP was associated with low or nearly absent fluid movement in tubules observed using micropuncture technique [102]. In sum, at the present time it is unknown how increased RVP might affect (proximal) tubular sodium transport. Furthermore, it seems that factors other than physical forces such as peritubular capillary pressure, the neurohormonal factors induced by increased RVP are determinant for the modulation of sodium reabsorption.

1.3.3 Increased RVP and RSNA

Some studies indicate that an increase in RVP can cause distension of intrarenal veins, which could stimulate mechanoreceptors, and thereby activate RSNA [103, 92]. It has been suggested that the reduction of RBF and GFR induced by increased RVP could be attributed to the activation of RSNA since in renal denervated animals, the reduction in GFR was prevented [92] as well as the vasoconstriction [103]. However, data are conflicting. Kopp has shown that increased RVP enhanced afferent renal nerve activity but decreased efferent renal nerve activity [104]. The enhanced afferent renal nerve activity has been shown to inhibit cardiopulmonary sympathetic efferent nerve activity and decrease ventricular contractility [103]. This is important in terms of understanding how increased

RVP could impact on remote organs such as the heart. Increased RVP not only increased ipsilateral RVR and urinary sodium excretion, but also increased contralateral urine flow and sodium excretion, presumably due to the reno-renal reflex, since the contralateral kidney responses were abolished by ipsilateral denervation [105]. From the above, we can see that although increased RVP enhanced the afferent sympathetic nerve activity, efferent sympathetic activity, while modulating renal hemodynamic and functional changes, might not be the primary mediator.

1.3.4 Increased RVP and RAS

It has been postulated that an increase in RVP leads to activation of the RAS [91, 106]. In 1982, Kastner *et al* showed in dogs that renin levels were profoundly increased due to RVP elevation [91]. Increased RVP would decrease NaCl delivery to the MD and induce the release of renin. In their study, inhibition of ANG II did not decrease MAP. However, increased ANG II level could increase filtration fraction [57], which prevented the decrease of GFR when RVP was elevated. That is why in the same study, GFR decreased to a major degree in response to RVP elevation after ACEi [91]. However, the activation of the RAS may lead to adverse outcomes such as increased vascular resistance. ANG II directly activates sodium transporters and consequently sodium reabsorption in both proximal tubules [107] and distal tubules [108]. Inhibition of ANG II might decrease arterial pressure which decreases GFR. Taken together, ANG II maintains the stability of GFR. However, overactivation of the RAS could result in sodium and water retention. It is still inconclusive whether inhibition of the RAS would benefit the kidney function in RVP elevation.

1.3.5 Increased RVP and NO

NO is essential in modulating RVC as well as renal function. There are no direct data about NO bioactivity in response to elevation of RVP. However, intrarenal NO activity and RPP are positively correlated [109]. Increased RVP results in a decrease of RPP, which could oppose the synthesis of NO by endothelial NOS [110]. An in vitro study has shown that NO release was inhibited when ambient

pressure was increased [111]. On the other hand, the decreased MD delivery upon RVP elevation could result in subsequent increased NO release by nNOS in MD cells [110], which might cause renin release resulting in vasoconstriction and sodium retention. In sum, the integrated effect of endothelial NO deficiency as well as increased MD NO release might contribute to the renal dysfunction in RVP elevation.

1.4 Pathophysiology

Increased RVP could either be due to increased intravascular pressure or extravascular factors (Figure 1.2). Increased intravascular pressure is most likely due to congestion in conditions such as CHF, CKD, and end-stage liver disease with ascites. In ascites, the fluid accumulation in the abdominal cavity leads to the increased abdominal pressure. Increased abdominal pressure in abdominal compartment syndrome is also a common cause of increases RVP. The obstruction of renal venous flow seen in renal vein thrombosis is an important intravascular factor. This section describes 1) the pathophysiology of several clinical conditions accompanied with increased RVP; 2) what we currently know about how increased RVP in these diseases affects kidney function.

1.4.1 Congestive heart failure (CHF)

CHF is characterized by reduced cardiac function and is accompanied by fluid volume overload. The impact of increased RVP on kidney function is important with respect to fluid volume regulation. There is only one study documenting the RVP in chronic CHF patients. In this study, pressures in the venous system were accessed by cannulation of the right renal vein, inferior vena cava, right atrium and peripheral veins in 17 patients with normal heart and kidney functions and 10 patients with chronic CHF. It showed that not only RVP, but pressure in all of the measured venous system were higher in chronic CHF patients [4]. Although there is rarely direct measurement of an increased RVP documented with CHF, the increased central venous pressure, which would translate into an increased RVP, is well documented [11, 5, 112, 113]. Furthermore, venous congestion, as estimated by the

central venous pressure, is an independent risk factor for impaired kidney function during episodes of acute heart failure [11].

If increased RVP is the cause of impaired kidney function, are there clinical and bench studies showing decongestion improves kidney function? The National Heart, Lung, and Blood Institute Heart Failure Clinical Research Network conducted the Diuretic Optimization Strategies Evaluation (DOSE) trial to look for better management of decongestion. In this trial, there was no significant improvement in kidney function upon higher doses of furosemide [114]. However, furosemide itself is a diuretic that influences kidney function in a complex way [115]. Furthermore, most patients in the trial had received moderate-to-high dose of diuretics as previous treatment. Another decongestion treatment is ultrafiltration. It is the mechanical removal of iso-osmotic fluid volume from the blood. The benefit of ultrafiltration in CHF is not conclusive. A recent trial showed increased serum creatinine and blood urea levels 72 hours after initiation of ultrafiltration (Cardiorenal Rescue Study in Acute Decompensated Heart Failure, CARRESS-HF, trial: [116]). However, the Ultrafiltration versus Intravenous Diuretics for Patients Hospitalized for Acute Decompensated Congestive Heart Failure (UNLOAD) trial, despite not showing differences in creatinine level, had significant lower rehospitalization rates in patients receiving ultrafiltration than receiving continuous diuretic infusion [117]. Furthermore, the effects of volume and cardiac function in these trials would complicate the interpretation of outcomes. In sum, aggressive volume removal in ultrafiltration without proportionate fluid refill from the interstitial space might result in a drop in arterial blood volume which could cause impaired kidney function in these ultrafiltration trials. This could indicate that 1) the study settings are not appropriate to evaluate the importance of increased RVP in kidney functions; 2) decongestion management we currently apply is not ideal and other optimized strategies are needed.

1.4.2 Chronic kidney disease (CKD)

In CKD, impaired sodium and water excretion results in extracellular fluid volume expansion [118]. Fluid overload becomes most evident at the advanced stage of kidney failure and in patients needing hemodialysis [119]. One cross-sectional study using bio-impedance to estimate volume status indicates that fluid overload is highly prevalent in hemodialysis population [119]. According to the inferior vena cava diameter measurement, which is another method to estimate volume status [120], there is clearly venous congestion in CKD [3]. However, it is not entirely clear how increased RVP contributes to CKD progression. Studies are needed to evaluate the correlation between renal venous pressure (venous congestion) and disease prognosis.

1.4.3 Ascites in advanced liver failure

In advanced liver failure, impaired hepatic synthesis of albumin leads to hypoalbuminemia, a shift of fluid into the intercellular space and often to ascites. Although there is no direct data to support that RVP is increased in ascites, the measurement of abdominal pressure provides a correlation with impaired kidney function and severity of ascites [121]. Decompression with paracentesis has been used widely in clinics to treat the refractory ascites [122]. There is one study showing that the large-volume paracentesis results in worsening kidney function despite removal of the ascitic fluid [123]. It might be due to the massive reduction in effective circulatory fluid volume and failure to refill from the intracellular space. A low-volume and continuous drainage of the ascites could be considered as a better strategy. Taken together, there are not enough studies to determine the optimal decompression strategy for the improvement of kidney function in states with ascites.

1.4.4 Abdominal compartment syndrome

Increased abdominal pressure can affect multiple organ functions and lead to abdominal compartment syndrome, which greatly increases mortality [124, 125]. Kidney function is especially vulnerable in abdominal compartment syndrome. Decompressive laparotomy is considered a potential treatment for

abdominal compartment syndrome. One meta-analysis and systematic review collected data from 286 cases shows that the decompression is linked to a decrease in intra-abdominal pressure and improvement in kidney function [126]. However, the direct assessment of RVP in this context is lacking. Furthermore, although the decompression improves kidney parameters, it does not decrease the mortality.

1.4.5 Renal vein thrombosis

Renal vein thrombosis can be a complication in patients with the nephrotic syndrome [127], In neonates, it is a potentially fatal disease [128, 129]. The obstruction of renal venous return would impair kidney function and lead to acute kidney injury. In fact, children with end stage renal failure with a compromised inferior vena cava have increased risk of renal vein thrombosis after transplantation due to the narrowing of renal venous outflow. Transplantation with a venous bypass to the splenic vein released the obstruction, and patients were able to maintain a stable kidney function [130]. This is important because it shows that after normalization of RVP, kidney function after transplantation was maintained.

Taken together, increased RVP can contribute to impaired kidney function in multiple disease states. Future studies are needed to elucidate the mechanisms, as well to optimize the management of increased RVP.

1.5 Outline of the thesis

This thesis is about the study of renal responses to increased RVP, the renal autoregulation, as well as the role of the two most powerful mediators, RSNA and RAS (Figure 1.3).

Chapter 1 (this chapter) is a general introduction about the significance of increased RVP, how increased RVP impacts kidney functions in both physiology and pathophysiology.

Chapter 2 reports on an animal model of isolated increased RVP. In male Lewis rats, the isolated constriction of the left renal vein simulates the increased RVP in the left kidney. It is an appropriate model to study mechanisms of kidney dysfunction without other confounders. In this chapter, we will illustrate the hemodynamic changes in response to the increased RVP both in rats on a normal salt diet and on a high salt diet.

In **Chapter 3**, we will explore one of the underling mechanisms involved in the response to increased RVP, renal nerve traffic. In this chapter, we will respectively use renal denervation and also direct nerve recording to illustrate the role of the renal nerves.

In **Chapter 4**, we will discuss the role of another potential mediator, the RAS, using the same animal model. When ANG II is clamped, the modulation of ANG II is absent, which is used to study the role of modulation of the RAS in response to increased RVP. Autoregulation is an important feature in the renal microcirculation, and is supposed to maintain the stability of kidney function upon RPP fluctuations. Therefore, it is important to study how autoregulation functions when RVP is increased.

Chapter 5 is about two unfinished stories: urinary sodium excretion and tubular pressure. Due to missing data and small sample size, the conclusions from the two stories are not strongly supported by statistics. However, the urinary sodium excretion story is valuable to study the impacts of increased RVP on the sodium handling. Measuring the free flow tubular pressure would be the first step to explore the microcirculation in increased RVP, which provides advantages for future TGF measurement.

Chapter 6 is the overall discussion and conclusion. As the complexity of increased RVP in clinical setting, more studies are needed to identify the culprit and explore better therapeutic targets.

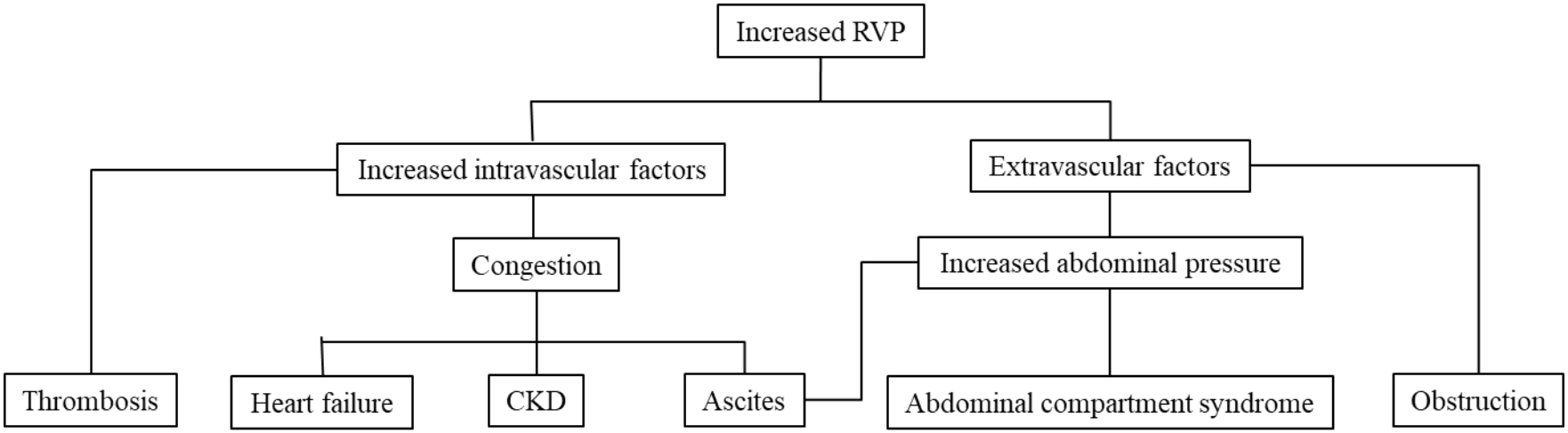

Figure 1.2 Diseases involving an increased RVP

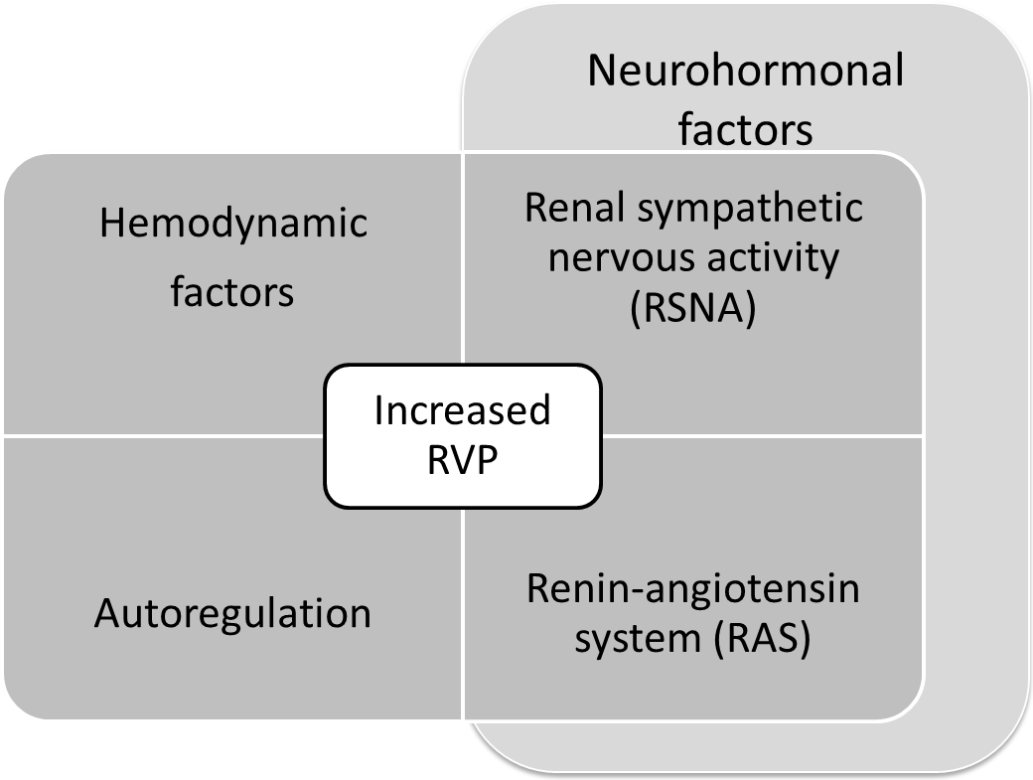

Figure 1.3 Main components of the thesis

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CHAPTER 2:

Sodium intake attenuates renal venous pressure-induced renal hemodynamic changes in rats

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Abstract

Increased central venous pressure and renal venous pressure (RVP) is associated with worsening of renal function in acute exacerbation of congestive heart failure (CHF). The kidney plays an essential role of maintaining the sodium and fluid balance. Kidney dysfunction in CHF would result in sodium and fluid retention, which can exacerbate the congestive state. In healthy individuals, excessive salt intake would lead to increased fluid and salt excretion by the kidney. However, the role of a high salt diet in kidney function has not been well addressed in RVP elevation. We test whether an acute, isolated elevation of RVP in one kidney leads to ipsilateral renal vasoconstriction and decreased GFR and whether this depends on dietary salt intake. Male Lewis rats receive a normal (1% NaCl, NS) or high salt diet (6% NaCl, HS) for ≥14 days before the acute experiment. Rats are then randomized into 3 groups: time control and RVP elevation to either 10 or 20 mmHg to assess heart rate, renal blood flow (RBF) and GFR. To increase RVP, the left renal vein is partially occluded for 120 min. Increasing RVP to 20 mmHg decreases ipsilateral RBF (7.5±0.4 to 4.1±0.7 ml/min, $p<0.001$) and renal vascular conductance (RVC, 0.082±0.006 to 0.060±0.011 ml/min·mmHg, $p<0.05$) and GFR (1.28±0.08 to 0.40±0.13 ml/min, $p<0.05$) in NS rats. The reduction of RVC is abolished by a high salt diet. Since the renin and aldosterone levels are significantly lower in HS rats, these suggests the suppression of the RAS in HS rats. Taken together, acute elevated RVP induces renal vasoconstriction and decreased GFR, which is likely mediated via the RAS.

2.1 Introduction

The complex syndrome of combined cardiac and renal failure comes with high mortality [1]. Failure of one organ can initiate or aggravate failure of the other [2, 3]. Mechanisms governing this reciprocal interaction are ill-defined, which constrains mechanism-based treatment [2]. One proposed mechanism is that increased central venous pressure leads to increased renal venous pressure (RVP) [4, 5], which, in turn, could impair renal function. This association between increased central venous pressure and worsening renal function in the context of acute heart failure is well established [6, 4, 5].

However, mechanisms mediating the decrease in renal blood flow (RBF) and glomerular filtration rate (GFR) upon increased RVP remain incompletely understood. A decrease in renal arteriovenous pressure difference (renal perfusion pressure) will decrease RBF. It is unclear whether renal nerves or endo/autocrine factors are also involved. The decrease in GFR upon increased RVP is even more complex: it could involve increased interstitial and tubular pressure [7, 8] as well as neural and endo/autocrine factors. Since the renin-angiotensin system (RAS) is likely involved in the response to RVP increases, the level of salt intake seems to be relevant but has not been studied.

We hypothesized that increased RVP will induce renal vasoconstriction with a concomitant decrease in RBF and GFR. In addition, we hypothesized this to be dependent on activation of the RAS. The observed RVP-induced renal hemodynamic changes would be suppressed in rats given a high salt diet.

2.2 Methods

Animals

Male Lewis rats (300 to 450 g, n=39) (Charles River, St. Constant, QC, Canada) were housed in a temperature and humidity-controlled room with a 12hr:12hr light/dark cycle. All rats received regular rat chow with 1% NaCl *ad libitum* (Canadian Lab Diets, Leduc, AB, Canada) and had free access to tap water.

Rats randomly assigned to a high salt diet received modified chow (Canadian Lab Diets, Leduc, AB, Canada) formulated with 6% NaCl *ad libitum* with free access to tap water for at least 2 weeks before the experiment. Intact rats on regular chow (n=23) served as normal controls for several experimental groups as follows: intact rats receiving high salt diet (n=16). Each of these experimental groups included subgroups of rats in which RVP was left unaltered (time controls) or increased to either 10 or 20mmHg. Experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care and received prior approval by the Animal Care and Use Committee of the University of Alberta.

Preparation

Rats were given buprenorphine (0.02mg/kg, i.m.) 30 minutes before anesthesia. Isoflurane anesthesia was induced in an induction chamber pre-charged with room air. Isoflurane was introduced in 0.5% increments up to 4% in 100% oxygen (1 L/min). Once the rat reached surgical plane of anesthesia, it was placed on a heated surgical table equipped with a thermo-feedback system to maintain rectal temperature between 36-37°C (Vestavia Scientific, Birmingham, AL, USA). Anesthesia was maintained through a nose cone and the isoflurane dose was gradually reduced in 0.5% increments to 2%. Rats were permitted to breathe spontaneously. Hair from the

neck, abdomen and left groin was removed by shaving and the surgical field was cleansed with alternating applications of 10% Povidone iodine and 70% ethanol. Following a midline neck incision to expose the trachea, the rat was intubated via tracheotomy using PE-240 tubing (BD Intramedic, Sparks, MD, USA). The tracheal tube was then fitted to the nosecone and isoflurane dose was adjusted to 1.5-1.75% to maintain surgical plane with the loss of the toe-pinch reflex. The toe-pinch reflex was assessed every 5 minutes to verify surgical plane during the surgery and experimental recording period. The left femoral vein was catheterized (Silastic tubing, 0.51mm ID, 0.94mm OD, Dow Corning, Midland, MI, USA) for intravenous infusion, which was immediately commenced (see below). The left femoral artery was cannulated (PE-50, BD Intramedic, Sparks, MD, USA) for direct recording of systemic arterial pressure and heart rate (HR).

Renal Hemodynamic Experiments

Following midline laparotomy, the left kidney was exposed. The left adrenal vein or supraspermatic vein was cannulated (Micro-Renathane MRE-025, Braintree Scientific, Braintree, MA, USA) and the cannula advanced until the tip rested in the main renal vein for direct measurement of RVP. A length of 3-0 prolene (Johnson-Johnson, San Lorenzo, Puerto Rico) was slipped around the left renal vein at its junction with the inferior vena cava and sheathed with a small piece of PE-50 tubing to create a sling. To increase RVP, the sling was tightened to constrict the renal vein. Pressures were acquired using PowerLab via disposable blood pressure transducers (ADInstruments, CO, USA). A 1RB transit-time flow probe was placed around the left renal artery for direct measurement of RBF (Transonic, Ithaca, NY, USA). The left ureter was catheterized for urine collection (PE-10, BD Intramedic, Sparks, MD, USA). The rat received supplemental fluids during surgical preparation (5% bovine serum albumin, BSA, A7906, Sigma, Oakville, ON,

Canada) with 250 µg/min FITC inulin (Sigma, Oakville, ON, Canada) at 1.5mL/hr. This infusion continued throughout the experiment with 1% BSA with 250 µg/min FITC inulin at 1.5mL/hr.

Experimental design

Following completion of surgical instrumentation, rats were stabilized for 60 minutes. Baseline MAP, HR, RBF and RVP were collected for 60 minutes, after which time RVP was selectively increased to either 10 or 20 mmHg by graded constriction of the left renal vein or not manipulated (time controls). Data collection continued for a further 120 minutes. For hemodynamic experiments, blood samples (200 µl) were obtained at the beginning of the baseline period and every 60 min thereafter. Timed urine samples were collected every 30 minutes.

Analytical methods

To determine GFR using FITC-Inulin, plasma and urine samples were diluted in 0.5 mol/l HEPES (pH 7.4) to maintain physiological pH. A 96-well black plate (Greiner, Monroe, NC, USA) was used for loading 50µl of each solution in duplicate. Fluorescence was determined using the Fluoroskan Ascent® Microplate Fluorometer (Thermo Fisher Scientific, Vantaa, Finland), at the excitation wavelength of 485nm and emission wavelength of 527nm. Terminal blood samples were obtained from the femoral catheter to measure plasma renin level by ELISA (NOVATEINBIO, Cambridge, MA, USA).

Analysis and statistics

Data are presented as the average of consecutive 30 min intervals. The baseline characterization was compared between both rats on the normal and high salt diet using General linear model multivariate (MANOVA) with Bonferroni post-hoc test. To evaluate the impact of elevated RVP,

Multiple linear model with repeated measurement was used to compare each time point of three groups in animals on both diets, using Bonferroni as post-hoc test. Plasma renin and aldosterone level were analyzed with 2-way ANOVA with Student Newman Keuls post-hoc test. Data were log-transformed or ranked if not normally distributed. Data were analyzed using SPSS 24 (IBM, Armonk, NY, US) and SigmaPlot 13 (Systat, San Jose, CA, USA). A p value less than 0.05 was considered significant. All data are presented as means ± SEM.

2.3 Results

Characterization of experimental groups

Average body weight of rats on high salt diet (HS Intact, n=16) was higher ($p<0.05$) than rats on normal salt diet (NS Intact, n=23, Table 2.1). The baseline heart rate was similar in the two diet groups. The high salt diet did not influence baseline mean arterial pressure (MAP) or RBF. HS rats had higher baseline RVP than NS rats ($p<0.001$). GFR was higher in intact HS rats than in NS rats (Table 2.1).

Increased RVP and renal hemodynamics

Increased RVP did not affect MAP, regardless of diet and presence or absence of renal nerves (Figure 2.1; all data are represented in Table 2.2). HR was decreased upon a major RVP elevation (Figure 2.2 A). A moderate increase of RVP (0.3±0.2 mmHg to 12.5±0.8 mmHg) induced a significant reduction in RBF to 73±5% ($p<0.05$, Figure 2.3 A). It did not affect RVC (Figure 2.4 A) or GFR (Figure 2.5 A). When RVP was elevated to a higher level (0.6±0.3 mmHg to 19.6±0.5 mmHg), RBF and RVC were reduced to 55±8% ($p<0.001$, Figure 2.3 A) and 75±12% ($p<0.05$, Figure 2.4 A) of baseline, respectively. GFR significantly decreased to 31±11% of baseline ($p<0.05$, Figure 2.5 A).

All these hemodynamic responses were either abolished or ameliorated by HS diet. Both moderate (from 1.4±0.3 to 10.8±0.4 mmHg) and major increases of RVP (from 1.6±0.4 to 19.3±0.7 mmHg) decreased RBF ($p<0.05$, Figure 2.3 B), in contrast to NS rats, RVC did not decrease in response to RVP elevation in HS animals (Figure 2.4 B). GFR appeared to decrease slightly when RVP was increased although the reduction was not statistically significant (Figure 2.5 B).

Increased RVP, plasma renin and aldosterone levels

Overall plasma renin levels in NS animals were higher ($p<0.001$) than HS rats at the end of the experiments (Table 2.3). RVP level did not affect plasma renin except in intact HS rats with moderate RVP, where the renin level was significantly lower than HS rats without increased RVP ($p<0.05$). Plasma aldosterone was lower in HS rats compared to NS rats ($p<0.001$).

2.4 Discussion

In the present study, we demonstrate that selectively increasing RVP reduced RBF and RVC in rats on the NS diet. This increased renal vascular tone in response to increased RVP was not observed in animals on the HS diet. In rats on the NS diet, high RVP resulted in an immediate and sustained decrease in GFR. In rats on the HS diet, GFR changes induced by high RVP occurred gradually and were non-significant. In sum, increasing RVP to 20 mmHg decreased RVC and GFR, a response that was dependent upon dietary sodium intake. Since the HS diet suppressed the RAS (Table 2.3), these results support the possibility of an active role of the RAS in controlling RVP-induced renal vasoconstriction.

