Aus dem Institut für Vegetative Physiologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Renal Vascular Function and Contrast Media Induced Acute Kidney Injury

zur Erlangung des akademischen Grades
Doctor rerum medicinalium (Dr. rer. medic.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Zhi Zhao Liu
aus Chongqing, China

Datum der Promotion: 25. 10. 2013

Table of Contents

Abstract (English)

[P1] — Contrast media induced acute kidney injury (CI-AKI) is the third most common cause of hospital acquired AKI. The purpose of this study was to determine whether contrast media modifies vasa recta tone and reactivity. We investigated whether there is different vasotonus to contrast media between rat and human vasa recta. Results showed that contrast media constricted rat and human vasa recta significantly and similarly. The response to angiotensin II was enhanced in rat vasa recta. Scanning electron microscopy showed the surface of endothelia damaged by contrast media in rat interlobar arteries. These phenomena were blunted by adrenomedullin. The study suggests that contrast media constrict rat and human vasa recta and this could be the result of an endothelial dysfunction induced by contrast media cytotoxicity.

[P2] — A key feature of CI-AKI is the decreased glomerular filtration rate. The study tested the hypothesis that contrast media impair the mice renal microvasculature function in a manner that contributes to decrease the effective glomerular filtration pressure. Results showed that afferent arteriolar diameter was significantly decreased and its response to angiotensin II was increased by contrast media application. However, neither the diameter nor the reactivity to angiotensin II of efferent arterioles was changed. Fluorescence experiments with 4-amino-5 methylamino-2, 7-difluorofluorescein (DAF-FM) showed that both contrast media and L-NAME (L-N^G-Nitroarginine methyl ester, a nitric oxide synthases inhibitor) impaired the nitric oxide bioavailability in afferent arterioles. Fluorescence experiments with dihydroethidium (DHE) showed that treatment with contrast media resulted in an increased superoxide concentration. This increase was blunted by the superoxide dismutase mimetic tempol. In conclusion, a stronger constrictive effect on afferent arterioles compared to efferent arterioles contributes to the reduced glomerular filtration rate. Decreased nitric oxide bioavailability and increased superoxide concentration might be the underlying mechanism.

[P3] — Numerous studies revealed gender differences in the cardiovascular control and the pathogenesis of cardiovascular events. Here we tested the hypothesis that renal interlobar arteries present gender differences in their response to angiotensin II. The angiotensin II type 2 receptors (AT_2R) may play a role in this context. Results showed that male interlobar arteries constricted more pronounced to angiotensin II compared to that of female. Blockade of AT_2R enhanced constriction to angiotensin II only in females. Endothelial dependent vasodilation was more dependent on nitric oxide in females. This study suggests that the nitric oxide mediated effect via AT_2R underlies the gender differences in the response of interlobar arteries to angiotensin II.

Abstract (German)

[P1] — Kontrastmitel induziertes akutes Nierenversagen (CI-AKI) ist die dritthäufigste Ursache einer krankenhauspflichtigen AKI. Das Ziel der vorliegenden Studie war, den Einfluss von Kontrastmitteln auf den Gefäßtonus und die Gefässreaktivität zu testen. Wir nahmen an, dass der mögliche Effekt durch die Wirkung auf das Gefäßendothel im Sinne einer endothelialen Dysfunktion entsteht. Es wurde weiterhin untersucht, ob es Unterschiede in der Wirkung auf humane und murine Vasa recta gibt. Die Ergebnisse zeigen vergleichbare Effekte von Kontrastmitteln auf die Gefäße verschiedener Spezies. Die Angiotensin II-Antworten waren in Vasa recta erhöht. Die Endotheloberfläche von kontrastmittelperfundierten Aa. interlobares wies Zeichen einer Schädigung auf. Beide Phänomene wurden durch Adrenomedullin abgeschwächt. Die Studien deuten auf einen konstriktorischen Effekt von Kontrastmitteln auf humane und murine Vasa recta infolge einer endotheliale Dysfunktion hin.

[P2] — Die Verminderung der glomerulären Filtrationsrate ist ein Hauptcharakteristikum der CI-AKI. Die Studie prüfte die Hypothese einer differentiellen Wirkung von Kontrastmitteln auf die glomerulären Arteriolen mit dem Ergebnis eines verminderten glomerulären Filtrationsdruckes. Die Studie zeigt, dass Kontrastmittelapplikation den Durchmesser afferenter Arteriolen vermindert und die Antwort auf Angiotensin II erhöht. Efferente Arteriolen reagierten nicht signifikant auf die Kontrastmittelapplikation. L-NAME (NO-Synthase-Hemmer)-Applikation als auch Kontrastmittelgabe führten zu einer Verminderung der Fluoreszenz des Stickstoffmonoxid (NO)-Indikators 4-amino-5 methylamino-2, 7-difluorofluorescein (DAF-FM). Dies weist auf eine verminderte Bioverfügbarkeit von NO nach Kontrastmittelgabe hin. Behandlung mit Kontrastmitteln erhöhte die Fluoreszenz des Superoxid-Indikators Dihydroethidium (DHE). Diese Wirkung wurde durch das Superoxid-dismutasemimetikum Tempol deutlich abgeschwächt. Wir schlussfolgern, dass die stärkere Aktion von Kontrastmitteln an den afferenten Arteriolen im Vergleich zu den efferenten Gefäßen zur Verminderung der glomerulären Filtrationsrate beitragen kann. Die Effekte von Kontrastmitteln an den afferenten Arteriolen werden durch die verminderte Bioverfügbarkeit von NO und die höhere Konzentration von Superoxid vermittelt.