Reports about RBF and GFR changes in response to increases in RVP are conflicting. In swine on a normal salt diet, RBF and GFR were reduced when RVP was increased to 30 mmHg [9]. In dogs,

graded elevation of RVP to ~20 mmHg decreased RBF, GFR and RVC [10]. Yet, another experiment in dogs showed stepwise increases in RVP up to 50 mmHg did not affect RBF and GFR, but increased RVC [11]. A potential explanation for the stability of RBF and GFR in the latter experiments is that RVP elevation triggered a correction by renal autoregulation [11]. In our study, increasing RVP to 10 mmHg in rats on the NS diet gradually decreased RBF but not RVC. A 20 mmHg increase in RVP resulted in a rapid, major decrease in RBF and RVC. This points towards other mechanisms besides the decrease in renal perfusion pressure, since a 20% decrease in renal perfusion pressure was accompanied by a ~50% decrease in RBF. The observation that a major increase (~20 mmHg) but not a moderate increase (~10 mmHg) of RVP decreased GFR suggests that compensatory mechanisms can stabilize GFR up to a certain threshold of RVP elevation.

Salt intake is relevant, since even increases in RVP up to 20 mmHg in HS animals did not affect RVC. These findings are novel, and we are unaware of previous studies about salt intake and the renal response to increased RVP. In our study, baseline RVP was significantly higher in HS rats, in line with a higher volume status. Information obtained in dogs without and with *acute* isotonic volume expansion via saline injection is opposite: a graded increase of RVP to 40 mmHg decreased RBF and GFR only in volume expanded dogs [7]. This was attributed to a more pronounced increase of renal interstitial pressure in response to RVP elevation in dogs with acute volume expansion. However, acute volume expansion is hardly comparable to chronic volume expansion by high dietary salt. The observation that a decrease in the arteriovenous pressure gradient in rats on a HS diet was not accompanied by decrease in RVC suggests that the compensatory mechanisms are related to the RAS. One possibility is that decreased activity of the RAS could be

partially responsible, as renin and aldosterone levels were significantly lower in rats on HS diet in our study.

Taken together, the present study shows that RVP elevation decreased RBF, RVC and GFR in animals on the NS diet. The HS diet attenuated the RBF reduction and abrogated the RVC reduction in response to increased RVP. Although the HS diet did not prevent the decrease of GFR, the reduction was attenuated. The differences in response to increased RVP between animals on NS versus HS diets implicates that physical reduction of the arteriovenous pressure gradient is not determinant, but other hormonal factors are involved. The remission of hemodynamic responses to increased RVP with HS diet suggested a role of the RAS. However, we are aware of that HS diet suppresses but does not abolish RAS activation, as well as that HS diet may affect other intrarenal mediators such as prostaglandins [12] and NO biosynthesis [13], or vasoconstrictor agents such as endothelin [14]. Therefore, given the complexity induced by the HS diet, this observation must be tested directly by targeted inhibition of the RAS in the further study.

Perspectives

The data are compatible with the situation of an increased RVP due to venous congestion such as combined cardiac and renal failure, where the increased RVP contributes to compromise of kidney function. Furthermore, in patients with chronic kidney disease, tubulo-interstitial damage could enhance the renal response to increases in RVP. Moreover, the decrease in HR in this respect suggested potential systemic influence of increased RVP which could further aggravate cardio-renal failure. Although multiple mechanisms might be involved, our data suggest an essential role of the RAS. The RAS over-activation in congestive heart failure could also exacerbate the hemodynamic effect of increased RVP, giving rise to marked cardiovascular and renal

dysfunction. Studies delineating the role of the RAS by inhibition of ACE and systemic infusion of ANG II to study this key mediator are needed.

Table 2.1 Baseline group characteristics of male Lewis rats maintained on a normal salt and high salt diet.

Baseline	Normal salt diet	High salt diet
n	23	16
BW (g)	363±9	382±9 *
HR (beats/min)	366±5	364±4
MAP (mmHg)	97.0±1.6	97.7±2.1
RVP (mmHg)	0.4±0.2	1.5±0.2 *
RBF (ml/min)	7.2±0.4	7.9±0.5
RVC (ml/min·mmHg)	0.076±0.005	0.082±0.006
GFR (ml/min)	1.39±0.08	1.67±0.14 *

n: number, BW: body weight, HCT: hematocrit, HR: heart rate, MAP: main arterial pressure, RVP: renal venous pressure, RBF: renal blood flow, RVC: renal vascular conductance, GFR: glomerular filtration rate. The baseline characterization is compared between both rats on the normal and high salt diet using general linear model multivariate with Bonferroni post-hoc test. * $p<0.05$ compared to normal salt diet, ** $p<0.001$ compared to normal salt diet, # $p<0.05$ compared to high salt diet, ## $p<0.001$ compared to high salt diet.

Table 2.2 All group characteristics of both baseline and endpoint from male Lewis rats maintained on a normal salt and high salt diet.

NORMAL SALT DIET						
	Control (n=9)		RVP 10 (n=6)		RVP 20 (n=8)	
	Baseline	End	Baseline	End	Baseline	End
HCT (%)	42.3±1.0	41.0±1.1	44.3±1.0	44.4±1.1	43.5±1.2	42.2±1.5
HR (Beats/min)	370±8	363±5	357±12	348±14	368±8	344±11*
MAP (mmHg)	100.2±2.2	91.4±2.3	96.1±3.0	90.2±4.6	94.0±2.7	88.1±3.0
RVP (mmHg)	0.5±0.4	0.3±0.4	0.3±0.2	12.5±0.8**	0.6±0.3	19.6±0.5**
RBF (ml/min)	6.8±1.0	6.2±0.8	7.4±0.7	5.5±0.8*	7.5±0.4	4.1±0.7**
RVC (ml/min·mmHg)	0.069 ±0.010	0.069 ±0.008	0.078 ±0.008	0.070 ±0.007	0.082 ±0.006	0.060 ±0.011*
GFR (ml/min)	1.55±0.08	1.53±0.10	1.21±0.19	1.01±0.21	1.28±0.08	0.40±0.13**
HIGH SALT DIET						
	Control (n=5)		RVP 10 (n=5)		RVP 20 (n=6)	
	Baseline	End	Baseline	End	Baseline	End
HCT (%)	44.4±0.7	43.4±0.8	45.5±0.6	45.7±0.6	44.7±0.8	43.6±1.1
HR (Beats/min)	362±8	352±7	364±8	341±11	365±7	348±10
MAP (mmHg)	96.5±4.5	90.3±3.3	97.5±3.5	90.4±2.5	98.8±3.5	93.6±2.8
RVP (mmHg)	1.4±0.3	1.2±0.3	1.4±0.3	10.8±0.4**	1.6±0.4	19.3±0.7**
RBF (ml/min)	7.7±0.3	8.1±0.5	9.3±1.3	8.5±0.9*	6.8±0.8	5.6±0.6*
RVC (ml/min·mmHg)	0.082 ±0.005	0.091 ±0.006	0.096 ±0.010	0.106 ±0.009	0.072 ±0.010	0.078 ±0.012
GFR (ml/min)	1.58±0.06	1.51±0.14	1.55±0.18	1.45±0.14	1.73±0.06	0.86±0.12**

Multiple linear model with repeated measurement was used to compare each time point of three RVP subgroups (control, RVP 10 and RVP 20) in animals on both normal and high salt diets, using Bonferroni as post-hoc test. * $p<0.05$ compared with the baseline value of the same RVP subgroup on the same diet; ** $p<0.001$ compared with the baseline value of the same RVP subgroup on the same diet.

Table 2.3 Plasma renin and aldosterone level of male Lewis rats maintained on a normal salt (NS) and high salt (HS) diet.

Plasma Renin level (pmol/L)	TC	RVP10	RVP20
NS	197±7 (n=7)	186±26 (n=5)	168±14 (n=7)
HS	112±3 (n=5) $^{\#\#}$	99±2 (n=5) $^{*,\ \#\#}$	112±4 (n=5) $^{\#\#}$
Plasma Aldosterone level (pg/mL)	**TC**	**RVP10**	**RVP20**
NS	89±28	57±14	48±28
HS	17±8$^{\#\#}$	22±4$^{\#\#}$	25±6$^{\#\#}$

Plasma renin and aldosterone levels were analyzed with 2-way ANOVA with Student Newman Keuls post-hoc test. Plasma aldosterone data were log-transformed because they were not normally distributed. * $p<0.05$ compared with control group on the same diet, ** $p<0.001$ compared with control group on the same diet; # $p<0.05$ compared with intact rats on a normal salt diet, ## $p<0.001$ compared with NS rats.

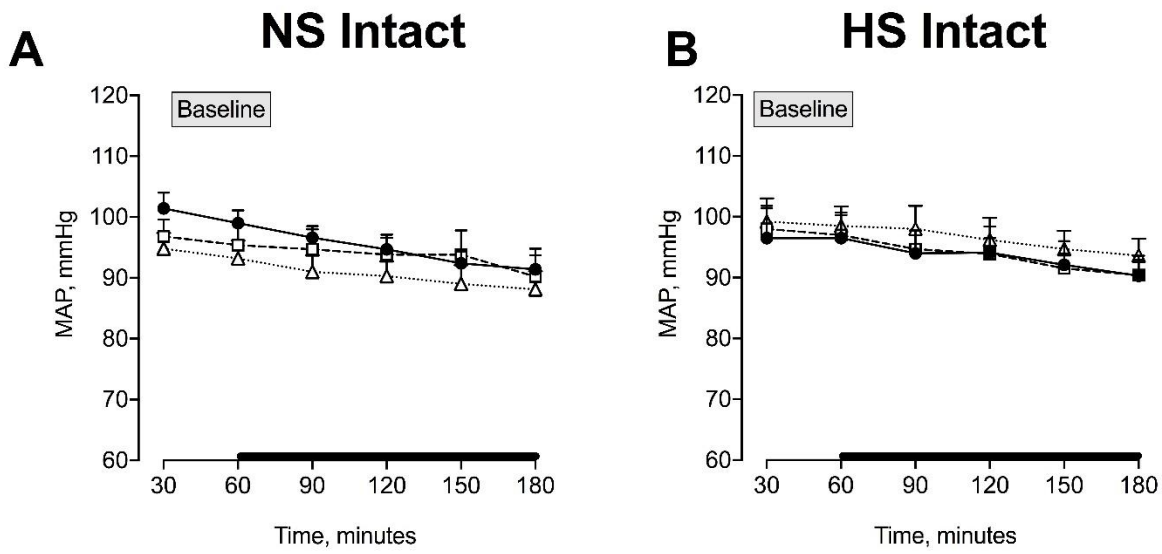

Figure. 2.1 Mean arterial pressure (MAP) in response to increased RVP in male Lewis rats maintained on a normal salt (NS) and high salt (HS) diet. The first two time points of each graph represent baseline. The latter four time points represent either control (●, control) , moderate RVP elevation (□, RVP 10) and major RVP elevation (△, RVP 20). The thick underline in y-axis represents the duration of time when RVP was manipulated. Multiple linear model with repeated measurement was used to compare each time point of three RVP subgroups (control, RVP 10 and RVP 20) in animals on both normal and high salt diets, using Bonferroni as post-hoc test. Increased RVP did not impact MAP in either of the NS (A), HS (B) rats.

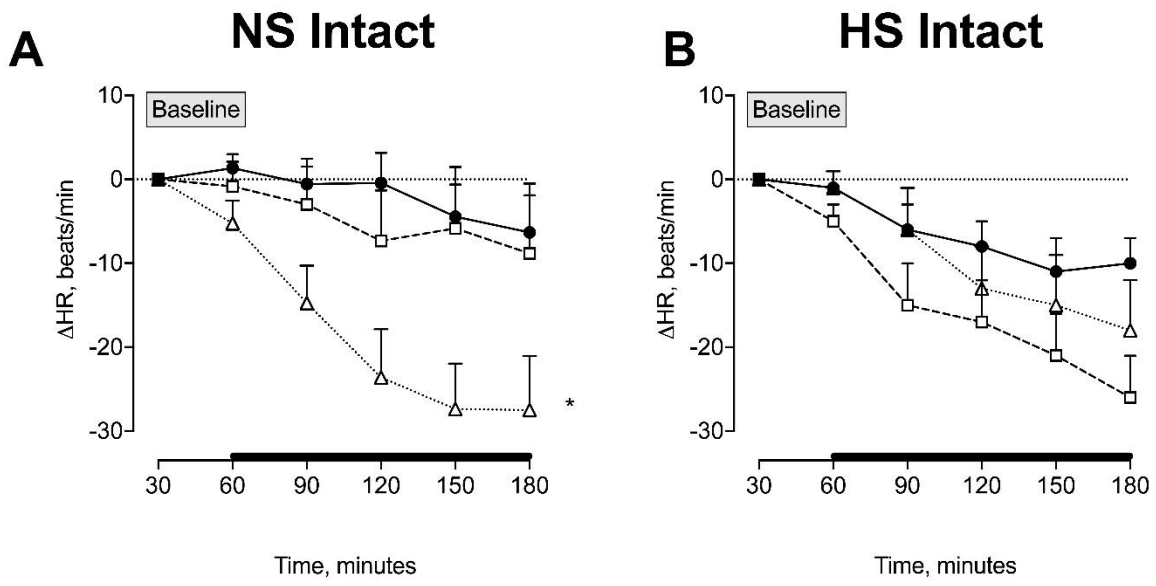

Figure 2.2 Changes in heart rate (Δ HR) in response to increased RVP in Lewis rats maintained on a normal salt and high salt (HS) diet. The first two time points of each graph represent baseline. The latter four time points represent either control (●, control) , moderate RVP elevation (□, RVP 10) and major RVP elevation (△, RVP 20). The thick underline in y-axis represents the duration of time when RVP was manipulated. Multiple linear model with repeated measurement was used to compare each time point of three RVP subgroups (control, RVP 10 and RVP 20) in animals on both normal and high salt diets, using Bonferroni as post-hoc test. Moderate RVP elevation did not impact HR in NS rats. Major RVP elevation induced a significant HR reduction compared to control animals (A, * $p<0.05$). However, no significant differences in HR were identified among different RVP subgroups in HS (B) rats.

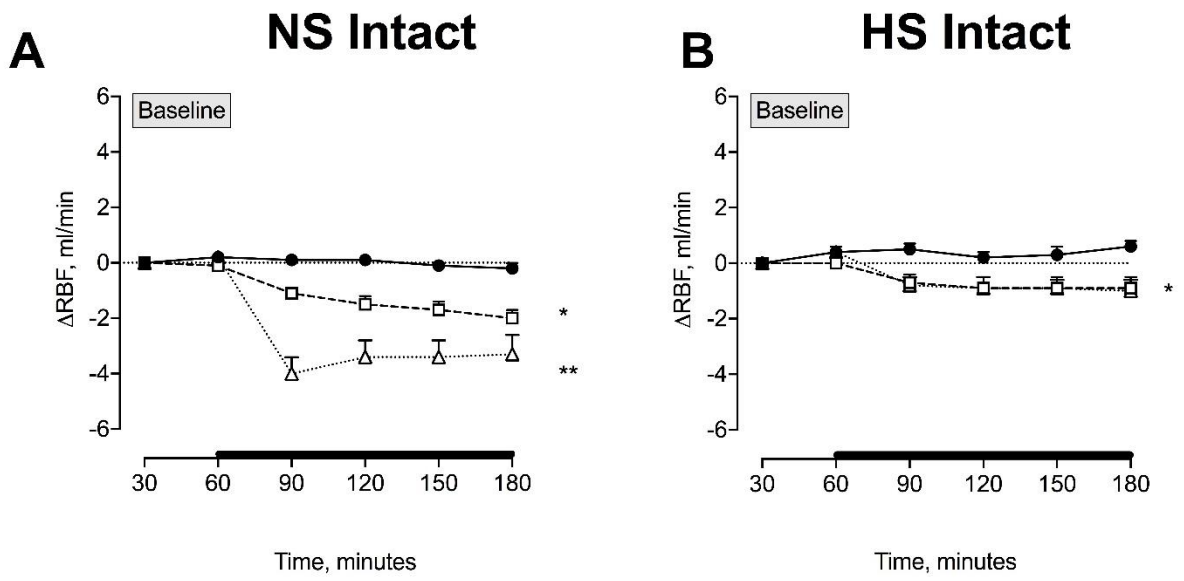

Figure 2.3 Changes in renal blood flow (ΔRBF) in response to increased RVP in Lewis rats maintained on a normal salt (NS) and high salt (HS) diet. The first two time points of each graph represent baseline. The latter four time points represent either control (●, control) , moderate RVP elevation (□, RVP 10) and major RVP elevation (△, RVP 20). The thick underline in y-axis represents the duration of time when RVP was manipulated. Multiple linear model with repeated measurement was used to compare each time point of three RVP subgroups (control, RVP 10 and RVP 20) in animals on both normal and high salt diets, using Bonferroni as post-hoc test. In intact rats, increase of RVP (RVP 10 and RVP 20) caused significant reduction of RBF in rats on a normal salt diet (A, * $p<0.05$, ** $p<0.001$) and on a high salt diet (B, * $p<0.05$).

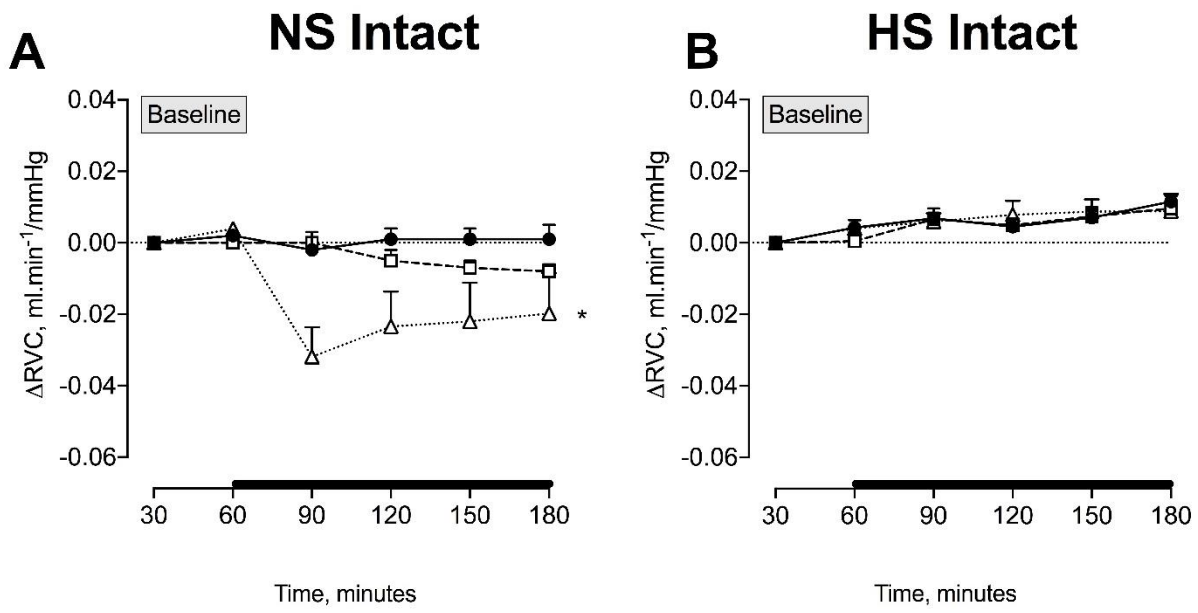

Figure 2.4 Changes in renal vascular conductance (ΔRVC) in response to increased RVP in Lewis rats maintained on a normal salt (NS) and high salt (HS) diet. The first two time points of each graph represent baseline. The latter four time points represent either control (●, control) , moderate RVP elevation (□, RVP 10) and major RVP elevation (△, RVP 20). The thick underline in y-axis represents the duration of time when RVP was manipulated. Multiple linear model with repeated measurement was used to compare each time point of three RVP subgroups (control, RVP 10 and RVP 20) in animals on both normal and high salt diets, using Bonferroni as post-hoc test. RVP 10 did not decrease RVC in rats on either diet. RVP 20 decreased RVC in NS intact rats (A, * $p<0.05$), The reduction of RVC was abolished in rats on a high salt diet (B).

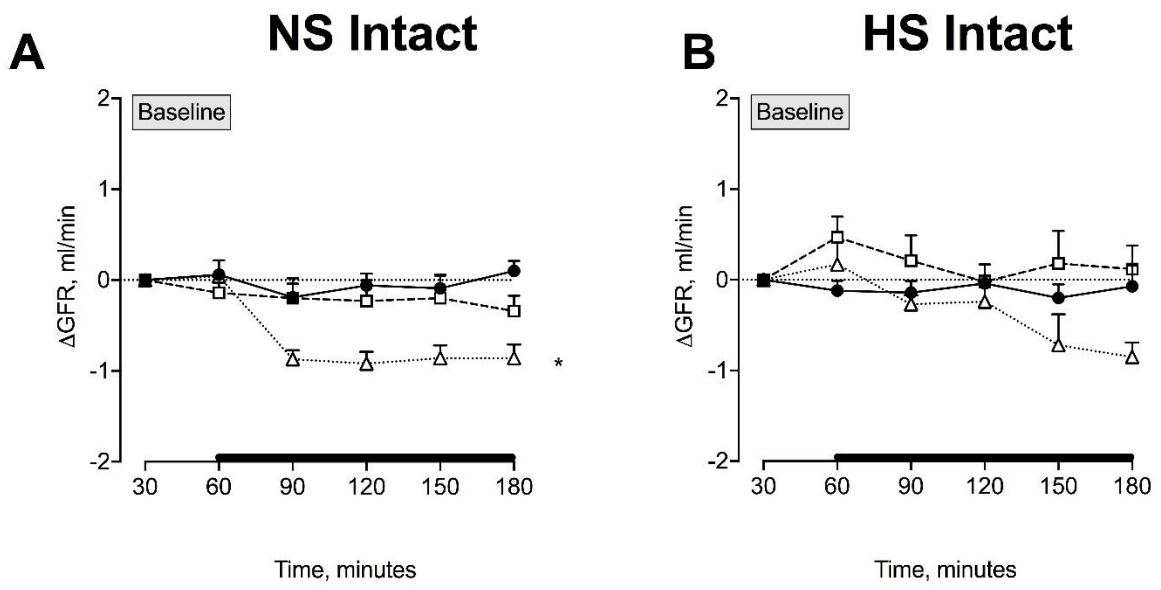

Figure 2.5 Changes in glomerular filtration rate (ΔGFR) in response to increased RVP in Lewis rats maintained on a normal salt (NS) and high salt (HS) diet. The first two time points of each graph represent baseline. The latter four time points represent either control (●, control) , moderate RVP elevation (□, RVP 10) and major RVP elevation (△, RVP 20). The thick underline in y-axis represents the duration of time when RVP was manipulated. RVP 20 decreased GFR significantly compared to time control animals (A, * $p<05$). However, no significant differences in GFR were identified among different RVP subgroups in HS (B) rats (B).

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CHAPTER 3:

Acute renal venous congestion-induced renal vasoconstriction is not mediated by increased renal sympathetic nerve activity in male Lewis rats

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Abstract

Acute increased renal venous pressure (RVP) induces immediate reduction in renal blood flow (RBF), renal vascular conductance (RVC) and glomerular filtration rate (GFR). In addition to the change in renal perfusion pressure, the renal nerves could well be involved in these renal hemodynamic responses. Experiments are conducted to characterize the involvement of the renal nerves and renal sympathetic nerve activity (RSNA). Male Lewis rats are randomized into 3 groups: time control and RVP elevation to either 10 or 20 mmHg to assess heart rate, RBF and GFR. To increase RVP, the left renal vein is partially occluded for 120 min. To determine the role of renal nerves, surgical denervation is conducted in rats on both normal (1% NaCl, NS) and high salt (6% NaCl, HS) diets. In addition, RSNA is recorded in a separate group of rats on a NS diet. Renal denervation does not prevent the hemodynamic changes induced by increased RVP. Following renal denervation, an increase in RVP from 0.5±0.1 to 20.1±0.2 mmHg causes a pronounced reduction in RBF ($p<0.001$) as well as a significant decrease in RVC ($p<0.05$). Renal denervation does not prevent the reduction in GFR following pronounced RVP elevation ($p<0.001$). HS deneravted rats have similar reductions in RBF, RVC and GFR in response to RVP elevation. Furthermore, a major increase in RVP (1.6±0.8 to 24.7±1.2 mmHg) immediately suppresses RSNA ($p<0.05$). Taken together, increasing RVP to 20 mmHg decreases ipsilateral RBF and renal vascular conductance and GFR in NS rats. The hemodynamic changes are not prevented by renal denervation. A major elevation of RVP significantly alters renal sympathetic input by suppressing both frequency and amplitude of action potentials. These observations suggest that the renal vasoconstriction and decreased GFR by acute elevation of RVP are not mediated via renal nerves.

3.1 Introduction

Previously in chapter 2, we observed that increased renal venous pressure (RVP) induces a direct modulation of renal hemodynamics in the form of a reduction in renal blood flow (RBF), renal vascular conductance (RVC) and glomerular filtration rate (GFR). The decrease in the arterio-venous pressure gradient cannot completely explain the impaired kidney function. Neurohormonal factors are likely involved. It is well known that the activation of renal sympathetic nerves activity (RSNA) can induce an immediate drop in RBF and GFR [1]. However, it is not clear whether vasoconstriction induced by increased RVP is mediated by the increased RSNA. Increased RSNA has been implicated in the RVP-induced reduction in RBF in rats [2] Other reports indicate suppression of renal sympathetic nerve activity (RSNA) [3, 4]. Therefore, it remains unclear how renal nerves are involved in RVP-induced renal response.

In the present study, we investigated the involvement of renal nerves in modulation of the renal hemodynamic response to increased RVP, by performing a bilateral surgical denervation in male Lewis rats on both normal and high salt diet, since both RSNA and the RAS are naturally suppressed under high-salt intake [5, 6]. Additionally, the ipsilateral renal electrophysiological response was recorded directly when RVP was increased.

3.2 Methods

Animals

Male Lewis rats (300 to 450 g, n=45) (Charles River, St. Constant, QC, Canada) were housed in a temperature and humidity-controlled room with a 12hr:12hr light/dark cycle. All rats received regular rat chow with 1% NaCl *ad libitum* (Canadian Lab Diets, Leduc, AB, Canada) and had free access to tap water. Rats randomly assigned to a high salt diet received modified chow (Canadian Lab Diets, Leduc, AB, Canada) formulated with 6% NaCl *ad libitum* with free access to tap water for at least 2 weeks before the experiment. Bilateral renal denervation was performed in rats on either normal salt

(NS, n=16) or high salt diet (HS, n=15). Each of these experimental groups included subgroups of rats in which RVP was left unaltered (time controls) or increased to either 10 or 20mmHg. Separate rats (n=14) fed regular chow were used for direct measurement of RSNA (time control: n=8); (RVP 10mmHg: n=9); (RVP 20 mmHg: n=7). Experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care and received prior approval by the Animal Care and Use Committee of the University of Alberta.

Denervation study

Rats were given buprenorphine (0.02mg/kg, i.m.) 30 minutes before anesthesia. Isoflurane anesthesia was induced in an induction chamber pre-charged with room air. Isoflurane was introduced in 0.5% increments up to 4% in 100% oxygen (1 L/min). Once the rat reached surgical plane of anesthesia, it was placed on a heated surgical table equipped with a thermo-feedback system to maintain rectal temperature between 36-37°C (Vestavia Scientific, Birmingham, AL, USA). Anesthesia was maintained through a nose cone and the isoflurane dose was gradually reduced in 0.5% increments to 2%. Rats were permitted to breathe spontaneously. Hair from the neck, abdomen and left groin was removed by shaving and the surgical field was cleansed with alternating applications of 10% Povidone iodine and 70% ethanol. Following a midline neck incision to expose the trachea, the rat was intubated via tracheotomy using PE-240 tubing (BD Intramedic, Sparks, MD, USA). The tracheal tube was then fitted to the nosecone and isoflurane dose was adjusted to 1.5-1.75% to maintain surgical plane with the loss of the toe-pinch reflex. The toe-pinch reflex was assessed every 5 minutes to verify surgical plane during the surgery and experimental recording period. The left femoral vein was catheterized (Silastic tubing, 0.51mm ID, 0.94mm OD, Dow Corning, Midland, MI, USA) for intravenous infusion, which was immediately commenced (see below). The left femoral artery was cannulated (PE-50, BD Intramedic, Sparks, MD, USA) for direct recording of systemic arterial pressure and heart rate (HR). Following midline laparotomy, the left kidney was exposed. The renal nerves coursing along the left

and right renal vessels were surgically removed and the renal vessels painted with 10% phenol in 70% ethanol. The left adrenal vein or supraspermatic vein was cannulated (Micro-Renathane MRE-025, Braintree Scientific, Braintree, MA, USA) and the cannula advanced until the tip rested in the main renal vein for direct measurement of RVP. A length of 3-0 prolene (Johnson-Johnson, San Lorenzo, Puerto Rico) was slipped around the left renal vein at its junction with the inferior vena cava and sheathed with a small piece of PE-50 tubing to create a sling. To increase RVP, the sling was tightened to constrict the renal vein. Pressures were acquired using PowerLab via disposable blood pressure transducers (ADInstruments, CO, USA). A 1RB transit-time flow probe was placed around the left renal artery for direct measurement of RBF (Transonic, Ithaca, NY, USA). The left ureter was catheterized for urine collection (PE-10, BD Intramedic, Sparks, MD, USA). The rat received supplemental fluids during surgical preparation (5% bovine serum albumin in normal saline, BSA, A7906, Sigma, Oakville, ON, Canada) with 250 μg/min FITC inulin (Sigma, Oakville, ON, Canada) at 1.5mL/hr. This infusion continued throughout the experiment with 1% BSA with 250 μg/min FITC inulin at 1.5mL/hr.

RSNA recording

Rats were prepared as described, except that the left abdominal flank was shaved and cleansed. Following femoral artery and vein cannulation, the rat was placed on its right side with left flank exposed. Rats received supplemental fluids as above, with the exception of FITC-Inulin. A left flank incision was made in the skin and underlying abdominal muscle, parallel and 1 cm caudal to the last rib. The left kidney was exposed and retracted. The left adrenal or supra-spermatic vein was cannulated, and a snare placed around the renal vein. A branch of the renal nerve bundle was identified; this was carefully isolated from surrounding tissue and placed onto a stainless steel bipolar electrode [7] for multifiber recordings. A piece of paraffin film [7] was slipped between the bipolar leads and a third ground wire, which was in direct contact with underlying tissue. The area was dried with

absorbent spears (FST, BC, CA) and a two-component silicone elastomer (Kwik-Sil, World Precision Instruments WPI, USA) was applied to the nerve-electrode unit for electrical isolation. Once hardened, the edges of the silicone were secured to the surrounding tissue with tissue adhesive (VetBond, 3M, MN, USA). The renal nerve signal was amplified (x10, 000) and filtered (Low pass: 100Hz; High pass: 1000Hz, Leaf electronics LTD QT-B, WPI LPF-30, FL, USA); online recordings were acquired at 10kHz (LabChart 6, PowerLab, AD Instruments, CO, USA). RSNA was verified by elevation of arterial pressure with a bolus i.v. injection of phenylephrine (20μg/g, Sigma-Aldrich) which elicited rapid suppression of RSNA. The postganglionic nature of RSNA was verified at the end of the experimental protocol by i.v. injection of hexamethonium (50μg/g, Sigma-Aldrich). Background noise was determined from postmortem recording.

Experimental design

Following completion of surgical instrumentation, rats were stabilized for 60 minutes. Baseline data were collected for 60 minutes, after which time RVP was selectively increased to either 10 or 20 mmHg by graded constriction of the left renal vein or not manipulated (time controls). Data collection continued for a further 120 minutes. In the denervation experiments, blood samples (200 μl) were obtained at the beginning of the baseline period and every 60 min thereafter. Timed urine samples were collected every 30 minutes. No blood or urine sampling was collected during nerve recording experiments.

Analytical methods

To determine GFR in the denervation study using FITC-Inulin, plasma and urine samples were diluted in 0.5 mol/l HEPES (pH 7.4) to maintain physiological pH. A 96-well black plate (Greiner, Monroe, NC, USA) was used for loading 50μl of each solution in duplicate. Fluorescence was determined using the Fluoroskan Ascent® Microplate Fluorometer (Thermo Fisher Scientific, Vantaa, Finland), at the

excitation wavelength of 485nm and emission wavelength of 527nm. Terminal blood samples were obtained from the femoral catheter to measure plasma renin level by ELISA (NOVATEINBIO, Cambridge, MA, USA).

To determine RSNA, the total number of spikes above background were quantified using Spike Histogram software (Lab Chart 8, ADInstruments, CO, USA). Six measurements were taken during the baseline recording period and averaged. Measurements were taken at 5-minute intervals for the first 30 min of RVP increase and at 30-minute intervals thereafter. Quantification of the RSNA response to increased RVP was calculated as the percent change RSNA from baseline.

Analysis and statistics

Data are presented as the average of consecutive 30 min intervals. The baseline characteristics were compared between both denervated rats on the normal and high salt diet using a general linear model multivariate (MANOVA) with Bonferroni as post-hoc test. To evaluate the impact of elevated RVP, Multiple linear model with repeated measurements was used to compare each time point of three groups in denervated animals on different diets, using Bonferroni as post-hoc test. Plasma renin and aldosterone level were analyzed with 2-way ANOVA with Student Newman Keuls as post-hoc test. Data were log-transformed or ranked if not normally distributed. RSNA data were analyzed with Two-Way Repeated Measures ANOVA with Student Newman Keuls post-hoc test. Data were analyzed using SPSS 24 (IBM, Armonk, NY, US) and SigmaPlot 13 (Systat, San Jose, CA, USA). A p value less than 0.05 was considered significant. All data are presented as means ± SEM.

3.3 Results

Characterization of experimental groups

The high salt diet did not influence baseline mean arterial pressure (MAP) or RBF. Baseline MAP was lower following RD in both NS ($p<0.05$) and HS rats ($p<0.001$). HS, denervated rats had higher renal vascular conductance (RVC) ($p<0.05$). (table 3.1)

Increased RVP and renal hemodynamics

Renal denervation did not prevent the hemodynamic changes induced by increased RVP. Following renal denervation, moderate elevation of RVP (1.1±0.3 to 11.3±0.4 mmHg) did not alter MAP, HR, RBF or RVC (Figure 3.1 C, 3.2 C, 3.3 C, 3.4 C). All data are presented in Table 3.2. In contrast, major elevation of RVP (0.5±0.1 to 20.1±0.2 mmHg) did not significantly decrease HR due to the substantial variation (Figure 3.2 C) but caused a pronounced reduction in RBF ($p<0.001$, figure 3.3 C) as well as a significant decrease in RVC ($p<0.05$, Figure 3.4 C). Similarly, renal denervation did not prevent the reduction in GFR following pronounced RVP elevation ($p<0.001$, Figure 3.5 C).

Modest RVP increase had no effect on HR, RBF or RVC (Figure 3.2 D, 3.3 D, 3.4 D) in renal denervated rats receiving HS diet. However, similar to NS and intact counterparts, major RVP increase reduced HR ($p<0.05$, Figure 3.2 D), RBF and RVC ($p<0.001$, Figures 3.3 D, $p<0.05$, Figure 3.4 D). Although moderate RVP elevation did not reduce GFR in these rats, the major RVP elevation reduced GFR ($p<0.05$, Figure 3.5 D).

Normal salt diet, increased RVP and RSNA

Upon a moderate increase of RVP from 1.1±0.1 to 10.6±0.2 mmHg, RSNA was unchanged compared to time control (Figure 3.6). In contrast, a major increase of RVP from 1.6±0.8 to 24.7±1.2 mmHg immediately and significantly suppressed RSNA ($p<0.05$). This response was progressive and ultimately sustained with continued elevation of RVP (Figure 3.6).

Increased RVP, plasma renin and aldosterone levels

Plasma renin levels in NS animals were higher ($p<0.001$) than HS rats at the end of the experiments (Table 3.3). RVP levels did not affect plasma renin level. Plasma aldosterone was lower in HS rats compared to NS rats ($p<0.001$). Major RVP increase elicited higher aldosterone levels in NS rats subjected to renal denervation ($p<0.001$) (Table 3.3).

3.4 Discussion

In our previous study, we demonstrated that selectively increasing RVP reduced RBF and RVC in rats on the NS diet. In the present study, renal denervation did not prevent the reduction in RBF and RVC in rats fed either NS or HS diet. Furthermore, denervation did not prevent the GFR reduction in rats fed either diet. In sum, increasing RVP to 20 mmHg decreased RVC and GFR, a response that was not dependent on renal nerve traffic. It is corroborated by our observation that increasing RVP to 20 mmHg induced an immediate and sustained suppression of RSNA, contrary to our initial prediction. Although MAP was unaffected by RVP increase, HR was reduced when RVP was increased to 20 mmHg, which suggests depression central sympathetic nerve activity. Altogether these results support that mediators other than RSNA are involved in RVP-induced renal vasoconstriction and decrease in GFR.

Regarding renal nerves, we saw no differences in the responses of RBF, GFR and RVC in the response to increased RVP in denervated versus innervated kidneys. Abildgaard demonstrated in dogs that a gradual increase in RVP increased RVC whereas an instantaneous increase reduced RVC. The reduction of RVC in response to an instantaneous elevation of RVP was abolished by surgical denervation [8]. Early studies in rats reported significant reductions in RBF and reduced RVC in response to unilateral RVP increase >20 mmHg. This response was attributed to renal nerves, since denervation prevented any reduction of RVC [2]. A recent study in rats demonstrated that an increase in central venous pressure to 10 mmHg (which presumably increases RVP to the same degree) increased RSNA by 285% [9]. However, the pneumoperitoneum induced in rats in that study could

activate RSNA by mechanisms other than increased RVP. Therefore, the increased RSNA observed in this case might not be due to an increased RVP.

In contrast, Kopp et al reported that increasing RVP to 22 mmHg reduced ipsilateral RVC, with which our results agree. They also showed that increased RVP increased afferent renal nerve activity but decreased efferent renal nerve activity and inhibited renorenal reflex [4]. Our results are in agreement, suggesting that the hemodynamic changes we observe with an acute, major increase in RVP are likely not mediated by renal nerves. An obvious remaining possibility is that in our renal denervated rats, intrarenal RAS activation could still take place and elicit the observed reduction of RBF and RVC. This is supported by our observations in denervated rats on HS diet in which this response is attenuated, and that in denervated rats on NS diet, major RVP increase elicited higher aldosterone release.

Taken together, despite previous reports suggesting a role for the renal nerves, our results indicate otherwise since renal denervation did not affect RVP-induced reduction of RBF, RVC and GFR on either diet. Additionally, we demonstrate that elevated RVP rapidly suppresses RSNA. Increased RVP seen in diseases such as congestive heart failure is an acknowledged factor for renal dysfunction. To explore the mechanisms involved is valuable for therapeutic approaches. Although over-activation of RSNA is commonly found in congestive heart failure, our data suggest it is not be involved in exacerbate the hemodynamic effect induced by increased RVP. Studies delineating other candidates such as the RAS to study this key mediator are needed.

Table 3.1 Baseline group characteristics of denervated Lewis rats maintained on a normal salt and high salt diet.

Baseline	Denervated normal salt diet	Denervated high salt diet
N	16	15
BW (g)	379±5 *	381±9 *
HR (beats/min)	330±7 *	350±7
MAP (mmHg)	81.3±1.6 *, #	79.0±2.6 **, ##
RVP (mmHg)	1.1±0.3 *	1.3±0.2 *
RBF (ml/min)	7.0±0.4	7.3±0.5
RVC (ml/min·mmHg)	0.088±0.005	0.094±0.007 *
GFR (ml/min)	1.44±0.07 (n=14)	1.51±0.18 (n=14)

n: number, BW: body weight, HCT: hematocrit, HR: heart rate, MAP: main arterial pressure, RVP: renal venous pressure, RBF: renal blood flow, RVC: renal vascular conductance, GFR: glomerular filtration rate. The baseline characteristics were compared between both denervated rats on the normal and high salt diet using a general linear model multivariate (MANOVA) with Bonferroni as post-hoc test. No significant differences were identified between the denervated rats on a normal salt diet and on a high salt diet.

Table 3.2 All group characteristics of both baseline and endpoint from Denervated Lewis rats maintained on a normal salt and high salt diet.

NORMAL SALT DIET (Denervated)						
	Control (n=5)		RVP 10 (n=5)		RVP 20 (n=6)	
	Baseline	End	Baseline	End	Baseline	End
HCT (%)	42.2±1.1	39.6±1.7	43.5±1.0	43.2±1.1	44.2±0.6	44.3±1.1
HR (Beats/min)	320±8	319±8	327±17	319±14	341±10	322±14
MAP (mmHg)	81.1±3.2	72.8±4.7	83.8±4.0	74.9±4.7	79.4±1.4	70.6±1.4
RVP (mmHg)	0.8±0.2	1.1±0.4	1.4±0.6	10.2±0.2**	1.0±0.6	20.3±0.7**
RBF (ml/min)	8.1±1.0	6.3±1.0	6.6±0.2	4.8±0.5	6.4±0.5	2.3±0.2**
RVC (ml/min·mmHg)	0.100 ±0.012	0.088 ±0.012	0.082 ±0.006	0.074 ±0.006	0.082 ±0.007	0.045 ±0.004*
GFR (ml/min)	1.41±0.10 (n=4)	1.09±0.15 (n=4)	1.44±0.15 (n=4)	1.04±0.14 (n=4)	1.47±0.14	0.04±0.04*
HIGH SALT DIET (Denervated)						
	Control (n=5)		RVP 10 (n=5)		RVP 20 (n=5)	
	Baseline	End	Baseline	End	Baseline	End
HCT (%)	45.7±1.2	43.0±0.3	45.8±0.7	45.4±1.1	45.3±1.2	45.2±0.9
HR (Beats/min)	337±17	333±16	347±5	335±4	368±4	339±10*
MAP (mmHg)	75.6±3.0	70.6±2.1	77.3±2.5	75.8±1.4	84.1±6.7	80.0±4.5
RVP (mmHg)	1.3±0.5	1.2±0.5	1.5±0.2	10.7±0.4**	1.0±0.2	20.4±0.8**
RBF (ml/min)	7.4±0.3	7.1±0.2	7.6±1.1	6.3±0.6	6.9±1.3	3.6±0.7**
RVC (ml/min·mmHg)	0.100 ±0.003	0.102 ±0.002	0.101 ±0.014	0.098 ±0.011	0.083 ±0.014	0.060 ±0.011*
GFR (ml/min)	1.31±0.11	1.18±0.34	1.48±0.27	1.32±0.21	1.41±0.35	0.40±0.17*

Multiple linear model with repeated measurement was used to compare each time point of three RVP subgroups (control, RVP 10 and RVP 20) in animals on both normal and high salt diets, using Bonferroni as post-hoc test. * $p<0.05$ compared with the baseline value of the same RVP subgroup on the same diet; ** $p<0.001$ compared with the baseline value of the same RVP subgroup on the same diet.

Table 3.3 Plasma renin and aldosterone level of denervated Lewis rats maintained on a normal salt (NS) and high salt (HS) diet.

Plasma Renin level (pmol/L)	TC	RVP10	RVP20
NS Denervated	129±8 (n=5)	134±5 (n=5)	120±4 (n=6)
HS Denervated	113±6 (n=5)	99±3 (n=5)	111±8 (n=5)
Plasma Aldosterone level (pg/mL)	TC	RVP10	RVP20
NS Denervated	40±8	77±11	271±100**
HS Denervated	26±6	21±5	27±5

Plasma renin and aldosterone levels were analyzed with 2-way ANOVA with Student Newman Keuls post-hoc test. Plasma aldosterone data were log-transformed because they were not normally distributed. ** $p<0.001$ compared with control group on the same diet.

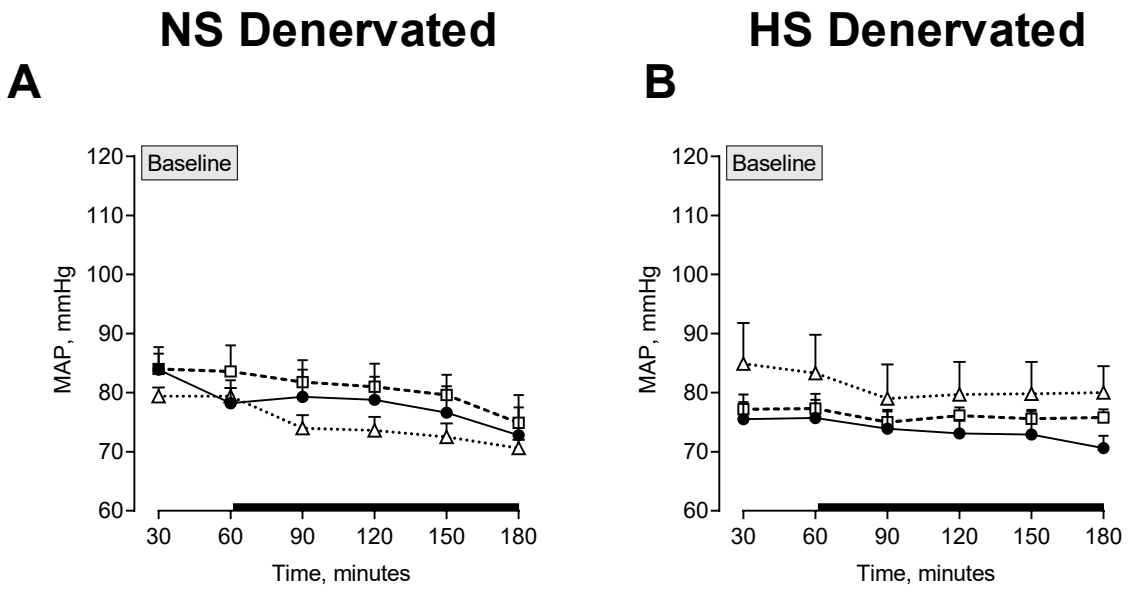

Figure 3.1 Mean Arterial Pressure (MAP) in response to increased RVP in denervated Lewis rats maintained on a normal salt (NS) and high salt (HS) diet. The first two time points of each graph represent baseline. The latter four time points represent either control (●, control) , moderate RVP elevation (□, RVP 10) and major RVP elevation (△, RVP 20). The thick underline in y-axis represents the duration of time when RVP was manipulated. Multiple linear model with repeated measurement was used to compare each time point of three RVP subgroups (control, RVP 10 and RVP 20) in in both NS denervated rats and HS denervated rats, using Bonferroni as post-hoc test. No significant differences in MAP were identified among different RVP subgroups in either of the NS (A) or HS (B) denervated rats.

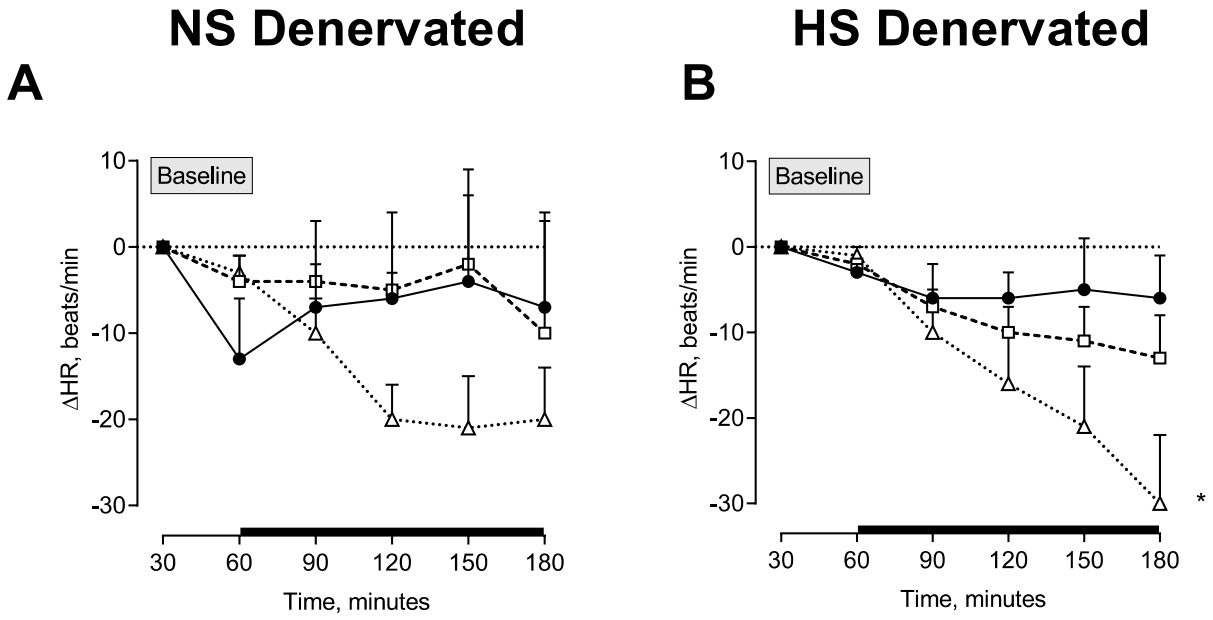

Figure 3.2 Changes in heart rate (Δ HR) in response to increased RVP in denervated Lewis rats maintained on a normal salt (NS) and high salt (HS) diet. The first two time points of each graph represent baseline. The latter four time points represent either control (●, control) , moderate RVP elevation (□, RVP 10) and major RVP elevation (△, RVP 20). The thick underline in y-axis represents the duration of time when RVP was manipulated. Multiple linear model with repeated measurement was used to compare each time point of three RVP subgroups (control, RVP 10 and RVP 20) in both NS denervated rats and HS denervated rats, using Bonferroni as post-hoc test. The reduction in HR in response to RVP elevation was not significant in NS denervated rats (A). RVP 20 but not RVP 10 in HS Denervated rats significantly decreased HR compared to its own time control animals (B, * $p<0.05$).

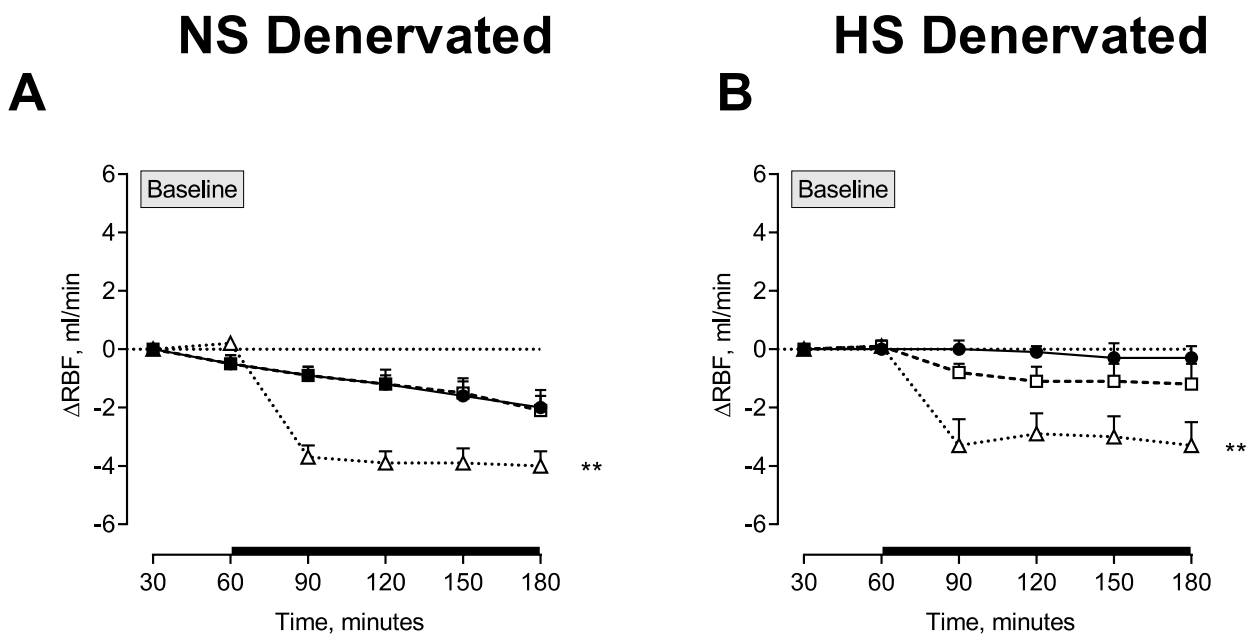

Figure 3.3 Changes in renal blood flow (Δ RBF) in response to increased RVP in denervated Lewis rats maintained on a normal salt (NS) and high salt (HS) diet. The first two time points of each graph represent baseline. The latter four time points represent either control (●, control) , moderate RVP elevation (□, RVP 10) and major RVP elevation (△, RVP 20). The thick underline in y-axis represents the duration of time when RVP was manipulated. Multiple linear model with repeated measurement was used to compare each time point of three RVP subgroups (control, RVP 10 and RVP 20) in in both NS denervated rats and HS denervated rats, using Bonferroni as post-hoc test. RVP 20 decreased RBF significantly in NS denervated rats (A, ** $p<0.001$) and HS denervated rats (B, * $p<0.05$) compared to their own control animals.

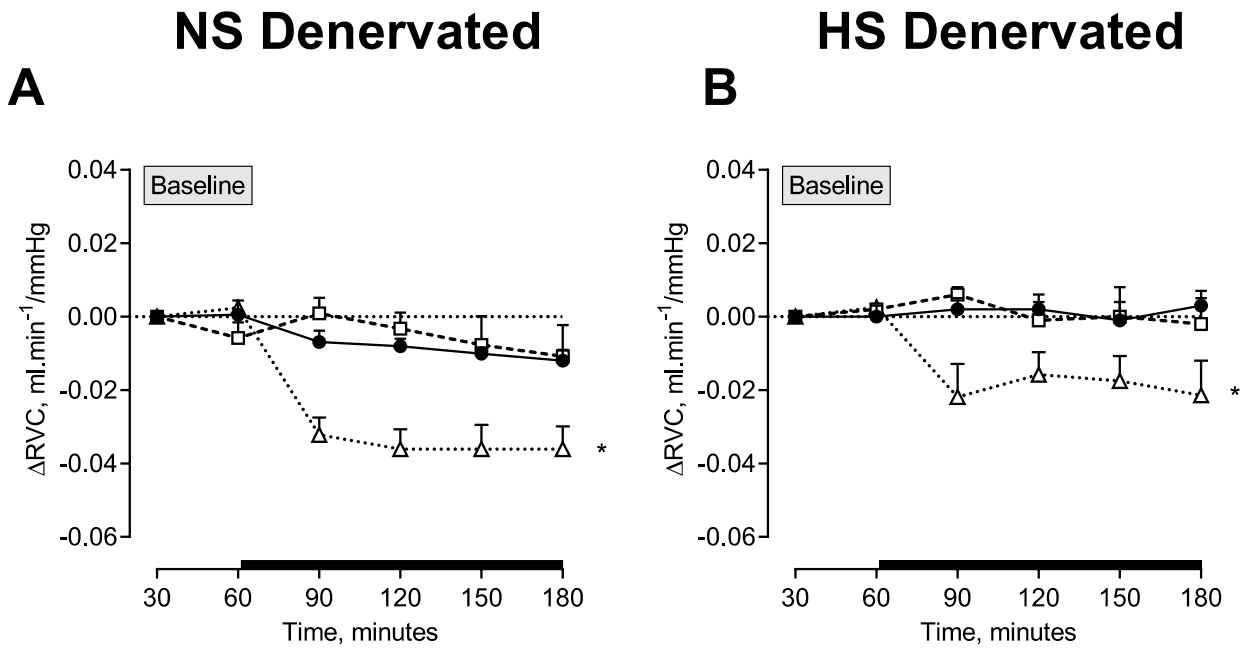

Figure 3.4 Changes in renal vascular conductance (Δ RVC) in response to increased RVP in denervated Lewis rats maintained on a normal salt (NS) and high salt (HS) diet. The first two time points of each graph represent baseline. The latter four time points represent either control (●, control) , moderate RVP elevation (□, RVP 10) and major RVP elevation (△, RVP 20). The thick underline in y-axis represents the duration of time when RVP was manipulated. Multiple linear model with repeated measurement was used to compare each time point of three RVP subgroups (control, RVP 10 and RVP 20) in in both NS denervated rats and HS denervated rats, using Bonferroni as post-hoc test. RVP 10 did not decrease RVC in all rats. RVP20 decreased RVC in NS denervated rats (A, * $p<0.05$) and HS denervated rats (B, * $p<0.05$) in comparison to their own control animals.

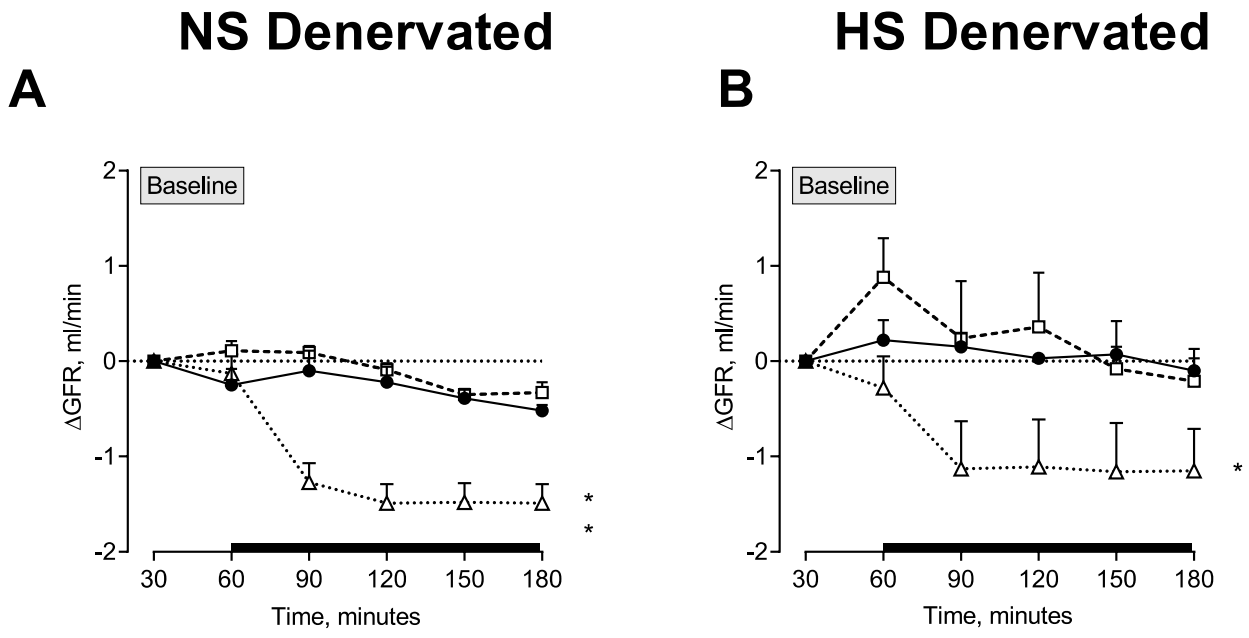

Figure 3.5 Changes in glomerular filtration rate (ΔGFR) in response to increased RVP in denervated Lewis rats maintained on a normal salt (NS) and high salt (HS) diet. The first two time points of each graph represent baseline. The latter four time points represent either control (●, control) , moderate RVP elevation (□, RVP 10) and major RVP elevation (△, RVP 20). The thick underline in y-axis represents the duration of time when RVP was manipulated. Multiple linear model with repeated measurement was used to compare each time point of three RVP subgroups (control, RVP 10 and RVP 20) in in both NS denervated rats and HS denervated rats, using Bonferroni as post-hoc test. In NS denervated rats, RVP 10 did not affect GFR but RVP 20 decreased GFR significantly (A, ** $p<0.001$). RVP 20 decreased GFR significantly in HS denervated rats (B, * $p<0.05$).

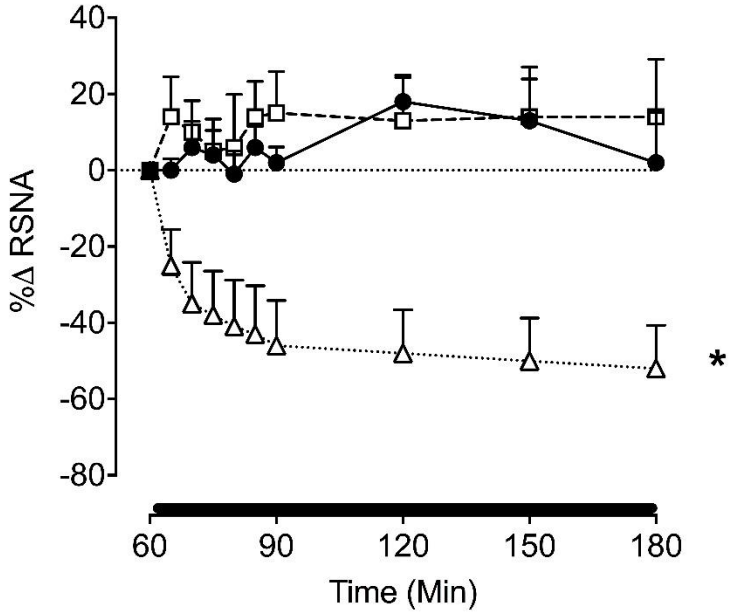

Figure 3.6 RSNA in response to RVP elevation in Lewis rats on a normal salt (NS) diet. RSNA data are presented using percentage of baseline. The ten time points from each group represent either control (●, control) , moderate RVP elevation (□, RVP 10) and major RVP elevation (△, RVP 20). The thick underline in y-axis represents the duration of time when RVP was manipulated. RSNA data were analyzed with Two-Way Repeated Measures ANOVA with Student Newman Keuls post-hoc test. Moderate increase of RVP did not impact RSNA. In contrast, augmented RVP increase immediately suppressed RSNA (* $p<0.05$).

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CHAPTER 4:

The renin angiotensin system decreases renal vascular conductance but maintains autoregulation during renal venous pressure elevation

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Abstract

Increased central venous pressure in congestive states leads to increased renal venous pressure (RVP), which can negatively impact on kidney function. This could be mediated by the renin-angiotensin system (RAS). In our previous study, we have shown that the increased RVP induced a reduction in renal blood flow (RBF), glomerular filtration rate (GFR) and renal vascular conductance (RVC) [1]. In the present study, we investigate the involvement of the RAS in the renal hemodynamic response to the increased RVP by a targeted inhibition using angiotensin-converting enzyme inhibition (ACEi). When endogenous angiotensin II (ANG II) is inhibited, the circulatory ANG II is clamped by a continuous and constant infusion of extraneous ANG II. The ANG II clamp does not prevent the decrease of RBF (-1.9±0.4ml/min, $P<0.05$), and ipsilateral GFR (-0.77±0.18 ml/min, $P<0.05$). However, the reduction in RVC seen in the untreated Lewis rats is completely abolished (0.011±0.005 ml/min.mmHg). Additionally, increased RVP from 0.2±0.2 to 19.7±0.3 mmHg induces a decline in MAP (-22.4±4.1 mmHg, $P<0.05$) and heart rate (HR, -23±4 bpm, $P<0.001$). Furthermore, we report the hemodynamic response in the absence of ANG II, in which vasopressin is given to maintain a comparable MAP when the ANG II function is inhibited with ACEi. In these rats without ANG II, an increase in RVP from 0.2±0.2 to 19.6±0.6 mmHg does not impact MAP or HR. The RVC does not decrease (0.018±0.008 ml/min.mmHg). Although RBF decreases by 1.2±0.5ml/min ($P<0.05$), the reduction of GFR is no longer significant (-0.54±0.15 ml/min); MAP or HR does not decrease in response to an increased RVP. Additionally, we test RBF autoregulation by stepwise arterial pressure reductions. RBF autoregulation remains intact and is reset at a lower level when RVP is increased. In conclusion, RVP-induced renal vasoconstriction is attenuated when ANG II is fixed or inhibited; renal autoregulation is intact in RVP elevation. This suggests a primary role for the RAS and autoregulation in the impaired kidney function induced by increased RVP.

4.1 Introduction

In our previous study, where we demonstrated that an acute isolated increase of renal venous pressure (RVP) lead to reduction of renal vascular conductance (RVC) [1]. Interestingly, the RVC reduction was abolished in rats receiving a high salt diet, but not by renal denervation. This suggested that the RAS suppression by the high salt diet is in favor of preserving kidney functions. In 1982, Kastner *et al* showed renin levels were profoundly increased due to RVP elevation. However, in their study, inhibition of the RAS led to a worse GFR response to an RVP elevation [2]. The role of the RAS in RVP elevation remains unclear. Taken together, over-activation of the RAS might be important to explain the worsening kidney function, but data are conflicting.

One aim of the present study was to test in rats that vasoconstriction caused by an isolated increase of RVP was due to activation of the RAS. We measured hemodynamic responses both in rats with ANG II clamped, in which endogenous ANG II was blocked using an angiotensin converting enzyme inhibitor (ACEi) but exogenous ANG II was administered at a constant level to restore arterial pressure. We also studied the situation where ANG II was absent, by complete blockade with an ACEi and arterial pressure was restored with vasopressin (AVP). Secondly, increased RVP causes a passive increase of the glomerular pressure (Figure 4.4). Thus, myogenic response likely contributes to the RVP-induced vasoconstriction in the untreated animals. Therefore, it is important to study whether autoregulation of renal blood flow (RBF) is intact when RVP was increased.

4.2 Methods

All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care and received prior approval by the Animal Care and Use Committee of the University of Alberta. Male Lewis rats (350 to 450g) (Charles River, St. Constant, QC, Canada) were housed in a temperature and humidity-controlled room with a 12hr:12hr light/dark cycle. All rats (n=55) received regular rat chow with 1% NaCl *ad libitum* (Canadian Lab Diets, Leduc, AB, Canada) and had free access to tap water.

General surgical preparation

Rats were given buprenorphine (0.02mg/kg, i.m.) 30 minutes before anesthesia. Isoflurane anesthesia was induced in an induction chamber pre-charged with room air. Isoflurane was introduced in 0.5% increments up to 4% in 100% oxygen (1 L/min). Once the rat reached surgical plane of anesthesia, it was placed on a heated surgical table equipped with a thermo-feedback system to maintain rectal temperature between 36-37°C (Vestavia Scientific, Birmingham, AL, USA). Anesthesia was maintained through a nose cone and the isoflurane dose was gradually reduced in 0.5% increments to 2%. Rats were permitted to breathe spontaneously. Hair from the neck, abdomen and left groin was removed by shaving and the surgical field was cleansed with alternating applications of 10% Povidone iodine and 70% ethanol. Following a midline neck incision to expose the trachea, the rat was intubated via tracheotomy using PE-240 tubing (BD Intramedic, Sparks, MD, USA). The tracheal tube was then fitted to the nosecone and isoflurane dose was adjusted to 1.5-1.75% to maintain surgical plane with the loss of the toe-pinch reflex. The left femoral vein was catheterized (Silastic tubing, 0.51mm ID, 0.94mm OD, Dow Corning, Midland, MI, USA) for intravenous infusion, which was immediately commenced (see below). The left femoral artery was cannulated (PE-50, BD Intramedic, Sparks, MD, USA) for direct recording of systemic arterial pressure and heart rate (HR). Following midline laparotomy, the left kidney was exposed. The left adrenal vein or supraspermatic vein was cannulated (Micro-Renathane MRE-025, Braintree Scientific, Braintree,

MA, USA) and the cannula advanced until the tip rested in the main renal vein for direct measurement of RVP. A length of 3-0 prolene (Johnson-Johnson, San Lorenzo, Puerto Rico) was slipped around the left renal vein at its junction with the inferior vena cava and sheathed with a small piece of PE-50 tubing to create a sling. To increase RVP, the sling was tightened to constrict the renal vein. Pressures were acquired using PowerLab via disposable blood pressure transducers (ADInstruments, CO, USA). A 1RB transit-time flow probe was placed around the left renal artery for direct measurement of RBF (Transonic, Ithaca, NY, USA). The left ureter was catheterized for urine collection (PE-10, BD Intramedic, Sparks, MD, USA). The rat received supplemental fluids during surgical preparation (5% bovine serum albumin in normal saline, BSA, A7906, Sigma, Oakville, ON, Canada) with 250 μg/min FITC inulin (Sigma, Oakville, ON, Canada) at 1.5mL/hr. This infusion continued throughout the experiment with 1% BSA with 250 μg/min FITC inulin at 1.5mL/hr.

Experiment 1: ANG II clamped experiment

Experiment 1 was designed to test the hemodynamic responses to increased RVP with a clamped ANG II (n=18). Enalapril was then administered i.v. in a bolus of 0.2 mg/kg BW and infused at 3 ug/min [3]. Following completion of surgical instrumentation, angiotensin I (ANG I) was administered i.v. in a bolus of 25 pmol to test the adequacy of ACEi treatment. ANG II (started at 0.25 ug/kg/min) was continuously infused to restore the MAP. Rats were stabilized for 60 minutes. Baseline data were collected for 60 minutes, after which, RVP was selectively increased to 20 mmHg (n=10) by partial constriction of the left renal vein, or not manipulated (time controls, TC, n=8).

Experiment 2: ANG II absent experiment

Experiment 2 was designed to demonstrate the hemodynamic responses without the presence of ANG II. An identical experiment was performed in another groups of rats (n=15). Enalapril was then administered i.v. in a bolus of 0.2 mg/kg BW and infused at 3 ug/min. Following completion of surgical

instrumentation, angiotensin I (ANG I) was administered i.v. in a bolus of 25 pmol to test the adequacy of ACEi treatment. AVP (started at 8 ng/kg/min) [4] was continuously infused to restore the MAP. Rats were stabilized for 60 minutes. Baseline data were collected for 60 minutes, after which, RVP was selectively increased to 20 mmHg (n=8) by partial constriction of the left renal vein, or not manipulated (TC, n=7).

Experiment 3: Autoregulation Experiment

Experiment 3 was designed to evaluate the RBF autoregulation in RVP elevation in a separated group of rats (n=22). Surgical preparation is the same as in hemodynamic studies. Additional sling around the aorta was placed above the left renal artery using a length of 3-0 prolene (Johnson-Johnson, San Lorenzo, Puerto Rico) and sheathed with a small piece of PE-50 tubing. The renal perfusion pressure (RPP) was decreased by step-wise decrease of 10mmHg via partial occlusion of sling around the aorta. Each step of the decrease was recorded for 5 min, then the sling around the aorta was released and the RBF was allowed to return to baseline value for 5 min. The stepwise decrease was repeated when the RVP was increased to either 10 mmHg (RVP10, n=8), 20mmHg (RVP20, n=8) or remained at baseline level (TC, n=6). An 1RB transit-time flow probe was placed around the left renal artery for direct measurement of RBF (Transonic, Ithaca, NY, US). The bladder (PE-50 tubing, BD Intramedic, Sparks, MD, US) was catheterized for urine drainage. The rat received supplemental fluids during surgical preparation (5% bovine serum albumin in normal saline, BSA, A7906, Sigma, Oakville, ON, Canada). This infusion continued throughout the experiment with 1% BSA at about 1.5mL/hr.

Analytic methods

To obtain the hemodynamic data, rats were stabilized for 60 minutes following completion of surgical instrumentation. Baseline data were collected for 60 minutes, after which time RVP was selectively increased to 20 mmHg by graded constriction of the left renal vein or not manipulated (TC). Data

collection continued for a further 120 minutes. For hemodynamic experiments, blood samples (200 µl) were obtained at the beginning of the baseline period and every 60 min thereafter. Timed urine samples were collected every 30 minutes. No blood or urine sampling was completed during autoregulation experiments.

To determine GFR using FITC-Inulin, plasma and urine samples were diluted in 0.5 mol/l HEPES (pH 7.4) to maintain physiological pH. A 96-well black plate (Greiner, Monroe, NC, USA) was used for loading 50µl of each solution in duplicate. Fluorescence was determined using the Fluoroskan Ascent® Microplate Fluorometer (Thermo Fisher Scientific, Vantaa, Finland), at the excitation wavelength of 485nm and emission wavelength of 527nm.

Analysis and statistics

Hemodynamic data are presented as the average of consecutive 30 min intervals. The baseline characterization was compared between both ANG II clamped and ANG II absent rats using General linear model multivariate (MANOVA) with Bonferroni post-hoc test. To evaluate the impact of elevated RVP, Multiple linear model with repeated measurement was used to compare each time point of different RVP groups, using Bonferroni as post-hoc test. Data were log-transformed or ranked if not normally distributed. Data were analyzed using SPSS 24 (IBM, Armonk, NY, US).

To evaluate the RBF autoregulation, a baseline of RPP and RBF was obtained before any constriction of the aorta. For each step of RPP reduction, the RBF was allowed to stabilize for 1min and the average valve of 4 min interval was used to calculate the autoregulation curves. The autoregulation curves were plotted and analyzed in SigmaPlot 13 (Systat, San Jose, CA, USA) using nonlinear regression analysis. The lower limit of RBF autoregulation was defined as the perfusion pressure, where the third derivative of the fitted curve was 0 [5]. The lower limit of RPP for autoregulation was compared using 2-way

ANOVA with SNK post-hoc test. Statistical significance was accepted at $p<0.05$. All data are presented as means ± SEM.

4.3 Results

Increased RVP and hemodynamics in ANG II clamped and ANG II absent rats

There were no significant differences in the BW, HCT and baseline MAP between the ANG II clamped and ANG II absent groups. In the absence of ANG II, the baseline RBF, GFR and RVC were higher compared to the ANG II clamped group. HR was lower in the ANG II absent rats (313±6 vs. 352±5 bpm, $p<0.05$) (Table 4.1)

Systemically, an acute increase of RVP to 20 mmHg induced a gradual reduction in MAP from 102±1 to 78±5 mmHg ($p<0.05$) and a decrease in HR from 359±5 to 337±8 bpm ($p<0.05$) in ANG II clamped animals. However, MAP and HR were not influenced by the RVP in the ANG II absent rats, shown in Figure 4.1.

In the ANG II clamped rats, the increase of RVP did not decrease the RVC, similarly in the ANG II absent rats. In both groups, the inhibition of RAS did not prevent the reduction of RBF in response to an increased RVP. The reduction of RBF was from 6.3±0.8 to 4.5±0.8ml/min in the ANG II clamped ($p<0.05$) and from 10.8±1.2 to 9.6±1.2ml/min in the ANG II absent rats ($p<0.05$). The inhibition of RAS did not prevent the decrease of GFR. In the ANG II clamped rats, GFR was decreased from 1.34±0.09 to 0.61±0.14 ml/min in response to RVP elevation ($p<0.05$). Although it is not statistically significant, the GFR was reduced from 1.57±0.06 to 1.05±0.13ml/min. (Figure 4.2)

Increased RVP and autoregulation of RBF

The lower limit of RPP for the RBF autoregulation was calculated from the fitted sigmoidal autoregulation curve. BRF would decline progressively below that pressure. The lower limit of RPP for

RBF autoregulation at baseline RVP was 59±2 mmHg. Moderate increased RVP from 0.1±0.4 to 11.0±0.4 mmHg left-shifted the autoregulation curve. The lower limit was reset to 52±3 mmHg (n=8; p=0.053). A major increase of RVP from 0.7±0.2 to 19.1±0.4 mmHg in separate experiments caused a significant decrease of the lower limit to 43.6±4.3 mmHg (n=8; $p<0.05$). (Figure 4.3)

4.4 Discussion

In our previous study, a substantial increase in RVP by 20 mmHg caused a significant reduction in RBF, RVC and GFR [1]. In the current study, the RVP-induced reduction in RVC was prevented in the absence of dynamic ANG II modulation. This suggests that RVP-induced vasoconstriction is dependent on the modulation of ANG II. The importance of ANG II in mediating vasoconstriction was further confirmed in our ANG II absent rats, in which the RVP-induced reduction in RVC was abolished in the absence of ANG II. In the untreated animals from the previous study (Chapter 2), a 20 mmHg increase in RVP decreased the renal perfusion pressure to 80% of control, causing the RBF to decrease to about 55%. In the present study, ANG II inhibition alleviated the reduction of RBF to 69% in the ANG II clamped rats and 89% in the ANG II absent rats. This further supports the dominant role of ANG II in the vasoconstriction caused by the increased RVP. Furthermore, we demonstrated that autoregulation of RBF was intact but was reset to a lower level when RVP was elevated. In sum, these data support the active role of the RAS and renal autoregulation in the mediation of vasoconstriction in RVP elevation.

Our finding is consistent with the findings from others that RVP elevation increased renin secretion. In a report in 1972, increased RVP resulted in renin secretion in dogs [6]. Kishimoto *et al* also showed in dogs that increased RVP to 30 mmHg caused an increase in renin secretion rate. They suggested it might be triggered by the intrarenal hemodynamic changes that blood flow shifted from the outer to inner cortex [7]. Similarly, Kopp *et al* showed that raising RVP to 28 mmHg increased ipsilateral renin secretion rate in dogs, which was related to the reno-renal reflex [8]. Kastner, Hall and Guyton also had similar findings

that increased RVP to 30mmHg increased renin secretion. The increase was steeper when RVP was over 30 mmHg and renin secretion rate was 16 times control at a RVP of 50 mmHg [2]. Remarkably, in their study, GFR remained stable over a range of RVP elevation from 0-50mmHg. Furthermore, inhibition of the RAS caused a greater reduction in GFR in response to RVP increase, which is an observation in contrast to our finding. In our study, when the ANG II level was fixed or completely absent, RVP-induced reduction of GFR was milder. This might be due to milder RBF change in rats with inhibition of the RAS, which is different from their findings in dogs that the increased RVP caused greater reduction in RBF with inhibition of the RAS. Regardless of the different species, the RAS is actively involved in mediating the RVP-induced vasoconstriction.

Systemically, increased RVP decreased MAP and HR in the ANG II clamped rats, which indicates an inhibitory effect of the baroreflex in the absence of ANG II modulation. The question here is: how does the kidney communicate with the baroreceptor? The effect of ANG II on baroreflex control of HR is still debated. In our current study, the baroreflex was preserved in the absence of ANG II. It might be attributed to the absence of ANG II, as well as the use of AVP, which might potentiate the baroreflex [9, 10]. However, in our ANG II clamped rats, the absence of dynamic ANG II secretion did not prevent the impairment of the baroreflex. The lack of consistency might be due to variations of ANG II resetting the baroreflex in different specifies. ANG II has been shown to attenuate the sympathetic baroreflex function and reduce the baroreflex gain (HR/MAP) [11] only in the reflex decrease of HR during hypertension but not in the reflex increase during hypotension. In the same study, it was shown that ANG II did not act directly on the baroreceptor. There are also data showing that ANG II reset the baroreflex without altering the sensitivity. This indicates that the interactions among ANG II and other mediators, other than ANG II itself, are dominant in the systemic impact of increased RVP.

Our previous study has shown that increased RVP decreased HR but not MAP in untreated rats [1]. The decrease of HR was not prevented by a high salt diet when endogenous RSNA and RAS were presumably suppressed. However, the decrease in HR was not significant in the denervated rats. In addition, we previously showed that RSNA was suppressed during RVP elevation [1]. Furthermore, endogenous ANG II impairs the renal mechanosensory nerves therefore suppresses the afferent renal nerve activity [12, 13]. Another potential candidate that cannot be overlooked is nitric oxide (NO). NO has been shown to blunt baroreflex control of HR in lambs [14]. The baroreflex depressant action might be related to the activation of endothelial nitric oxide synthase [15]. In sum, increased RVP tends to blunt the baroreflex, probably through the suppression of RSNA and increased ANG II secretion, and NO might also be involved.

In this study, we also studied function and resetting of RBF autoregulation in response to RVP elevation. Increased RVP would cause an increase in afferent arteriolar pressure, which could trigger an autoregulatory adjustment that constricts the afferent arterioles. Increased glomerular capillary hydrostatic pressure might increase GFR, which would result in an increase in distal delivery, which could also trigger vasoconstriction in the afferent arterioles via TGF, thus reducing single nephron GFR. However, the increased RVP also increases the tubular pressure as well as interstitial pressure, which reduces the distal delivery and makes the overall response of TGF uncertain. Studies in rats and dogs have shown TGF was unaffected by an increase in RVP to 20 mmHg [16, 17]. Myogenic response on the other hand, has rarely been studied. Myogenic response could also contribute to vasoconstriction due to the increased afferent arteriolar pressure when RVP is increased. In conclusion, the role of autoregulation in RVP elevation has not been clearly demonstrated in the literature. Our finding that autoregulation of RBF is not impaired by the increased RVP indicates that autoregulation is another contributor to the RVP-induced vasoconstriction. This also shows that during acute venous congestion, the kidney is able to maintain relatively stable RBF in response to further fluctuation in RPP. However, in chronic venous congestion, more mediators might get involved to the complexity. For example, NO

contributes to the vasoconstriction that occurs when MAP is restored after a prolonged reduction for at least 10 min in spontaneously hypertensive rats [18]. Our finding indicates that autoregulation participates in the acute vasoconstriction response to the RVP elevation.

Taken together, our previous and current findings are consistent with a primary role of the RAS in the renal hemodynamic responses to increased RVP. The renal vasoconstriction induced by elevated RVP was prevented by inhibition of ANG II. In addition, RBF and GFR were better preserved without dynamic modulation of ANG II or in the absence of ANG II compared to the untreated animals. Increased RVP tends to blunt the baroreflex by the interaction between RAS and the other mediators. The intact RBF autoregulation and decreased lower limit in renal venous congestion is important because it protects the kidney in case of a sudden arterial pressure insufficiency, but it also causes vasoconstriction.

Perspectives

Our current study consists of two parts of investigations in the mechanisms behind RVP-induced vasoconstriction as well as possible systemic impacts. The role of RAS is of interest because pharmacological inhibition of the RAS is widely used in the treatment of congestive heart failure together with pharmacological decongestion. However, the impact of RAS inhibitors on renal hemodynamics and excretory function is not well studied in combination with diuretics. The intact autoregulation is also important in maintaining GFR at the cost of vasoconstriction. These mechanisms are studied under an acute increase of RVP. It is commonly assumed that inappropriate activation of autoregulation, particularly of TGF, contributes to the impaired renal function seen in heart failure. However, there is currently little evidence to support this assumption. Further investigations are needed to study the endogenous adaptations of the kidney in chronic venous congestion as well as in disease model.

Table 4.1 Baseline information in untreated control, ANG II clamped and ANG II absent rats.

	Untreated control #	ANG II clamped	ANG II absent
N	17	18	15
BW, g	373±8	395±11	382±7
HCT, %	43.8±0.5	46.2±0.7	45.5±0.4
MAP, mmHg	97±2	102±1	99±2
HR, bpm	370±5	352±5	313±6*‡
RBF, ml/min	7.2±0.6	6.1±0.5	10.2±0.8*‡
RVP, mmHg	0.4±0.1	0.2±0.2	0.2±0.2
RVC, ml/min. mmHg	0.075±0.006	0.060±0.006	0.103±0.008*‡
GFR, ml/min	1.42±0.07	1.37±0.08	1.64±0.06*‡

*Data were significantly different from untreated control ($p<0.05$)

‡Data were significantly different from the ANG II clamped group ($p<0.05$)

#Data from this group were published in (Huang et al., 2018)

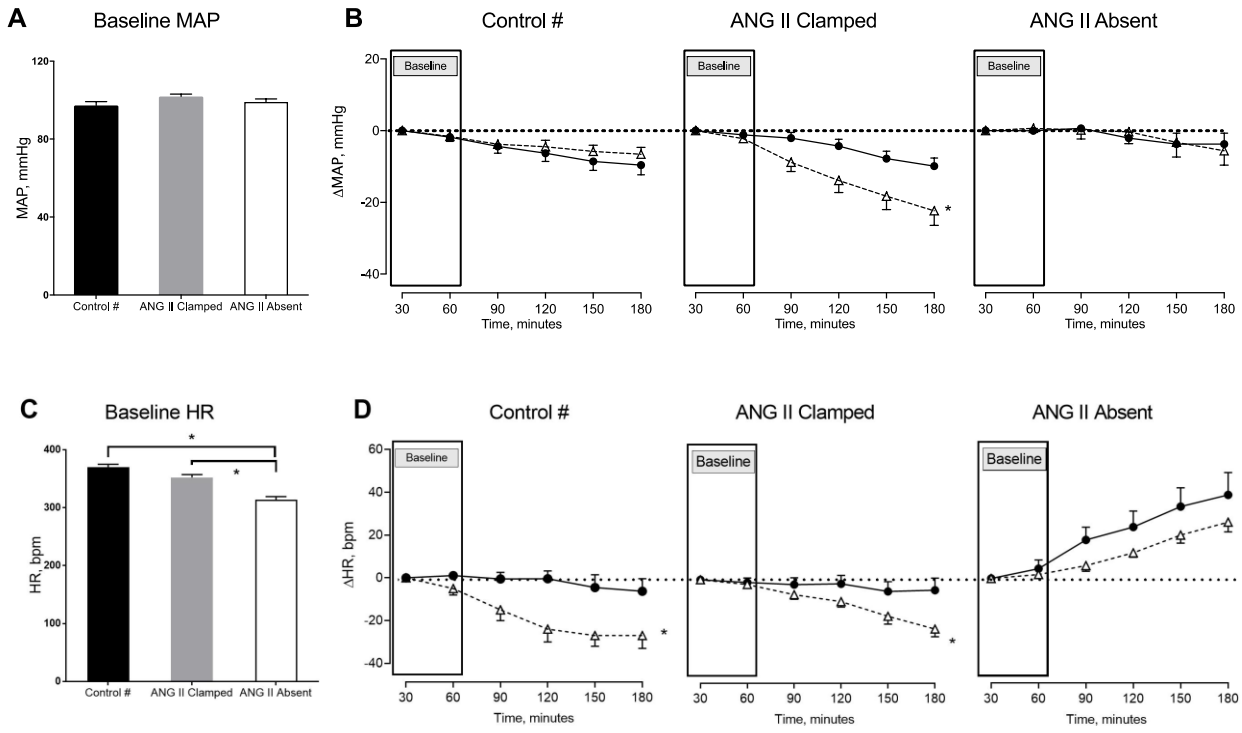

Figure 4.1 Changes in mean arterial pressure (MAP) and heart rate (HR) in response to increased RVP. The bar grafts represent the baseline MAP (A) and HR (C) comparisons among untreated control (black, control), ANG II clamped (grey, ANG II Clamped) and ANG II absent (open bar, ANG II Absent) rats. The line charts represent changes from baseline, ΔMAP (B) and ΔHR (D). In the line chart, the first two time points in each line represent baseline. The latter four time points represent either control (●, control) and major RVP elevation (△, RVP 20). There is no significant difference in the baseline MAP (A). Increased RVP caused a significant decrease in MAP in the ANG II clamped group (B) (*, $p<0.05$) but not in untreated control group or ANG II absent group. Baseline HR was significantly lower in the ANG II absent group compared to untreated control and ANG II clamped group (C) ((*, $p<0.05$). Increased RVP induced HR reduction in untreated control group and ANG II clamped group (*, $p<0.05$) but not in the ANG II absent group (D). #Data from these were published in (Huang et al., 2018)

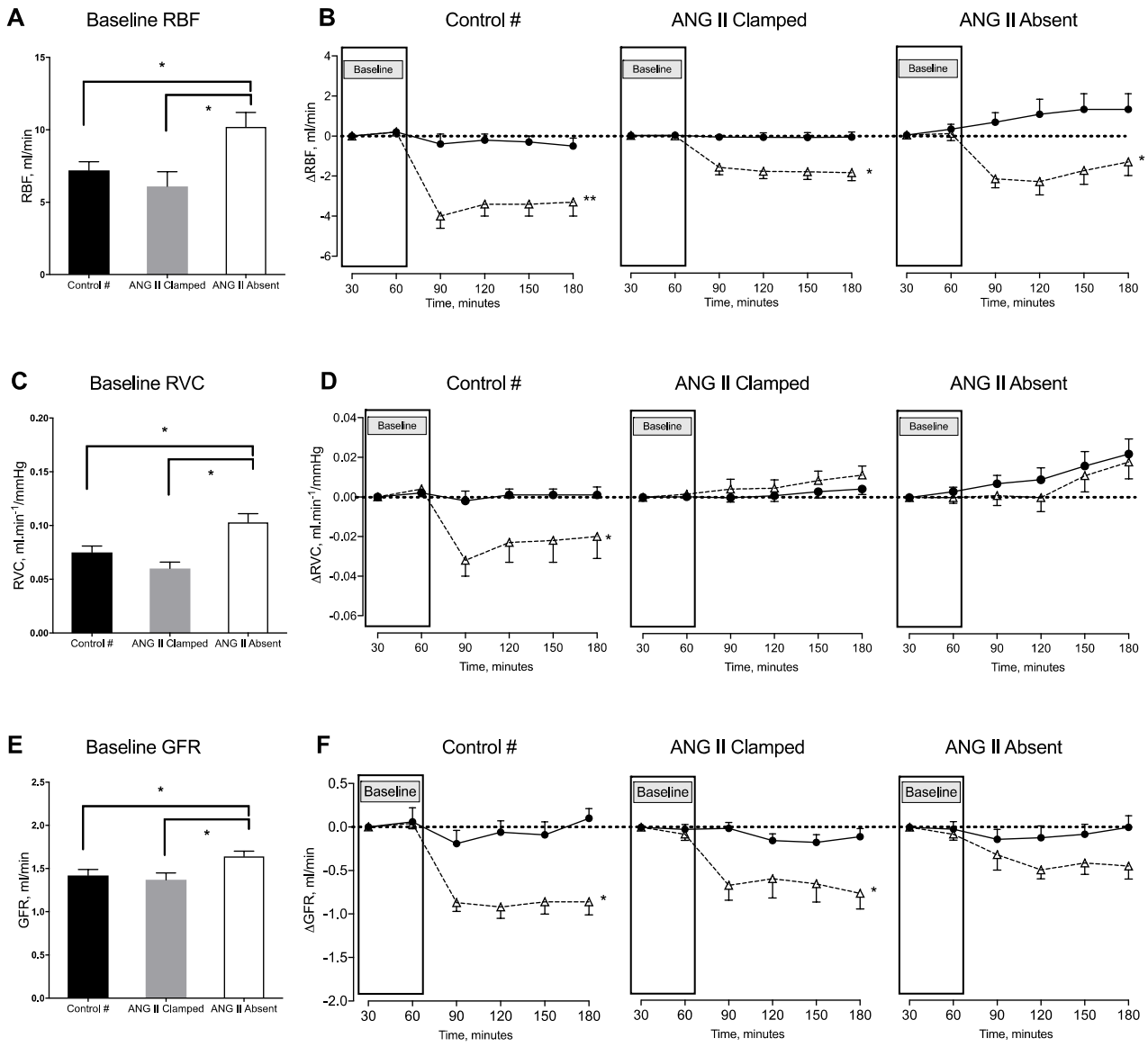

Figure 4.2 Changes in renal blood flow (RBF), Renal vascular conductance (RVC) and Glomerular filtration rate (GFR) in response to increased RVP. The bar grafts represent the baseline RBF (A), RVC (B) and GFR (C) comparisons among untreated control (black, control), ANG II clamped (grey, ANG II Clamped) and ANG II absent (open bar, ANG II Absent) rats. The line charts represent changes from baseline, ΔRBF (B), ΔRVC (D) and ΔGFR (F). In the line chart, the first two time points in each line represent baseline. The latter four time points represent either control (●, control) and major RVP elevation (Δ, RVP 20). Baseline RBF is significantly higher in the ANG II absent group compared to

untreated control and ANG II clamped group (A) (*, $p<0.05$). Increased RVP caused immediate decrease of RBF in untreated control group (**, $p<0.001$), RBF decreased in response to RVP increase in ANG II clamped or ANG II absent group in both groups (B) (*, $p<0.05$). Baseline RVC was higher in ANG II absent group (C) (*, $P<0.05$). Increased RVP caused a reduction in RVC in untreated control group (D) (*, $p<0.05$), but did not impact RVC in either ANG II clamped or ANG II absent group. Baseline GFR was higher in ANG II absent group (E) (*, $p<0.05$). Increased RVP caused significant reduction in GFR in both untreated and ANG II clamped rats (F) (*, $p<0.05$), while the decrease in ANG II absent rats was not statistically significant ($p=0.081$). #Data from these were published in (Huang et al., 2018)

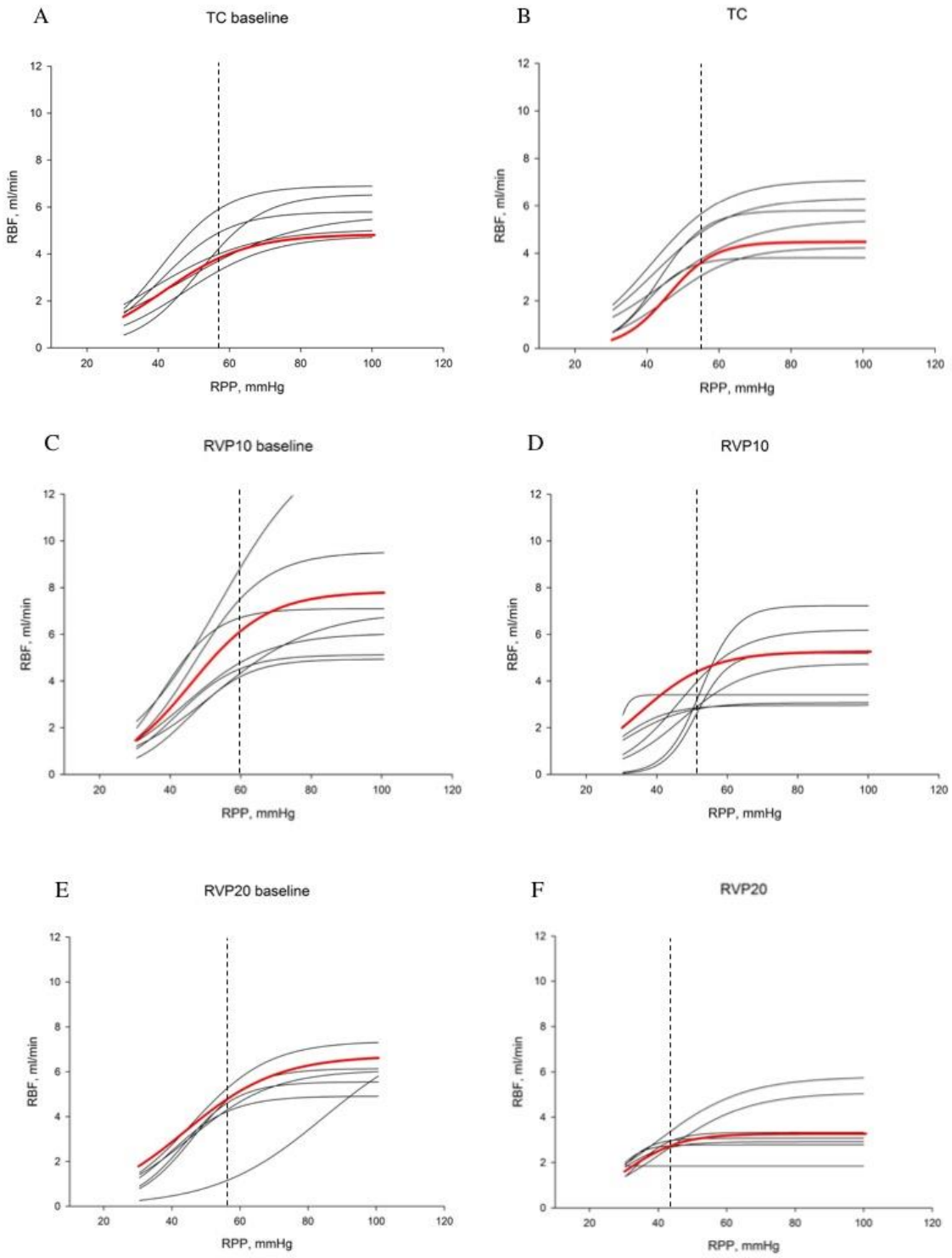

Figure 4.3 Renal blood flow (RBF) autoregulation curves. Each curve is generated by using nonlinear regression analysis. Three panels in the left (A, C and E) represent baseline autoregulation curves before RVP manipulation. Charts in the right represent the autoregulation curve without RVP elevation (B, TC),

in RVP elevation of ~10mmHg (D, RVP 10) and in RVP elevation of ~20mmHg (F, RVP 20). The red curve indicates the average of the curves. The lower limit of RBF autoregulation is indicated by a dash line.

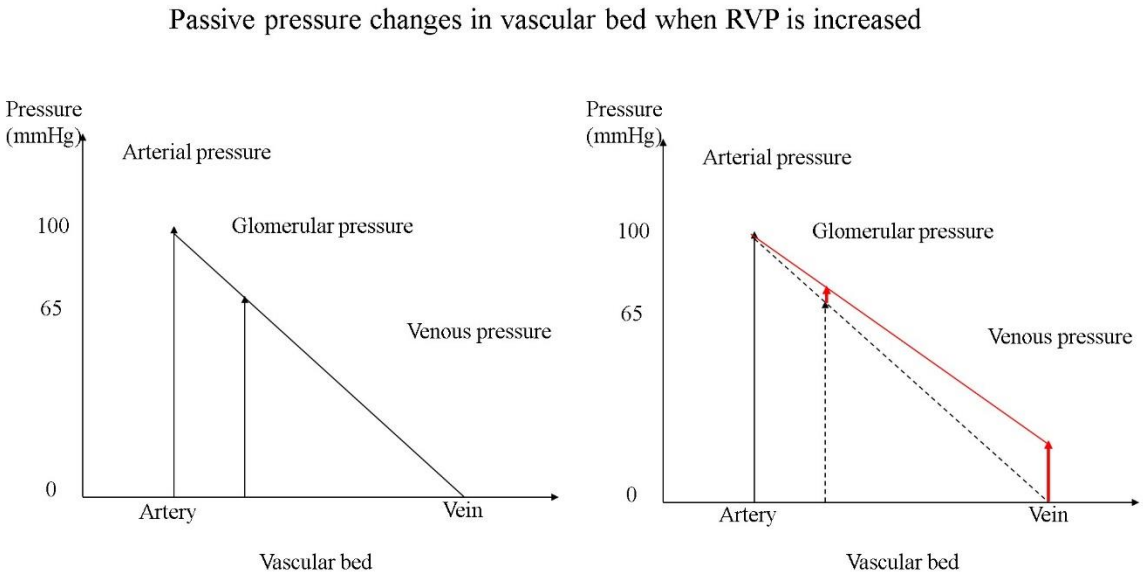

Figure 4.4 Passive pressure changes in vascular bed when RVP is increased. When pressure is increased in the venous system without neurohumoral adjustment, the glomerular capillary hydrostatic pressure is increased passively.

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CHAPTER 5:

Changes in urinary sodium excretion and tubular pressure in response to increased renal venous pressure

Introduction

This chapter contains data of urinary sodium excretion and tubular pressure. Although strong conclusions cannot be drawn due to technical challenge and missing data, we find that the results are adding to the comprehension of the renal response to increased RVP.

5.1 Increased RVP and sodium handling

Methodology

Urine samples collected from experiments described in Chapter 2, 3 and 4 (shown in fig 5.1) were used to measure the urinary sodium excretion ($U_{Na}V$). Urine flow data were analyzed using 2-Way Repeated Measures ANOVA with Bonferroni post-hoc test. Data were analyzed using SigmaPlot 13 (Systat, San Jose, CA, USA). A p value less than 0.05 was considered significant. All data are presented as means ± SEM.

Urinary (100 µl) and plasma (50 µl) sodium levels were determined by flame photometry (PFP7/C Clinical Flame Photometer, Stone, Staffordshire, UK). Increased RVP tended to increase sodium excretion in our experiments. However, the urinary sodium excretion could only be calculated in animals with sufficient urine output.

Results

Increased RVP caused a significant reduction in urine output in untreated rats (Figure 5.1 A, $p < 0.05$), denervated rats on a normal salt diet (Figure 5.1 C, $p < 0.001$), denervated rats on a high salt diet (Figure 5.1 D, $p < 0.05$) and ANG II absent group (Figure 5.1 F, $p < 0.05$).

In the absence of ANG II modulation, increased RVP enhanced natriuresis. In the untreated rats, 5 out of 8 untreated rats on the normal salt diet had increased fractional excretion of sodium (FE_{Na}) upon increased RVP (Figure 5.5 A) without significant changes in $U_{Na}V$ (Figure 5.3 A). The conclusion

that increased RVP enhanced natriuresis cannot be drawn, because there was substantial variation due to different urine output. In the untreated rats, 3 out of 8 rats did not have urine output after increasing RVP, which made assessment of $U_{Na}V$ impossible. Similar results were observed in the denervated rats, in which due to the low MAP, the net ultrafiltration pressure must have been quite low at baseline compared to the untreated rats. In the denervated rats, 4 out of 6 denervated rats had no urine output at the end of the experiment due to the increased RVP.

Rats on a high salt diet expectedly had higher baseline $U_{Na}V$ than rats on a normal salt diet (Figure 5.2). Increased RVP did not change $U_{Na}V$ in rats on a high salt diet (Figure 5.3 B) After an increase in RVP we observed a progressive increase in FE_{Na} in 3 out of 6 rats on a high salt diet. In denervated rats on either normal or high salt diet, increased RVP did not cause significant changes in $U_{Na}V$ (Figure 5.3 C, D). Denervated rats had progressively increased FE_{Na} (Figure 5.5 C). The increase in FE_{Na} was abrupt in denervated rats on a high salt diet (Figure 5.5 D). Both $U_{Na}V$ and FE_{Na} were increased after RVP elevation in the ANG II clamped rats (Figure 5.3 E and Figure 5.5 E). The ANG II absent rats had higher $U_{Na}V$ than other groups (Figure 5.2). In the ANG II absent rats, increased RVP induced an immediate initial increase of $U_{Na}V$ and FE_{Na} (Figure 5.3 F and Figure 5.5 F). Both $U_{Na}V$ and FE_{Na} then gradually decreased over time to baseline at the end of the experiment.

Discussion

Our findings in untreated rats are in line with one experiment done in dogs that acute increased RVP showed marked depression of water and sodium output [1]. However, there are studies showing opposite results. In one experiment done in dogs [2], a positive correlation was reported between $U_{Na}V$ and renal interstitial pressure upon stepwise, 10-15 min increases in RVP to 40 mmHg. Experiments

performed in isolated perfused rat kidneys [3] also demonstrated that stepwise, 10 minute increases in RVP to 25 mmHg increased sodium excretion. However, in the ANG II absent rats, the sodium excretion gradually returned to baseline level, which might be because that the natriuresis was prevented by treatment with vasopressin [3]. The abrupt increase in FE_{Na} in untreated rats and the abrupt natriuresis in the ANG II clamped / absent rats in our study showing an enhanced natriuresis after short duration increases in RVP would suggest rapidly responding factors such as autoregulation or renal nerves might be involved. We have shown that RSNA was suppressed in RVP elevation and renal denervation did not abrogate the increase in FE_{Na}. In addition, there are studies about acute increased arterial pressure eliciting similar rapid natriuresis enhancement [4, 5]. They showed that the elevation in arterial pressure without altering GFR is able be enhance natriuresis via trafficking of sodium transporters out of the apical membrane. Their findings are in line with ours. Although ANG II did not cause the pressure-dependent response, it would induce the redistribution of NHE3 transporters into brush-border microvilli [6] which increased sodium reabsorption. That explains our finding in the absence of ANG II modulation, increased RVP enhanced natriuresis. Taken together, our studies have shown that acutely increased RVP impacts the renal sodium handling through the depression of GFR, possibly the RAS and active sodium transport. Further steps would be to evaluate the segmental sodium handling and segmental sodium transporters as well as to evaluate the sodium handling in a chronic setting.

5.2 Increased RVP and tubular pressure

Introduction

The knowledge of tubular pressure in RVP elevation is essential for understanding of RVP-induced renal dysfunction, since the tubular pressure is a prime determinant of net ultrafiltration pressure, defined as the hydrostatic pressure gradient of glomerulus and Bowman's capsule minus glomerular oncotic pressure, where the hydrostatic pressure in the Bowman's capsule is exerted by tubular

pressure. Determinants of tubular pressure are tubular flow and resistance. Increased RVP might first increase tubular pressure due to the increased resistance. A positive correlation between increased RVP and intratubular pressure has been reported in the literature [7, 8]. However, increased RVP causes reduction in RBF and single nephron GFR. Eventually during high RVP, the very low proximal flow would offset the increase in tubular resistance and lead to a decrease in tubular pressure. In this context, we tested the free-flow intratubular pressure in RVP elevation.

All general aspects surgery and approach for this study was similar to the studies presented in Chapters 2-4. In this study, however, following exposure of the kidney, the kidney was placed in a Lucite kidney cup (Vestavia Scientific, Birmingham, AL, USA) and covered with warm moist cotton to avoid drying out. Great care was taken to avoid twisting of the renal pedicle and malposition of the kidney. The kidney in the Lucite cup was sealed with 6% agar. Once sealed, the cup was filled with warm normal saline to prevent evaporative fluid loss [9]. The rod of kidney cup was then clamped to the surgical table to stablize the kidney. Under the microscope, the first step is to map the tubule by injecting artificial tubular fluid (in mM: 135 NaCl, 5 KCl, 10 $NaHC0_3$, 1 $MgS0_4$, 1 $CaC1_2$, 1 Na_2P0_4/NaH_2P0_4, and 4 urea, pH 7.40) [10] with 0.2% fast green into a random tubule segment on the surface of the kidney using a localization pipette (5-10 μm tip diameter). The proximal and distal segments of the tubule was indentified by carefully observing the flow of the green bolus. The intratubular pressure was measured by direct puncturing the upstream segment of the localization pipette with the servo-null pressure pipette filled with 2 M NaCl (2-5 μm tip diameter) [11]. The servo-null system (Model 5A pressure system, Instrumentation for physiology and medcine, CA, USA)was used to measure the intratubular pressure. Before each experiment, the system was calibrated using a pressure pipette filled with 2 M NaCl inserting into a sealed well filled with normal saline and connected to a sphygmomanometer. Due to the different NaCl concentration between the inside and outside of the pressure pipette tip, the electrical resistances were different. The resistances were monitered by the

servo-null system. When the pressure pipette was inserted into the normal saline and the pressure at the tip changed mannuly by the sphygmomanometer, the servo-null system generated a counterpressure to maintain the resistance constant. The pressure was then transduced by the disposable blood pressure transducers (ADInstruments, CO, USA). During the experiment, baseline tubular pressure was measured for an average of 1 minute during 60 min baseline phase of the experiment. Tubular pressure at RVP elevation was measured after RVP was increased from 0.6±0.3 mmHg to 19.6±0.5 mmHg for another 120min. Tubular pressure increased in response to RVP elevation right after RVP was increased to ~20mmHg (Figure 5.6). However, there was not a significant correlation between increased RVP and tubular pressure due to the drop in tubular pressure during the last hour of the experiments.

Discussion

The increased tubular pressure opposes glomerular filtration and decreases net ultrafiltration pressure, which could contribute to the decreased GFR. Eventually, the low proximal flow leads to a decrease in tubular pressure. However, these data are not complete due to technical issue. The swelling of kidneys due to congestion leads to the leakage of seals around the kidney and forces the removal of micropipettes. Thus limited data can be acquiared at the end of the experiments.Taken together, our data support that increased RVP causes an acute increase in intratubular pressure. To better verify the segments of the tubules, to increase the samples size of the free-flow introtubular pressure measurement, as well as to measure the TGF response using stop-flow pressure are needed in the furture study.

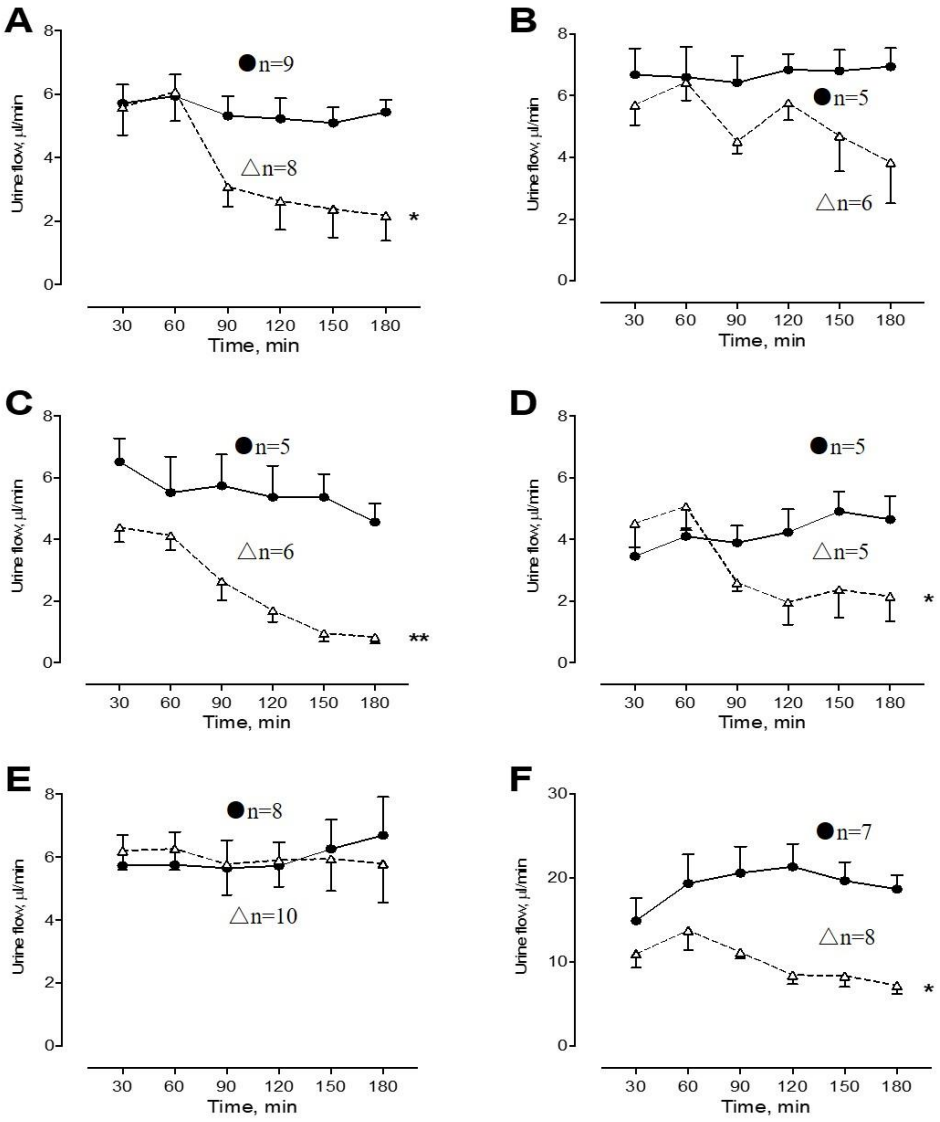

Fig 5.1 Urine flow in resposne to increased RVP. Urine samples were collected from experiments described in chapter 2, 3 and 4 (n: animal number). The line charts represent urine flow. The first two time points represent baseline. The latter four time points represent control (●, control) and major RVP elevation (△, RVP 20). Increased RVP resulted in a significant reduction in urine output in untreated rats (A, * $p < 0.05$), denervated rats on a normal salt diet (C, ** $p < 0.001$), denervated rats on a high salt diet (D, * $p < 0.05$) and ANG II absent group (F, * $p < 0.05$). In the denervated rats on a normal salt diet (B) and ANG II clamped rats (E), the urine reduction in response to RVP elevation were not statistically significant. Data were analyzed using 2-Way Repeated Measures ANOVA with

Bonferroni post-hoc test. Please note that the scale for the y-axis in ANG II absent group (F) is different due to the wide range of urine flow.

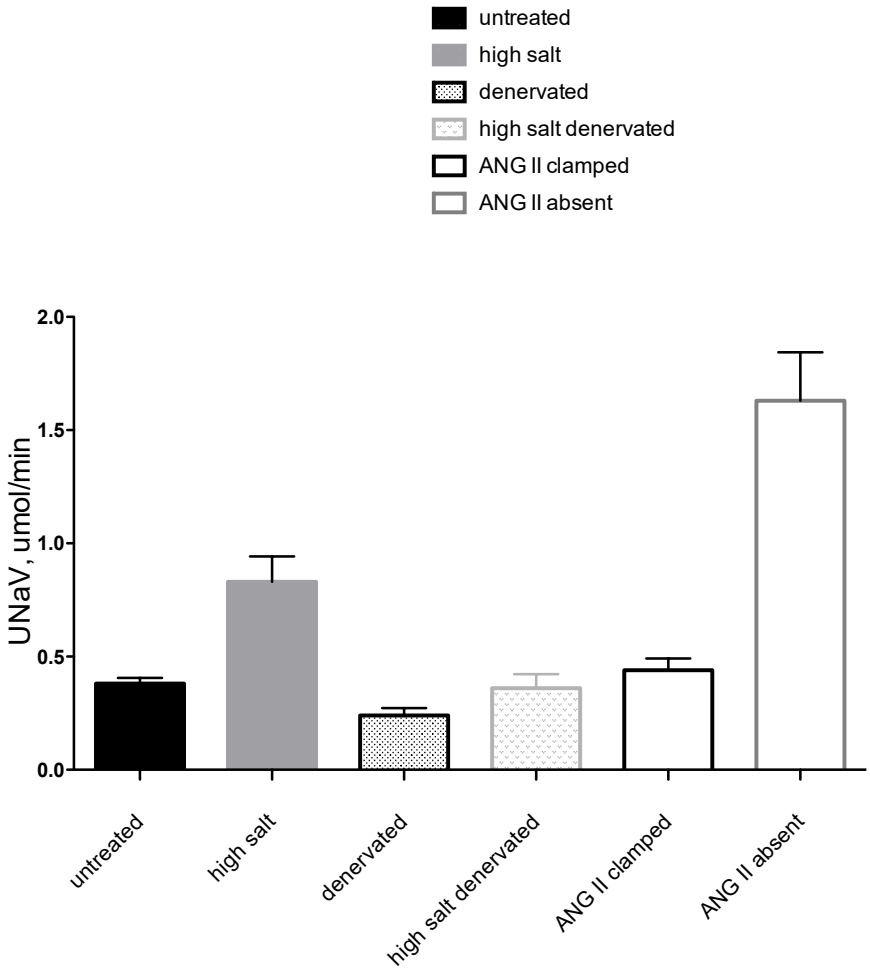

Fig 5.2 Baseline urinary sodium excretion ($U_{Na}V$). In comparison to the untreated rats (black box), rats on high salt diet (grey box) had higher baseline $U_{Na}V$. Denervated rats (black dash box) had lower $U_{Na}V$. Denervated rats on high salt diet (grey dash box) and ANG II clamped rats (black open box) had similar baseline $U_{Na}V$ as the untreated rats. $U_{Na}V$ was higher in the ANG II absent rats (grey open box).

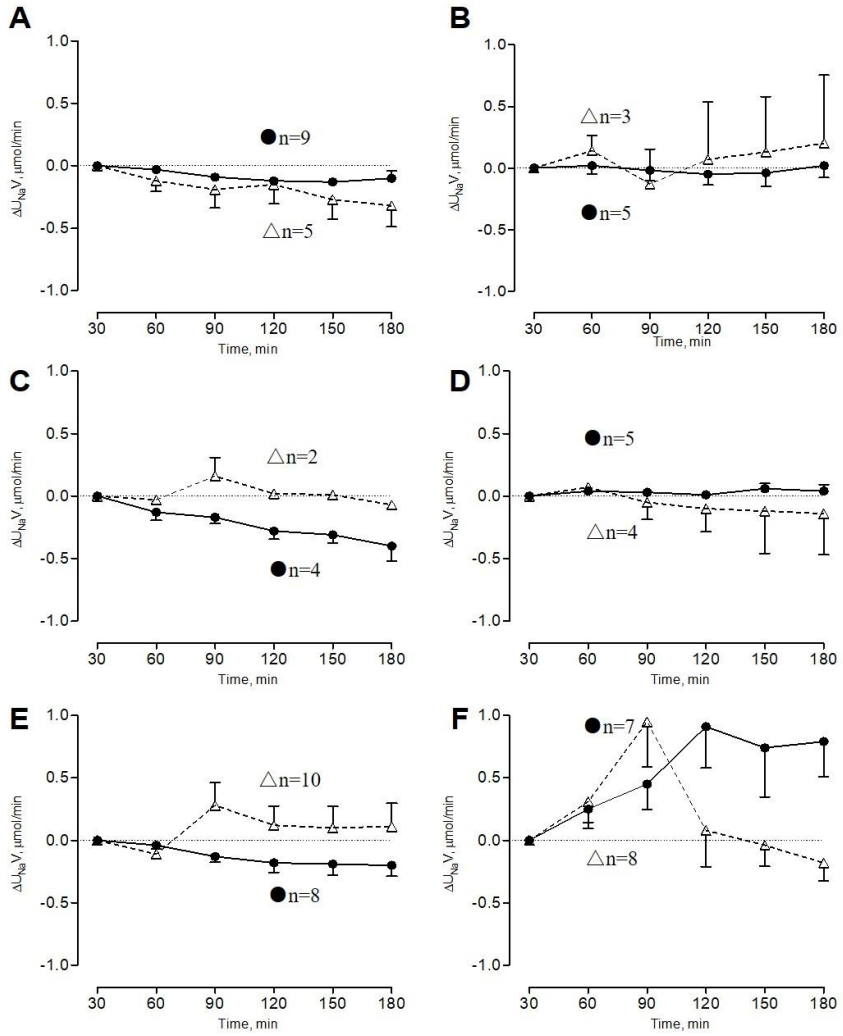

Fig 5.3 Changes in Urinary sodium excretion ($\Delta U_{Na}V$) in response to increased RVP. The line charts represent the changes from baseline $U_{Na}V$ (n: animal number). The first two time points in each line represent baseline. The latter four time points represent either control (●, control) and major RVP elevation (△, RVP 20). There is no difference in untreated rats (A), rats on high salt diet (B), denervated rats on normal (C) or high salt diet (D). Increased RVP increased $U_{Na}V$ in the ANG II clamped rats (E). There was an acute increase in $U_{Na}V$ after increased RVP followed by a progressive decrease in the ANG II absent rats (F).

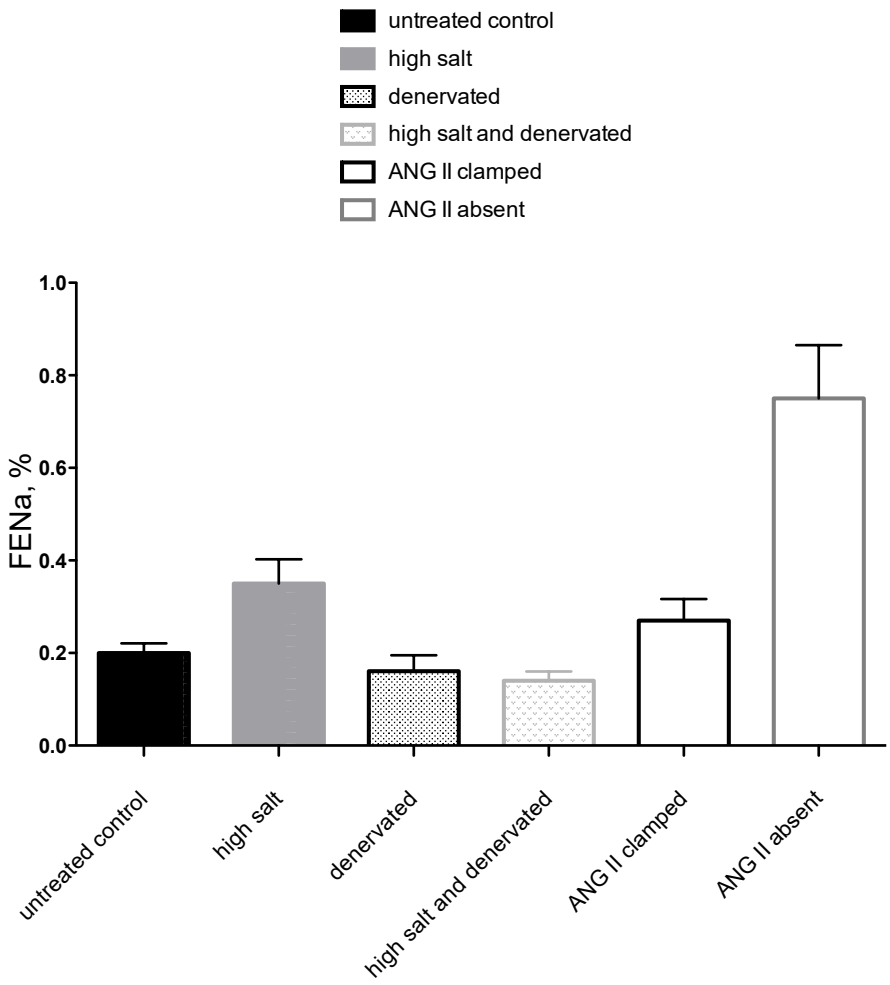

Fig 5.4 Baseline fractional excretion of sodium (FE_{Na}). In comparison to the untreated rats (black box), rats on high salt diet (grey box) had higher baseline FE_{Na}. Denervated rats (black dash box) had lower FE_{Na}, similar to the denervated rats on high salt diet (grey dash box). ANG II clamped rats (black open box) had higher baseline FE_{Na} than the untreated rats. FE_{Na} was higher in the ANG II absent rats (grey open box).

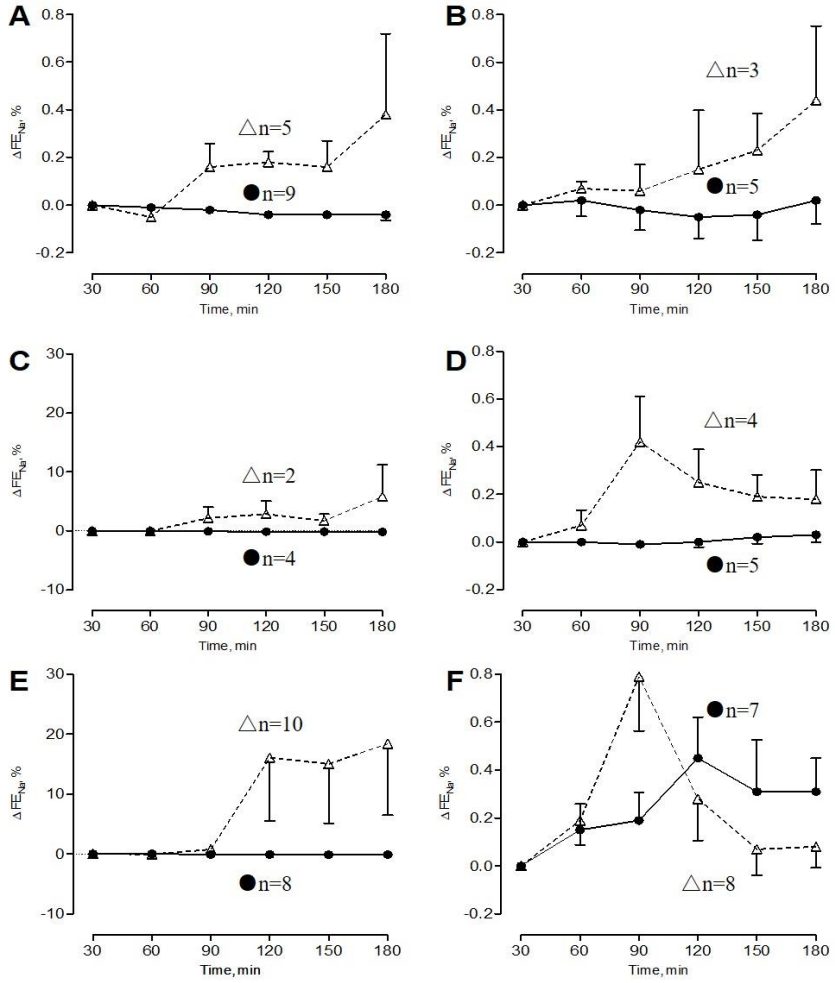

Figure 5.5 Changes in fractional excretion of sodium (ΔFE$_{Na}$) in response to increased RVP. The line charts represent the changes from baseline FE$_{Na}$ (n: animal number). The first two time-points in each line represent baseline. The latter four time points represent either control (●, control) and major RVP elevation (△, RVP 20). RVP elevation caused an immediate increase in the untreated rats (A), and a progressive increase in rats on high salt diet (B). The FE$_{Na}$ increased in response to RVP elevation in denervated rats (C), denervated rats on high salt diet (D). Increased RVP tended to increase FE$_{Na}$ in ANG II clamped rats (E) and ANG II absent rats (F). The acute increase in FE$_{Na}$ after increased RVP was followed by a progressive decrease in denervated rats on a high salt diet (D) and

ANG II absent rats (F). Please note that the scale for the y-axis is different for the groups due to the wide range of FE_{Na}.

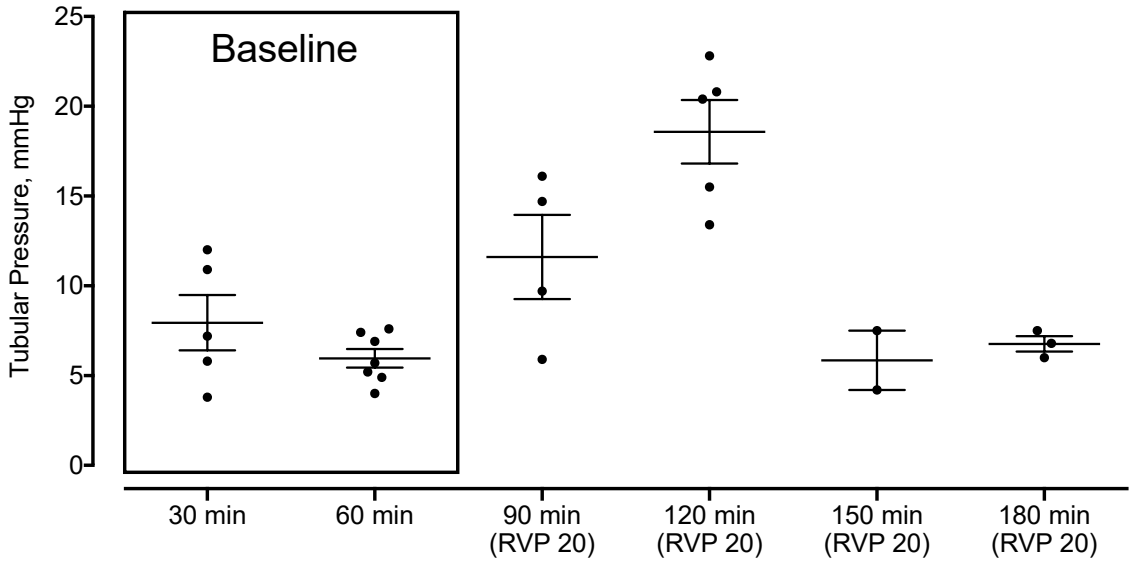

Figure 5.6 A scatter plot of tubular pressure in RVP elevation. Experiments were done in 7 rats on a normal salt diet. Each column gathered tubular pressure gained in 30 min. The first two columns represent the baseline when RVP was about 0.6±0.3 mmHg. The latter four columns represent tubular pressure in RVP elevated to 19.6±0.5 mmHg. The tubular pressure increased in response to RVP elevation. Although RVP was maintained about 20 mmHg through the whole procedure, tubular pressure returned to baseline level at the end of the study.

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CHAPTER 6:

Summary, Overall Discussion, Conclusion and Perspectives

Preamble

The research in this thesis was designed to study the impacts of increased RVP from several aspects including the renal responses to the increased RVP, potential mediators: RSNA and the RAS, as well as RBF autoregulation. This chapter is a summarized discussion of our major findings, limitations of our current study and future directions.

6.1 General discussion

Our data show that an acute isolated increase in RVP results in decreases in RBF, RVC and GFR. Surprisingly, RSNA is suppressed at higher levels of RVP. The result aligns with our data from the denervated rats, in which the bilateral denervation of renal sympathetic nerves does not prevent the decreases in RBF, RVC or GFR. RAS is probably mediating the renal vasoconstriction to an increased RVP. This is firstly supported by the results that a high salt diet attenuates the hemodynamic changes. Further experiments, in which clamped ANG II prevents the decreases in RBF, RVC and GFR at high RVP, also support the role of the RAS. Furthermore, we show that RBF autoregulation is intact but is reset to a lower perfusion pressure level during the RVP elevation, which is probably mediated by ANG II [1, 2]. This might also contribute to the RVP-induced vasoconstriction. In addition, an isolated increase of RVP decreases HR but not MAP in the untreated rats. The decrease in HR is not prevented by a high salt diet. However, the decrease in HR is not significant in the denervated rats. Increased RVP decreases MAP and HR in the ANG II clamped rats, but not in the ANG II absent rats. These indicate that increased RVP tends to impair baroreflex, which might be due to the supression of RSNA, inappropriate activation of the RAS and interaction with other modulators such as NO.

Regarding sodium handling, increased RVP modulates natriuresis through the impacts on GFR and active sodium transport. In the absence of ANG II modulation, increased RVP induces immediate natriuresis, which indicates an active role of the RAS in RVP-induced sodium retention. In the preliminary micropuncture experiments, increased RVP tends to increase proximal tubular pressure. It

is not clear whether and how this increase in the tubular pressure would impact on sodium transporters. For example, could the increased RVP cause the internalization of the sodium transporters? An acute increase in arterial pressure can induce redistribution and internalization of NHE3 [3] which suggests that changes in pressure might induce transporter changes. If the increased tubular pressure has similar influence, this could be another reason why the diuretics fails to reach ideal outcomes when there is increased venous pressure.

6.2 Conclusion and perspectives

First of all, increased RVP induces decreased RBF, GFR and RVC, as well as possible systemic influence through interactions among multiple modulators. Although RSNA is suppressed during RVP elevation, it is likely related to the systemic impacts on MAP and HR. RAS is primary in mediating the local vasoconstriction, which the intact autoregulation also contributes to. The inappropriate activation of RAS is also responsible for the sodium retention in RVP elevation. These suggest that the RVP-induced kidney dysfunction is complex and pharmacotherapy should aim at multiple pathways.

The increase in RVP turns out to have a significant systemic impact on the modulation of MAP and HR. Especially in the absence of ANG II modulation, the increased RVP blunts the baroreflex, which means that HR is not able to increase responsively when there is a drop in MAP. RBF autoregulation remains intact when RVP is increased. Although RBF decreases at a high RVP, it is autoregulated at a lower level. This suggests that the kidney in congestion is still able to maintain the stability in response to the blood pressure fluctuation at the cost of vasoconstriction.

The acute isolated increased RVP model is comparable to the venous congestion commonly found in heart or/and kidney disease. In a clinical situation, patients with congestion often present with oliguria. One of the contributors can be the inappropriate activation of the RAS which is due to the RVP elevation. Inhibition of the RAS might be beneficial for kidney function in congestive states. However,

inhibition of the RAS might have potential adverse impacts on MAP and HR control. Furosemide is widely used in congestive patients to remove eccessive fluid volume. It targets the NKCC2 and reduces sodium reabsorption in the thick ascending loop of Henle [4]. However, furosemide has potential negative effects on the renal hemodynamics, which we have extensively reviewed [5] (See Appendix section).

6.3 Limitations and future direction

We have used Lewis rats based on previously well-established model in combined heart and kidney disease [6, 7]. However, it is important to note the variations among different strains and species, which might be a limitation of our study. Another limitation is that these studies are only done in the male rats. Females could respond differently to RVP due to gender differences, such as in tubular tranport [8] and in the RAS [9]. Male rats have been shown to have greater ANG II level than female rats [10]. It has been shown in experimental studies that estrogen replacement reduces plasma ACE activity and circulating levels of ANG II [10]. Males and females also respond differently to ANG II stimulation [11]. It will be beneficial to explore the potential different responses in female sex. Involvements of other modulators such as NO, prostaglndin, endothelin and inflammatory factors could also be affected by sex.

Furthermore, the study is done using an acute isolated increased RVP. It is not sure whether the kdiney would respond similarly to a chronic increased RVP or compensate. Lastly, since isolated increased RVP is only the simplified model of venous congestion, further studies are needed to explore the renal response in systemic congestive states with associated increases in RVP such as CHF and CKD.

Increased venous pressure is now recognized as an important risk factor to the impaired kidney function and worse outcomes in patients with CHF. The clinical management for the renal congestion is still far from satisfactory. So far, there are no data regarding direct measurement of renal congestion in clinic. We recognize that while the exploration for physiological and pathophysiological

mechanisms shall be continued, to seek a safe method to measure and monitor RVP in clinical practice is also needed.

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Appendix

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TRANSLATIONAL PHYSIOLOGY

Everything we always wanted to know about furosemide but were afraid to ask

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Huang X, Dorhout Mees E, Vos P, Hamza S, Braam B. Everything we always wanted to know about furosemide but were afraid to ask. *Am J Physiol Renal Physiol* 310: F958–F971, 2016. First published February 24, 2016; doi:10.1152/ajprenal.00476.2015.—Furosemide is a widely used, potent natriuretic drug, which inhibits the Na^+-K^+-$2Cl^-$ cotransporter (NKCC)-2 in the ascending limb of the loop of Henle applied to reduce extracellular fluid volume expansion in heart and kidney disease. Undesirable consequences of furosemide, such as worsening of kidney function and unpredictable effects on sodium balance, led to this critical evaluation of how inhibition of NKCC affects renal and cardiovascular physiology. This evaluation reveals important knowledge gaps, involving furosemide as a drug, the function of NKCC2 (and NKCC1), and renal and systemic indirect effects of NKCC inhibition. Regarding renal effects, renal blood flow and glomerular filtration rate could become compromised by activation of tubuloglomerular feedback or by renin release, particularly if renal function is already compromised. Modulation of the intrarenal renin angiotensin system, however, is ill-defined. Regarding systemic effects, vasodilation followed by nonspecific NKCC inhibition and changes in venous compliance are not well understood. Repetitive administration of furosemide induces short-term (braking phenomenon, acute diuretic resistance) and long-term (chronic diuretic resistance) adaptations, of which the mechanisms are not well known. Modulation of NKCC2 expression and activity in kidney and heart failure is ill-defined. Lastly, furosemide's effects on cutaneous sodium stores and on uric acid levels could be beneficial or detrimental. Concluding, a considerable knowledge gap is identified regarding a potent drug with a relatively specific renal target, NKCC2, and renal and systemic actions. Resolving these questions would increase the understanding of NKCCs and their actions and improve rational use of furosemide in pathophysiology of fluid volume expansion.

extracellular fluid volume; natriuresis; renal function; chronic kidney disease; heart failure

DIURETICS BLOCKING THE Na^+-K^+-$2Cl^-$ cotransporter (NKCC) have an important place in the treatment of fluid overload, specifically in the context of kidney disease and heart failure (64). Of the drugs that inhibit the NKCC2 in the loop of Henle (furosemide, bumetanide, torsemide, ethacrynic acid), furosemide is most commonly applied (66) and >40 million prescriptions are dispensed every year in the USA (66a). However, many uncertainties remain about intrarenal and systemic actions of furosemide, including its two main targets NKCC1 and NKCC2.

Clinically, there are a number of undesirable consequences of the use of furosemide, of which the pathophysiological mechanisms have not been sufficiently investigated. Furosemide has been associated with worsening of kidney function in patients treated for volume overload admitted for acute heart failure (104) and even glomerular filtration rate (GFR) responses to furosemide in healthy subjects are variable (5, 13, 14, 31, 42, 51, 71, 91, 100, 102, 115, 120, 121, 133, 147, 158, 169). The very strong and short actions of furosemide have been associated with rebound sodium retention, and it has been brought to question whether furosemide would allow the reaching of a new steady state. Furthermore, knowledge about interactions between factors affecting loop of Henle transport and macula densa sensing of chloride is incomplete. This is important in relation to the function of the tubuloglomerular feedback (TGF) mechanism (72, 167) and to regula-

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tion of renin release (81). Altogether, this makes the impact of furosemide on natriuresis, on volume status, and on renal hemodynamic function unpredictable. Scientifically, these and other observations teach us that NKCC inhibition has substantially more consequences than merely inhibiting loop of Henle sodium and potassium reabsorption.

What makes furosemide an interesting scientific tool is that it has a specific target and impacts multiple aspects of integrative physiology. This paper reviews the actions of furosemide, focuses on unanswered questions, and provides areas where more scientific information could lead to a better understanding of targeting NKCC with furosemide. This eventually could lead to a better knowledge of NKCC transporters and of extracellular volume control. Understanding the entire profile of actions of the inhibition of NKCC better would eventually make furosemide a more effective clinical tool.

Pharmacology, Pharmacodynamics, Pharmacokinetics of Furosemide

Furosemide, 4-chloro-*N*-(2-furyl-methyl)-5-sulfamoyl-anthranilic acid (94), is a 330.7 mol wt member of the sulfonamides, which inhibits the NKCC in the thick ascending limb of the loop of Henle (114). Although insoluble in water, it remains stable in gastric and duodenal juice, bile, and urine (11). Furosemide strongly binds to plasma proteins (91–99%) (34), particularly to anionic sites on albumin (20). Most intestinal furosemide absorption occurs in the stomach and small intestine (30, 58, 160). Although the mean availability of oral furosemide is ~60%, absorption ranges from 10–100%. This is due to differences in product formulation, gastric pH and emptying, to timing of dosing in relation to food ingestion, and to disease conditions, like congestive heart failure (59). Renal actions peak within 1 h after oral and within 5 min after intravenous administration. There is substantial interindividual variability in bioavailability. The half-time (T½) of furosemide ranges from 0.5–2 h (160), but can be prolonged in renal failure. The duration of natriuretic effect is supposedly ~6 h after oral administration (lasts Six) and ~2 h after single-dose intravenous administration (165), yet can vary substantially. Of note, the tubular concentration of furosemide determines its natriuretic effect, and the urine concentration of furosemide has been used as a surrogate for the tubular concentration (165). Furosemide mainly enters the proximal tubular lumen via secretion by the organic anion transporter-1 (OAT1), while glomerular filtration is limited due to the high protein binding (164). Approximately 65% of furosemide is excreted unchanged in urine (11). Furosemide is also metabolized by renal and hepatic glucuronidation and subsequent secretion in urine and in bile (82, 126). The renal glucuronidation by renal UDP-glucuronosyltransferases UGT1A9 and UGT1A1 and clearance has been suggested to be most important (82). Furosemide directly increases urinary Na^+, K^+, and Cl^- excretion. Furosemide increases kaliuresis indirectly by promoting K^+ secretion by increased distal tubular fluid flow (99). Distal Ca^{2+} reabsorption is facilitated by the reduced luminal charge created by diminished sodium reabsorption and potassium recycling, effectively transforming NKCC into an electrogenic NaCl transporter (92). Furosemide can inhibit proximal tubular carbonic anhydrase (CA), leading to increased urinary excretion of HCO_3^- and phosphate (26).

Furosemide Targets the NKCC Transporters, CA, and GABA Receptors

The gene encoding for the NKCC has two isoforms, NKCC1 and NKCC2. The NKCC1 isoform is present in a wide variety of tissues, including the basolateral membrane of secretory epithelia, smooth muscle cells, fibroblasts, and red blood cells (55). Interestingly, in vascular endothelial cells, an increase in extracellular tonicity as small as 10 mOSm can cause significant stimulation of NKCC activity, which in turn increases cell volume (113). In contrast, the NKCC2 isoform is exclusively localized in the kidney, where it resides in the luminal membrane of the tubular epithelial cells of the thick ascending limb of the loop of Henle and of the macula densa (56). At least three different full-length isoforms of NKCC2 are derived from differential splicing: NKCC2A, NKCC2B, and NKCC2F. Each isoform is expressed differently along the thick ascending limb of Henle. Although the functional significance of these three isoforms is uncertain (117, 136), and factors affecting the level of expression are not fully understood, differential splicing of NKCC2 seems to be modulated by dietary salt intake. Dietary sodium restriction enhanced the expression of the high-affinity NKCC2B isoform and reduced the low-affinity NKCC2A isoform (136). Reports regarding selectivity between NKCC1/2 of furosemide are limited, yet indicate that furosemide has no selectivity for the two isoforms when NKCC1 is measured in the active state (60).

Both in vitro and in vivo studies show that furosemide inhibits CA by SO_2NH_2 moieties acting as an effective zinc-binding property of CA (130). Inhibition of different isoforms of CA I, II, and XI by furosemide varies (26, 130, 148, 150). The inhibition of CA I has been reported to result in vasodilation and a reduction in arterial blood pressure (BP) (129). Furosemide is also an antagonist at $GABA_A$ receptors (67), perhaps by allosteric modulation (88). $GABA_A$ receptors have been implicated in certain signal transduction cascades, such as mitogen-activated protein (MAP) kinase cascade (119). Reports of all of these actions of furosemide are limited, and the relevance of these effects is not well defined.

Questions Pertaining to the Natriuretic Response to Furosemide

Are renal or systemic hemodynamic effects of furosemide important for its natriuresis? Furosemide could limit or enhance its own diuretic actions in several ways. Since this subject has not been extensively investigated, scientific underpinning is relatively poor. A bolus injection of furosemide results in a strong natriuresis with fractional sodium excretion in healthy individuals exceeding 25% (13) and with peak Na^+ excretion of ~5 mmol/min. Obviously, this would decrease plasma volume (PV), if refill rates from the extracellular fluid volume (ECFV) are not sufficient to keep up with the rate of sodium and volume loss, resulting in *1*) a decrease in BP and pressure natriuresis; *2*) activation of the renin-angiotensin system (RAS) (on top of what already happens if the NKCC2 transporter in the macula densa is blocked); and *3*) activation of the sympathetic nervous system. Indeed Tucker and Blantz (159) reported a decrease in mean arterial pressure in animals

treated with furosemide, but without volume repletion (159); this was accompanied by a decrease in single nephron glomerular filtration rate (SNGFR), surprisingly mediated by a decrease in glomerular permeability coefficient LpA, but not by a decrease in net ultrafiltration pressure. Surprisingly, Costa et al. reported similar decreases in MAP after furosemide administration in rats in normovolemic vs. volume-expanded animals (33). To complicate this further, furosemide could elicit a natriuresis in an individual with decompensated heart failure, leading to a more favorable end-diastolic pressure and increased cardiac output (98). Clearly, a decrease in BP and renal perfusion pressure will limit the diuretic actions of the drug, yet whether this happens under normovolemic and hypervolemic states is not well characterized.

Activation of the RAS is similarly complex. While there is ample data to support that furosemide leads to increases in circulating levels of renin, data documenting how furosemide (and other diuretics) affects components of the intrarenal RAS is absent. This is particularly relevant with respect to intratubular and intrarenal levels of ANG II, which shows very substantial compartmentalization and have been reported to be up to 1,000 and 100 times plasma levels of ANG II, respectively (19, 143). Conversely, data about the natriuresis in response to furosemide in the absence and presence of inhibitors of the RAS is limited and not consistent. Two studies indicated that the fractional sodium excretion was diminished when the angiotensin-converting enzyme (ACEi) captopril was acutely administered before furosemide in humans with heart failure; however, the captopril dose led to substantial hypotension (48, 101). Motwani et al. (107) observed that an ultralow dose of captopril (1 mg), which did not decrease MAP in heart failure patients, enhanced the natriuretic actions of furosemide, whereas a higher dose (25 mg) decreased MAP and attenuated the natriuresis. In a chronic setting, ACEi seems to enhance the natriuretic response to furosemide in heart failure (53). It would be an interesting option to specifically target the renal RAS [e.g., by using lysozyme-modified captopril (61)] to prevent the acute BP effects of ACEi. Altogether, there is uncertainty about the regulation of systemic and renal RAS in response to furosemide and, conversely, about the renal response to furosemide in the presence and absence of an intact RAS.

Data about the renal sympathetic nervous system are more complex. Several studies have demonstrated that furosemide can directly activate the renal sympathetic nervous system (124), independent from the RAS (123). This could well be elicited by a change in the local micro-environment of the afferent nerve ending in the renal interstitium (52). Nevertheless, renal denervation did not affect the natriuresis evoked by furosemide acutely (146) in lambs or chronically (122) in rats under physiological conditions. Other studies revealed an enhanced natriuretic response to furosemide in healthy animals with acute unilateral denervation (125). This, however, might be difficult to interpret, since unilateral denervation will lead to a reno-renal reflex. Data on renal nerves, furosemide, and natriuresis in (experimental) chronic kidney disease (CKD) or heart failure is absent. Altogether, it remains unclear under which conditions direct activation of the afferent renal nerve endings by furosemide inhibits the natriuretic response to furosemide.

The two remaining issues in this section concern the two mechanisms that form the basis for autoregulation of renal blood flow (RBF) and GFR, myogenic response (MR) and TGF. The group of Loutzenhiser has shown that furosemide affects MR, so that autoregulatory behavior is impaired (163). Although others reported less attenuation of autoregulation (75), any attenuation of MR would render sodium excretion upon furosemide administration more dependent on renal perfusion pressure. This is particularly relevant in conditions with fluid congestion and hypotension, such as advanced heart failure, in which furosemide would be applied. Altogether, furosemide can impair renal autoregulation; whether this is relevant for sodium excretion is unknown. As mentioned, furosemide can block the TGF system by inhibiting the sensing of the Cl^- concentration in the macula densa. This would deactivate the TGF system and increase single nephron GFR and thereby filtered load. Conversely, furosemide increases the macula densa NaCl delivery and could activate the TGF system, depress GFR, and diminish filtered load. It is not clear whether tubular concentrations achieved by pharmacological use of furosemide tip the balance between these opposing forces toward an attenuated or activated TGF response. To illustrate this further, Fig. 1 shows the response to low-dose administration of furosemide and of the CA inhibitor acetazolamide in healthy subjects. Acetazolamide led to a consistent increase in lithium clearance (which can be used as estimate of distal delivery) and a consistent decrease in GFR. Furosemide led to a highly variable response in GFR, although never clearly increased GFR. How GFR would respond to furosemide in patients on multiple medications and with multiple co-morbidities is entirely unclear.

What determines the braking phenomenon and is there a fundamental difference with short-term furosemide resistance? The braking phenomenon is the decrease in the response to furosemide after the first dose (165) and is considered a physiological response to avoid ECFV contraction. Some data suggest that it can be prevented by restoring the diuretic-induced loss of ECFV (21). Confusingly, other data suggest that the braking phenomenon induced by the loop diuretic, bumetanide, is volume independent (4). In short-term therapy, more pronounced volume depletion will trigger more compensatory mechanisms to preserve the ECFV, which has also been denoted as acute diuretic resistance (57). Both the braking phenomenon and short-term resistance could be caused by postdiuretic Na^+ retention (7). This also happens when furosemide is suddenly withdrawn (106). Dietary Na^+ is a critical factor (43): during high Na^+ intake, the compensatory increase in Na^+ reabsorption between doses can lead to neutral Na^+ balance; to achieve a negative Na^+ balance, a dietary Na^+ restriction is required (7, 165).

Although a potential mechanism explaining rebound sodium retention is that furosemide could induce hyperaldosteronism, increases in aldosterone similar after 1 and 3 days of furosemide administration and 4 wk administration furosemide with or without spironolactone had similar effects on sodium balance (96). Moreover, neither ACEi nor ARB treatment could prevent acute diuretic resistance, suggesting that activation of RAAS is not responsible (81, 90). Altogether, it seems more plausible that acute volume depletion is to a certain extent related to acute resistance (57), yet the available data do not firmly rule out other mechanisms (90).

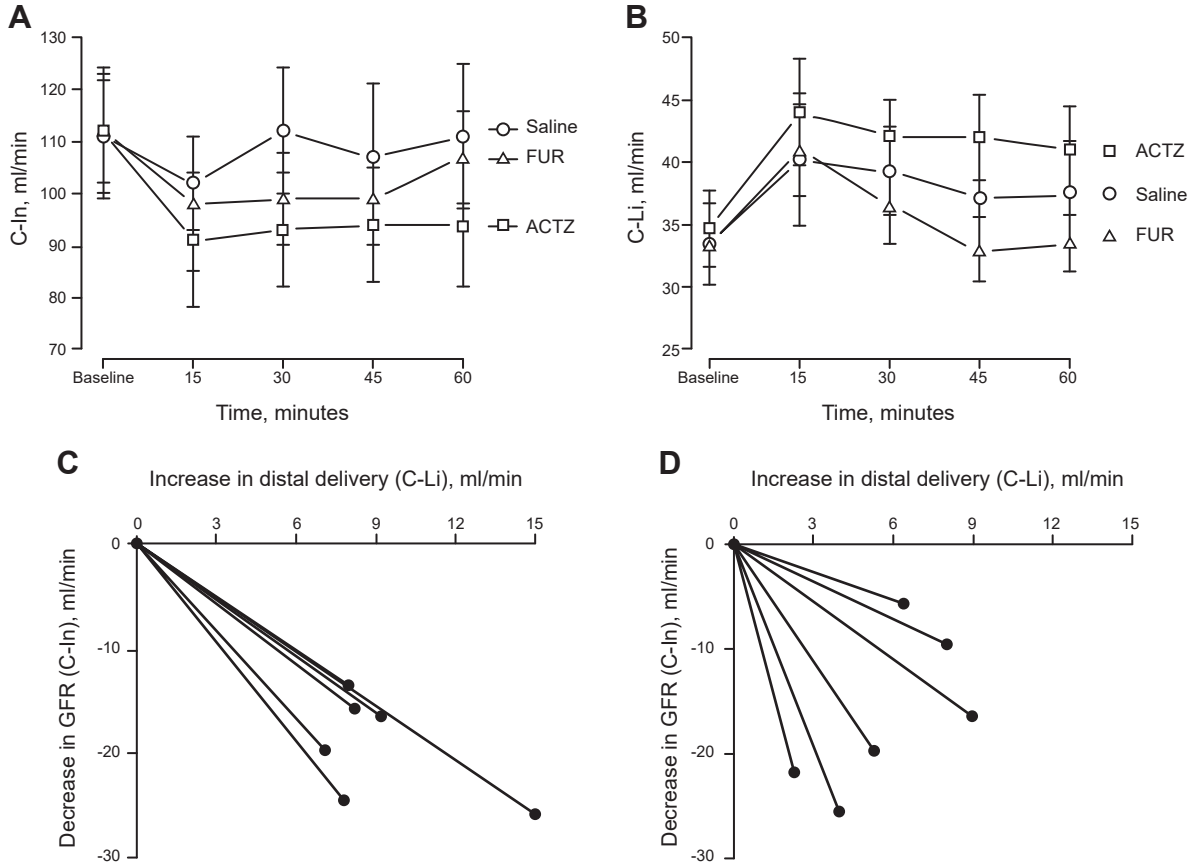


Fig. 1. Responses in inulin clearance (C-In; estimate for GFR); *A*) and lithium clearance (C-Li; estimate for distal delivery); *B*) in humans acutely treated with acetazolamide (ACTZ) 250 mg iv (proximal diuretic, activates TGF), furosemide (FUR) 2.5 mg iv (loop diuretic, variable response), and 2 liters iv NaCl 0.9% administered in 30 min (Saline). ACTZ quickly decreased C-In, as did FUR. Saline had only a quick effect on C-In. This together with literature data suggests that ACTZ can be used to test whether the TGF system can be further activated. When one relates the increase in distal delivery, assessed using C-Li, and the decrease in GFR, assessed by C-In, ACTZ results in a very consistent decrease in GFR (*C*) and FUR was less consistent (see text for explanation; *D*) (P. Vos, B. Braam, H. Koomans, unpublished data).

Incomplete understanding of braking and acute resistance is illustrated by the absence of good strategies to prevent these phenomena. A strategy could be to administer furosemide continuously and intravenously, allowing stable tubular drug delivery. Indeed, some studies report continuous furosemide infusion to be more effective to reduce ECFV in heart failure and severe fluid overload (118, 128). A large study in heart failure patients, however, was unable to demonstrate that continuous intravenous furosemide was more effective in decreasing volume overload than bolus injections (46). Altogether, this leaves the nature of the mechanisms of braking and resistance unanswered as well as how this could be prevented.

Could long term use of furosemide lead to enhanced reabsorption in other nephron segments? Several mechanisms could lead to enhanced reabsorption in other segments than the loop of Henle during long-term use of furosemide. Empirically this has led to the notion of diuretic synergism: thiazides are used to block the adapted sodium reabsorption in the distal nephron to enhance the diuretic effect of chronic furosemide therapy (7, 43).

First of all, furosemide could increase the expression of Na^+ transporters in other segments. Indeed, long-term use of furosemide can increase the abundance of NKCC2 in the thick ascending limb and OAT1 in the proximal tubule (84). Furthermore, furosemide infusion increases the abundance of all three subunits of the epithelial Na^+ channel in connecting tubules, cortical collecting ducts, outer medullary collecting ducts, and inner medullary collecting ducts (108). Chronic furosemide administration has also been associated with an increased Na^+-K^+-ATPase expression in the distal convoluted tubule and in cortical collecting duct (135), suggesting the possibility of enhanced capacity in sodium reabsorption in these segments. However, using micropuncture furosemide has been shown to enhance reabsorptive capacity of distal segment only in rats during sodium depletion (144). The question remains whether this adaptation is significant for the effect of chronic furosemide?

Second, furosemide could lead to hypertrophy of tubular segments. Furosemide administration for 6–7 days causes hypertrophy of the distal tubule (44, 77), connecting tubule and principal cells of the collecting ducts (78). This hypertrophy is associated with increases in Na^+ transport capacity (43). Continuous infusion of furosemide results in a substantial increase in the size of distal cells (43). The same adaptation of distal convoluted tubule has been demonstrated in humans during long-term use of furosemide (96). As a compensatory process, Na^+ that escapes from the loop of Henle could, therefore, be partially reabsorbed at more distal sites, decreasing overall

diuresis (84). According to Kaissling and Stanton (78), furosemide-induced increases in distal Na^+ concentration is a stimulus for epithelial cell growth in distal tubules in animal models.

Proximal tubular Na^+ reabsorption in response to furosemide treatment could, on the one hand, be diminished by direct furosemide actions, or, on the other hand, be enhanced by indirect actions of furosemide to stimulate renin and ANG II. In dogs, furosemide did not affect proximal tubular sodium reabsorption, yet decreased GFR. Meanwhile, furosemide inhibited the reabsorption of a saline drop in the proximal tubule so that the decrease in GFR might have obscured the effects of furosemide on proximal tubular reabsorption (86). Micropuncture experiments in dogs (39) and monkeys (12) do not show diminished proximal tubular reabsorption during short-term furosemide administration. However, administration of furosemide inhibits proximal tubular reabsorption in the rat, if great care is exercised to prevent retrograde flow of tubule fluid (22). Another study in rats indicates no inhibition of reabsorption, unless filtration rate was reduced to 50% of normal (37). All of these studies come with some limitations. For example, repetitive sampling of tubular fluid using stop flow techniques to analyze segmental tubular reabsorption have limitations, since they might alter reabsorption in downstream segments (39). Moreover, assessment of segmental tubular sodium handling during prolonged furosemide therapy in the absence, but also the presence of CKD or heart failure, has not been studied. Therefore, results regarding inhibition of proximal tubular transport are conflicting in physiological situations and incomplete in pathophysiology.

This leaves several issues. First, furosemide affects expression of transporters, not only in the loop itself, but also in other segments. This means that, if NKCC2 activity varies, this links to stimuli that modulate the expression of other transporters. What the exact stimuli are, is not resolved. Moreover, at a more physiological level, it seems that there is an intrinsic set point of the kidney for a certain degree of Na^+ reabsorption related to total body sodium (132); while this might affect very local phenomena, such as tubular fluid, tubular osmolality, and interstitial composition, this might also feed back to the kidney by neurohumoral mechanisms.

Why is the effect of furosemide on sodium balance, ECFV, and BP unpredictable? ECFV expansion can increase total peripheral resistance and BP by evoking total body autoregulation in response to overperfusion of tissues. A logical approach to reduce BP is to reduce ECFV using diuretics. Verification of this concept would require reliable and easily applicable ECFV measurements. Radioisotope measures are reliable but not easy to use. Mono- and multifrequency bioimpedance measurements of ECFV are reliable but have hardly been employed to research the subject. Only one study documented a 1.1-liter decrease in ECFV after initiation of furosemide therapy in patients with CKD (compared to subjects treated with nondiuretic antihypertensives) (170). To obtain insight into how furosemide affects ECFV regulation, further studies are needed to better characterize who will benefit from furosemide therapy and which regimen (dose, dose frequency, route of administration) is effective to reduce ECFV to normal.

Several studies in the 1970s indicate that dosing of furosemide once per day induces a natriuresis over ~6–8 h followed by sodium retention during the rest of the day (165). The mechanism of this sodium retention has not been entirely resolved; it could be related to *1*) activation of the RAS; *2*) activation of the SNS; and *3*) acute reduction in PV followed by decreased renal perfusion pressure and "pressure natriuresis." A stronger natriuretic response to furosemide would lead to a more pronounced decrease in PV. A decrease in PV of 16% (580 ml) was observed in hypertensive subjects after 100- to 200-mg intravenous furosemide (36). Of note, the stability of the effective circulating volume also is related to the refill rate from the interstitial space and the dynamics of the venous capacitance (see below). A study in heart failure patients demonstrated a net refill volume (and perhaps also venous-to-arterial fluid redistribution volume) exceeding the diuresis induced by furosemide (140). Refill rate cannot be easily assessed, although a methodology has been developed to measure refill rate in hemodialysis (24). Starling forces primarily determine refill rate and include the permeability of the capillary membrane, which is not fixed but influenced by numerous factors, including ANG II (168), atrial natriuretic peptide (142, 153), inflammatory cytokines (23, 93), and nitric oxide (NO) (28, 149). Characterization of capillary refill in complex states like advanced kidney failure would, therefore, contribute to the understanding of consequences of rapid alterations in volume status with furosemide.

Unpredictable responses to furosemide can also result from differences between the PV/ECFV and the PV/BP relationships of patients. Patients with non-nephrotic CKD have a steeper slope of the PV/ECFV and PV/BP relationship compared with patients with nephrotic syndrome (Fig. 2). Therefore, an acute

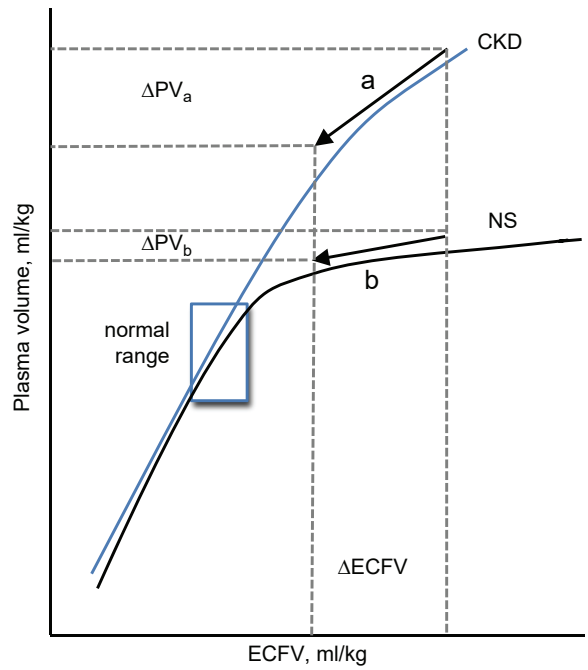


Fig. 2. Effects of furosemide in patients with CKD or nephrotic syndrome (NS). Both groups of patients have expansion of the extracellular fluid volume (ECFV); however, the relationship between ECFV and plasma volume (PV) is different. *a*: In CKD patients, the decrease in ECFV evoked by furosemide would be accompanied by a sharp decrease in PV (PV_a), because of the steep relationship between ECFV and PV. *b*: In patients with NS, however, the same decrease in ECFV would only result in a small decrease in PV (PV_b). Consequently, effects on blood pressure will be different. [Adapted from Koomans et al. (89).]

reduction in PV upon diuretic treatment in non-nephrotic patients is more likely to cause an acute reduction in BP compared with nephrotic CKD. In heart failure, the PV/ECFV and PV/BP relationships have hardly been studied. One study reported decreases in blood volume measured by labeled albumin and Cr-51 labeling of red cells in subjects with acute heart failure and pulmonary edema treated with furosemide (47). Unfortunately, methodology is not readily available to easily assess PV/ECFV and PV/BP relationships in humans or animals, and there is a little knowledge of these relationships in various disease states, including the response to diuretic therapy.

Closely related to this subject is the distribution of blood volume between the venous and arterial compartments. Schuster et al. (140) compared patients with acute heart failure with and without a diuretic response after furosemide. Remarkably, in both groups, there was a decrease in colloid osmotic pressure and a decrease in central venous pressure. This could be explained if furosemide increased venous capacitance, decreased venous pressure, thereby facilitating fluid reabsorption from the interstitial space and subsequent decreases in plasma oncotic pressure (140). Others have reported such an acute increase in venous capacitance upon furosemide (38). The mechanism, as well as the physiological importance of variations in venous capacitance by furosemide is unknown; it is even not known whether this is mediated via the NKCC transporters. In one study, in human umbilical vein endothelial cells, NKCC2 gene expression was induced by inflammatory cytokines, but whether such induction is present in intact veins and is physiologically significant is unknown (157).

Questions Regarding Hemodynamics and Vascular Regulation and the RAS

What determines the effects of furosemide on GFR and RBF? In 18 studies about the actions of furosemide on GFR and RBF in healthy subjects, changes in GFR and/or RBF were reported. In the majority of studies, GFR increased (5, 13, 14, 71, 120, 121, 133, 169) or remained stable (31, 42, 102, 147); in five studies GFR decreased (51, 91, 100, 158). RBF increased in five (71, 102, 115, 121, 169), remained stable in three (42, 51, 120), decreased in one (51), and was not reported in the other studies (5, 13, 14, 31, 91, 100, 133, 147, 158). This clearly illustrates how complex the actions of furosemide on renal hemodynamics are. Furosemide can affect GFR and RBF by acting on BP, on hydrostatic pressure in Bowman's space, on afferent and efferent resistance, on the glomerular surface area and permeability, and on plasma colloid osmotic pressure. Obviously, furosemide can decrease BP by causing a brisk natriuresis and, consequently, a decrease in ECFV and PV (36). The impact of furosemide on renal function might be different from other hypotensive agents, since furosemide can affect autoregulation (see below), and subjects treated with furosemide, typically patients with heart and/or renal failure, might have impaired autoregulation. Furthermore, furosemide can cause a direct vasodilation by acting on NKCC1 in the vascular wall. The extent to which this can cause relevant changes in BP in disease models and in humans with heart failure or CKD is not well determined.

Furosemide can increase tubular pressure and thereby increase pressure in Bowman's space, which will directly decrease net ultrafiltration pressure. Determinants of tubular pressure are tubular flow and resistance. Resistance, in turn, is determined by the tubular diameter, depending on tubular compliance and on renal interstitial pressure. Since the kidneys have a tight capsule, Starling forces governing fluid fluxes between the capillaries and the interstitium determine interstitial fluid volume and thereby interstitial pressure. The net response of all factors on tubular pressure after furosemide is unpredictable. Holstein-Rathlou and Leyssac (63) reported an acute increase in tubular pressure upon intraluminal administration of furosemide of ~5–7 mmHg, which is relevant with respect to an estimated net ultrafiltration pressure of 20–25 mmHg. The increase is likely due to an increase in tubular flow due to the combined effects of inhibition of loop of Henle reabsorption, and an increase in SNGFR due to inhibition of TGF. Since this was a single nephron study, it lacks effects on systemic hemodynamics and on the interstitium. Tucker and Blantz (159) reported the intrarenal responses to systemic administration of furosemide in rats with and without supplement with saline solution to compensate for urinary sodium excretion. Without volume repletion, SNGFR decreased substantially; with volume repletion it remained stable. Interestingly, tubular pressure was slightly decreased after furosemide without volume repletion (from 13 to 11 mmHg), but increased during volume repletion (from 13 to 18 mmHg). Remarkably, the permeability coefficient decreased after furosemide with volume repletion. Finally, Oppermann et al. (116) reported a very strong increase in free flow proximal tubular pressure upon systemic administration of furosemide, which was attenuated by decapsulation. These studies underscore that relevant changes in tubular pressure can occur after (intraluminal) furosemide administration. Nevertheless, the effects of furosemide on tubular pressure (and GFR and RBF) during high dietary sodium, fluid volume expansion, or disease models have hardly been studied.

Regarding afferent resistance, Oppermann et al. (116) reported NKCC1-dependent vasodilation of isolated perfused afferent arterioles preconstricted with ANG II or the NO synthesis inhibitor N^G-nitro-L-arginine methyl ester. Wang et al. (163) were able to demonstrate NKCC1, but not NKCC2, protein expression in afferent arterioles and showed diminished MR after furosemide and bumetanide. Bumetanide also strongly inhibited the vasoconstrictor response to ANG II; furosemide was not tested. Despite this, Oppermann et al. (116) reported an acute decrease in RBF in response to systemic furosemide, which was explained by compression of the intrarenal vasculature after increased tubular volume. Altogether, these studies leave the possibility that furosemide can decrease GFR through increased interstitial pressure and decrease RBF due to compression of the intravascular vasculature and the inability of the MR to compensate for this. That said, effects of furosemide on MR and vascular contractility have not been studied during relevant physiological perturbations and in disease models of fluid volume expansion (heart failure and CKD).

Generally, in patients with heart failure, plasma oncotic pressure is decreased (6), and PV and ECFV are substantially expanded (166). Hemoconcentration due to diuretic therapy could, therefore, increase plasma oncotic pressure and thereby decrease GFR. Whether increases in plasma oncotic pressure

as a consequence of decreases in PV upon furosemide treatment can decrease GFR has not been studied.

Are direct vascular actions of furosemide important and what is the mechanism? Multiple factors could contribute to the controversy surrounding direct vasodilator effects of furosemide (41). Inconsistencies about this subject can be traced back to the 1970s. Furosemide has been documented to cause vasodilation in animal experiments (1). In humans, an immediate fall in left ventricular filling pressure was detected preceding the natriuresis after furosemide administration in congestive heart failure after acute myocardial infarction, presumably due to a markedly increased venous capacitance (38). Patients with pulmonary edema reportedly have immediate relief of symptoms by furosemide before any diuretic effect is observed (127). Whether or not furosemide causes increases in venous capacitance are likely also strongly related to the dose being administered. In this context, it is relevant to emphasize that, in healthy subjects, low doses already exert natriuretic effects (e.g., 5 mg iv; see Fig. 1), yet in patients with advanced CKD or heart failure, very substantial dosages are applied (up to 1,000 mg/24 h iv).

Furosemide can induce vasodilation indirectly via the synthesis of prostaglandins (70) since co-administration of indomethacin with furosemide can abolish a change in venous capacitance (127, 131). Conversely, furosemide induced a decrease in medullary hypertonicity, decreased intramedullary prostaglandin activity, and caused a fall in medullary perfusion (40). Confusingly, other studies showed that renal hemodynamic effects of furosemide were not mediated by prostaglandins but were, in fact, direct (29). Furosemide also induced a direct relaxation of the renal, iliac, and carotid vasculature, independent of prostaglandins and persisting after bilateral nephrectomy (9). The direct vasorelaxing effect of furosemide on isolated vessel segments was suggested to be endothelium independent (152). Furosemide was unable to cause vasodilation in afferent arterioles of *NKCC1*$-/-$ mice (116). Paradoxically, acute administration of furosemide has also been reported to cause vasoconstriction, presumably due to increased angiotensin II (41, 116), norepinephrine, and AVP levels (50). Altogether, the exact mechanism and vascular site (arterial, arteriolar, venous) of the vascular actions of furosemide remain ambiguous, although it seems to involve a direct action on the NKCC1 transporter.

Does furosemide differentially affect the systemic and intrarenal RAS? Recapitulating, furosemide can increase renin release by activation of the sympathetic nervous system (15, 32), a decrease in afferent arterial perfusion pressure, and by direct inhibition of NKCC2 in the macula densa (27, 151). In addition, macula densa neuronal NO synthase (nNOS)-mediated NO generation can stimulate renin release (18). The increase in renin release on furosemide reportedly is inhibited in the presence of nonspecific or specific nNOS blockers (137), yet furosemide did not affect renal nNOS gene expression in healthy rats (139). Conversely, furosemide can reduce renin release by increased luminal Na^+ concentration at the level of the macula densa (105, 138). That leaves uncertainty about the net effect of furosemide on renin release.

To assess the intrarenal RAS, one can measure RAS components in whole kidney (cortex) tissue (17, 49) or in fluids thought to reflect the activity of the intrarenal RAS: lymph (83), interstitial fluid (112), tubular fluid (19, 109, 143), and urine (16, 87). Activity of the systemic RAS does not always parallel activity of the intrarenal RAS (16, 25, 49, 103). We have been unable to find any studies on kidney vs. systemic, or tubular fluid vs. systemic RAS levels during furosemide administration. However, Khuri et al. (83) demonstrated that intravenous furosemide increased renal lymphatic renin more substantially than plasma renin in Mongrel dogs. This could indicate that the macula densa sodium sensing was blocked, and the resulting renin release increased the interstitial and lymph renin levels selectively. Urinary ANG I and ANG II excretion have been suggested to reflect intrarenal RAS activity, although limited evidence is available (162), and there is substantial ANG I and II breakdown. The same group reported increased urinary ANG I and II excretion after furosemide (16). This still does not resolve the question whether there can be divergent responses in systemic and renal RAS. There is currently considerable interest in urinary angiotensinogen as an indicator of the intrarenal RAS, yet we have been unable to find studies reporting the responses to furosemide.

Why would it be important if furosemide causes a disproportionate or diverging increase in renal RAS levels? Here we are left with one of the most intriguing observations in the RAS literature: ANG II infusion increases renal ANG II levels (161). Yet what this means in terms of renal function is obscure. Is the ANG II buffered? Does it reach levels where ANG II might become natriuretic (141)? All in all, the effects of furosemide on the intrarenal RAS are not well documented, and the implications for renal function remain unclear.

Questions Regarding Furosemide in Kidney Disease, Urate, and the Third Compartment

Does kidney disease affect the actions of furosemide? Whether furosemide is equally potent in healthy individuals vs. patients with CKD is related to the pharmacology of furosemide and the tubular mechanisms that lead to Na^+ retention in CKD. Regarding the pharmacology, intestinal reabsorption of 40–90% has been reported in subjects with advanced CKD (65), yet altogether reabsorption seems diminished (154). Although a clear relationship between reabsorption and measures of GFR has not been documented in CKD, this could contribute to furosemide resistance. Moreover, furosemide T½ is increased in renal failure (10) and is highly variable, up to >20 h in some individuals. This could contribute to "resistance", since in some subjects it could accumulate so that additional dosages do not elicit a natriuresis. Conversely, having plasma levels over a more sustained period could prevent resistance. Another pharmacological issue is that, in states with high-grade proteinuria, the glomerular filtrate might contain so much albumin that it diminishes the free furosemide levels to such an extent that this diminishes its actions (145). In that regard, it is noted that Agarwal et al. (2) showed that displacing furosemide from plasma proteins with a sulphonomide (sulfisoxazole) did not correct resistance in nephrotic patients. There is still controversy about whether and how albumin infusion could increase the natriuretic actions of furosemide in the nephrotic state (45). Taken together, diminished reabsorption, increased T½, and high-grade proteinuria in patients with CKD could limit the natriuretic response.

A more physiological issue is that, when GFR declines in CKD, fractional excretion of Na^+ has to increase to maintain

Na^+ balance. A healthy subject with an estimated GFR of 120 $ml \cdot min^{-1} \cdot 1.73\ m^{-2}$, therefore, has a 10× lower fractional sodium excretion (0.5–1.0%) than a subject with advanced CKD and an estimated GFR of 12 $ml \cdot min^{-1} \cdot 1.73\ m^{-2}$ (5–10%), assuming similar intake and assuming sodium balance. This leads to the important question how the remnant nephrons in CKD decrease their Na^+ reabsorption. Since NKCC2-mediated reabsorption is so substantial, one option is that NKCC2 is suppressed. Data about CKD and expression of NKCC2 are limited. One study demonstrated an initial increase in NKCC2 expression after 5/6th nephrectomy in rats, later returning toward normal (85). Two studies in rats after uninephrectomy and salt loading demonstrated decreased expression of the NKCC2 (73, 74). In deoxycorticosterone acetate-salt hypertension (8), NKCC2 seems to decrease, whereas, in ANG II-induced hypertension (110), NKCC2 seems only decreased in the renal medulla. Altogether, data in renal failure about NKCC2 expression and activity are limited.

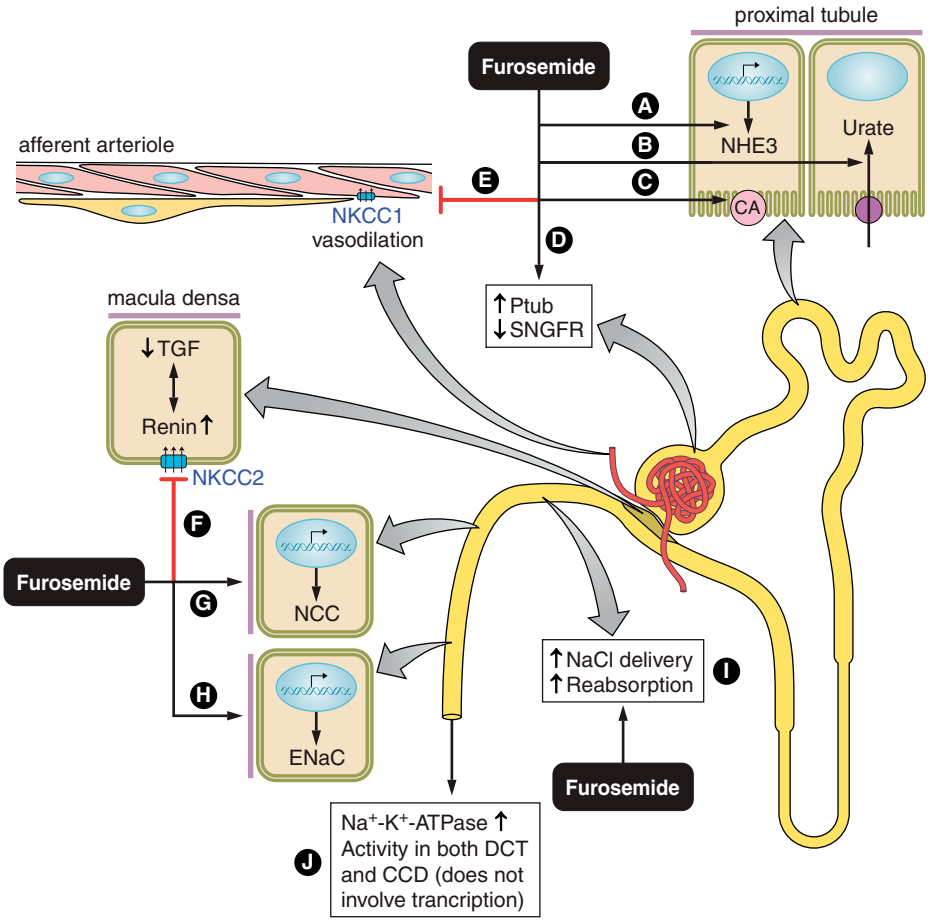


Fig. 3. Mechanisms of furosemide action in the kidney where questions are remaining. NHE3, Na^+/H^+ exchanger 3; ENaC, epithelial Na^+ channel; DCT, distal convoluted tubule; CCD, cortical collecting duct.

ISSUE

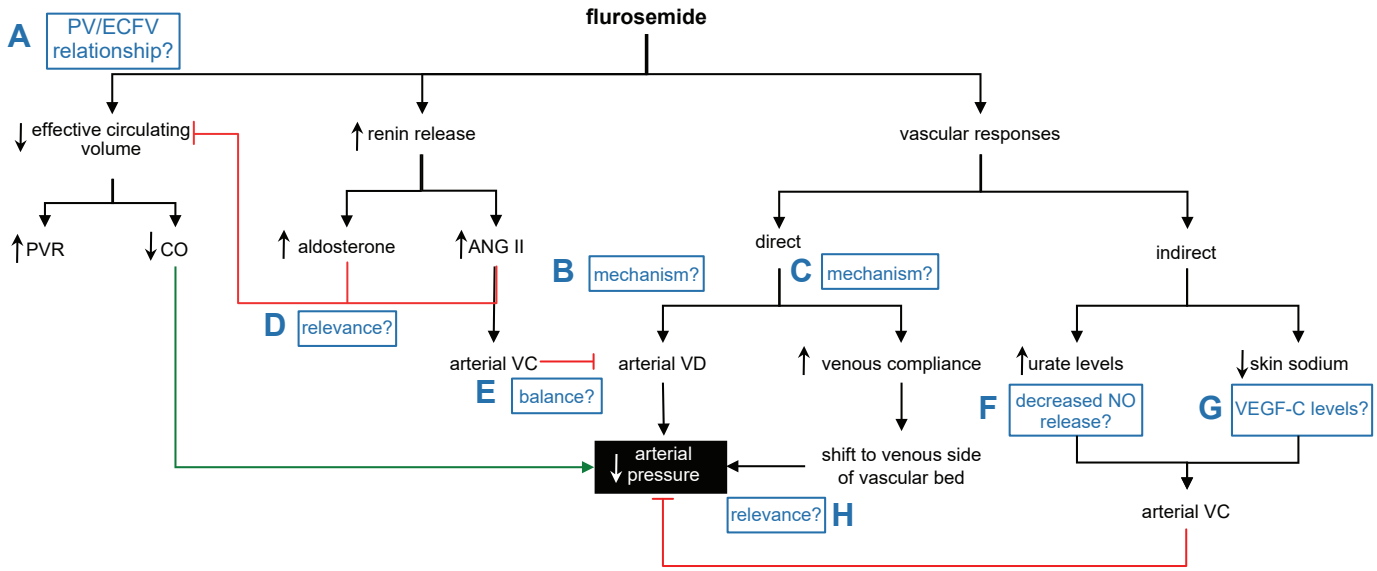
- **A** FUR can inhibit NHE3; mechanism and relevance unclear
- **B** FUR increases proximal tubular urate transport; direct or indirect?
- **C** FUR can inhibit carbonic anhydrase; mechanism and relevance unclear
- **D** Under which circumstances does tubular pressure increase upon FUR?
- **E** Can FUR cause an NKCC1-mediated decrease in afferent arteriolar tone?
- **F** Can FUR differentially inhibit TGF responses and enhance renin release?
- **G** Does FUR lead to a relevant increase in NCC gene and protein expression?
- **H** Similarly, can FUR increase in ENaC gene and protein expression?
- **I** Is FUR induced increased distal tubular fluid load and consequently increased distal tubular reabsorption similar under all circumstances?
- **J** Does FUR increase DCT and CCD Na^+-K^+-ATPase activity?

At a translational level, in relatively severe CKD, the natriuresis to 1.5 mg/kg furosemide in CKD was directly proportional to GFR (inulin clearance), yet the increase in fractional Na^+ and water excretion upon furosemide did not decline with lower GFR (54). Another report indicated an increase in fractional Na^+ excretion to >40% upon high-dose furosemide in patients with very low GFR ($4-8$ ml·min^{-1}·1.73 m^{-2})(3). Lastly, one report indicated maintained responses in urinary Na^+ excretion after furosemide given orally or intravenously in patients with mild and with advanced (134) CKD, indicating an enhanced response in fractional Na^+ excretion to furosemide. Therefore, experimental and human data seem in conflict: whether there are humans with clearly decreased NKCC2 expression and function and, consequently, diminished response to furosemide is unclear, and whether there is regulation of sodium transporters in CKD with different severity and etiology is unclear.

Are effects of furosemide on urate levels detrimental for BP regulation and kidney integrity? One of the most important side effects of diuretics is that it can provoke a gout attack. Several mechanisms have been suggested. Furosemide inhibits the human sodium phosphate transporter 4 (hNPT4) in proximal tubules, which releases urate into the tubular lumen (76). Moreover, hyperuricemia often coexists with hypertension, CKD, and cardiovascular disease (CVD). Yet it is hard to discriminate whether these issues are induced by elevated uric acid level directly or secondary to the coexistent conditions or drugs (69). Notably, in animal experiments, increased uric acid level resulted in systemic and glomerular hypertension due to elevated renal vascular resistance and reduced RBF (69). Similarly, in the 5/6 remnant kidney model, rats with hyperuricemia displayed more renal hypertrophy, arteriolosclerosis, glomerular injury, and interstitial fibrosis than those with similarly elevated BP but without hyperuricemia (80). Uric acid was also found to induce endothelial dysfunction via impairing NO release, which would impair vasodilation (68). This poses an intriguing problem: could furosemide aggravate hypertension and CKD and even associated CVD?

Data on the negative effects of uric acid are not consistent. This inconsistency can be due to the duality of uric acid. Uric acid can also act as an antioxidant. Increased serum antioxidant capacity was found in hyperuricemia in individuals with atherosclerosis. This finding could indicate compensation of the oxidative stress caused by CVD and CKD (111). The antioxidant activity of uric acid could be mediated by increasing the activity of SOD1 and SOD3 (62). Others have suggested that uric acid only functions as an antioxidant in the extracellular space, and indeed a reciprocal relationship has been demonstrated between uric acid and NO level in serum (79). So, returning to the effects of furosemide on uric acid levels, what determines whether furosemide could cause aggravation of CKD and CVD?

How does furosemide affect the "third compartment"? In recent years, the notion developed that Na^+ could distribute to cutaneous tissue, where it could bind to glycosaminoglycans in a nonosmotic fashion (156). This nonosmotic sodium possibly



	Issue
A	A brisk natriuresis after FUR can affect PV and ECFV differently depending on the clinical syndrome
B	The mechanism of direct vasodilation in the systemic circulation in unknown
C	Whether and how FUR can increase venouse compliance is not well known
D	Can stimulation of tubular reabsorption by ANG II and Aldosterone counteract the natriuresis after FUR?
E	Can renin-induced vasoconstriction supersede the direct FUR-induced vasodilation?
F	How does FUR-induced hyperuricemia leads to vasoconstriction?
G	Does FUR affect the skin sodium stores and the systemic VEGF-C levels?
H	Is a volume shift towards the venous site of the vascular bed after FUR relevant for blood pressure control?

Fig. 4. Mechanism of furosemide action in the systemic environment where questions are remaining. PVR, pulmonary vascular resistance; CO, cardiac output; VC, vasoconstriction; VD, vasodilation.

elicits an inflammation response, with release of VEGF-C as a consequence, which would offset hypertensive consequences of high sodium intake (155). Gradually, evidence is accumulating that the subcutaneous sodium storage is expanded in different disease states, such as hypertension (97) and end-stage renal disease (35). A question that is unanswered at this moment is whether any diuretic therapy can mobilize the subcutaneous sodium, and whether this would improve or deteriorate systemic hemodynamic function. A similarly interesting question is how sodium transport takes place between the subcutaneous stores and the lymphatic vessels. It is imaginable that this recruitment of salt from these stores is a regulated process (95). In that case, it may very well involve Na^+ transporters that are similar to transporters in the kidney to facilitate movement of sodium from the intercellular space back into the lymphatic vessels. If furosemide would exclusively remove sodium from the extracellular space and not recruit sodium from the subcutaneous stores, would furosemide be beneficial or harmful for the skin-VEGF axis?

Summary

This evaluation has revealed important knowledge gaps, involving furosemide as a drug itself, the function of NKCC2 (and NKCC1), and renal and systemic indirect effects of NKCC inhibition. Regarding the kidney, remaining questions are indicated in Fig. 3, regarding the systemic circulation; remaining issues are indicated in Fig. 4. Major themes are as follows: *1*) diuretic resistance and the braking phenomenon; *2*) systemic and intrarenal activation of the RAS; and *3*) systemic vascular effects, specifically furosemide-induced increases in venous capacitance. An important aspect of the analysis of the actions of furosemide is that the brisk and short diuresis strongly disturbs the steady state with numerous and heterogeneous consequences. Resolving the many remaining questions could help to better understand NKCCs and their actions and improve the application of furosemide in the pathophysiology of fluid volume expansion.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: X.H., S.H., and B.B. conception and design of research; X.H., S.H., and B.B. drafted manuscript; X.H., E.J.D.M., P.F.V., S.H., and B.B. edited and revised manuscript; X.H., E.J.D.M., P.F.V., S.H., and B.B. approved final version of manuscript; B.B. prepared figures.

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