[P3] — Zahlreiche Studien weisen auf eine geschlechtsspezifische kardiovaskuläre Regulation und Pathogenese kardiovaskulärer Ereignisse hin. In dieser Studie gingen wir davon aus, dass interlobare Arterien der Niere geschlechtsspezifische Differenzen in der Antwort auf Angiotensin II aufweisen und dass der AT_2-Rezeptor hierfür eine Rolle spielen könnte. Wir zeigten eine stärkere Kontraktion von interlobaren Arterien männlicher Mäuse in Vergleich zu weiblichen Tieren. Hemmung des AT_2-Rezeptors führte nur in weiblichen Mäusen zu einer stärkeren Kontraktion der Gefäße. Die enodthelvermittelte Dilatation war in weiblichen Tieren deutlich mehr abhängig von NO als in männlichen Tieren. Diese Ergebnisse lassen vermuten, dass den Geschlechtsunterschieden eine vermehrte AT_2-Rezeptor-vermittelte NO-Freisetzung durch Angiotensin II in weiblichen Tieren zugrunde liegt.

Introduction

The publications of [P1] and [P2] are both about the pathophysiology of contrast media induced acute kidney injury. It is the main focus of my doctorate study. For this reason, an additional submitted manuscript [MS] about contrast media and renal tubules is discussed and attached below. Given the word-limitation, the following summary lays stress on the effects of contrast media on the kidney, and little is mentioned about my work in gender differences presenting in vessels, which was published as [P3].

Contrast media are widely used in radio diagnosis. The frequency of contrast-induced acute kidney injury (CI-AKI) is around 10%, which makes CI-AKI to be the third most common cause of hospital-acquired AKI (Briguori et al. 2003). Although there are hundreds of studies trying to shed light on the pathophysiology of CI-AKI, it is not full understood yet. Reduced glomerular filtration rate and direct tubular damage are generally accepted as the key features of CI-AKI (Persson et al. 2005). Contrast media may induce acute renal vasoconstriction by causing imbalance between renal vasodilators and vasoconstrictors (Tumlin et al. 2006). The property of cytotoxicity, sharing within all types of contrast media, may directly damage the endothelial and epithelial cells in the kidney (Sendeski 2011). Hydration, the only widely recommended prophylaxis, diminishes but does not eliminate the risk of CI-AKI. We hypothesize that contrast media may influence the two main determinants of glomerular filtration rate - afferent and efferent arteriolar diameters. Contrast media affect vasa recta and tubules, and then cause damage in the medulla. Because of the interaction between tubules and afferent arterioles, contrast media interrupt the function of tubuloglomerular feedback. As a consequence, contrast media lead to renal dysfunction.

Goals

The study investigated the underlying mechanisms of CI-AKI in a new *in vitro* model. The focus was to examine the effects of contrast media on the tone and reactivity of mice afferent and efferent arterioles, rat and human vasa recta to vasoactive substance [microperfusion and microscopy], as well as the tubulo-vascular interaction in rabbits [double perfusion and microscopy], and the toxicity of contrast media on the rat thick ascending limbs (TALs) [staining of propidium iodide and trypan blue] plus interlobar arteries [scanning electron microscopy]. The protective effect of adrenomedullin was studied [microperfusion and microscopy, scanning electron microscopy]. Moreover the underlying cellular mechanisms were investigated with special attention to superoxide [dihydroethdium (DHE) fluorescence] and nitric oxide [4-amino-5 methylamino-2, 7-difluorofluorescein (DAF-FM) fluorescence]. The expression of 84 genes related to oxidative stress [qPCR] and the activity of superoxide dismutase [activity assay] in rat renal tubules were tested.

Since the main part of my doctorate study was to examine the functions of distinct vessels in the kidney, I performed several experiments on the mice interlobar arteries. In order to examine whether gender differences to angiotensin II exist in renal interlobar arteries, and whether angiotensin II type 2 receptors (AT_2R) play a role in such differences, isometric constriction and relaxation of female and male mice interlobar arteries were measured under blockade of nitric oxide synthases and AT_2R.

Methods and Results

Experiments were performed in accordance with the regulations of the Office for Health and Social Matters of Berlin (Berlin, Germany).

A. The Effects of Contrast Media on Renal Vasculature and Tubules

We chose iodixanol to perform experiments because it belongs to the last generation of iodinated contrast media. The dose of concentration was set to 23mg iodine/ml to mimic the effect what occurs *in vivo* during coronary angiography. In some parts of experiments, 11mg iodine/ml iodixanol was used and indicated if necessary.

Preparation of Arteries, Arterioles, Vasa recta and Tubules

All experiments were performed in the following kinds of vessels: renal interlobar artery, afferent arteriole, efferent arteriole and vas rectum. One special kind of the tubules - medullary TAL - was used. Adult male C57BL/6 mice, male Sprague-Dawley rats or young male New Zealand White rabbits were sacrificed. Kidneys were removed and sliced. Human non-malignant renal tissue was obtained from nephrectomized kidneys in the Charité hospital. The local review board approved of the study. Afferent or efferent arterioles attached to their glomeruli, descending vasa recta, single glomeruli with its afferent arteriole and adherent tubule, interlobar arteries, and TALs were dissected at 4°C with sharpened forceps and transferred to a temperature-controlled chamber assembled on the stage of an inverted microscope.

Microperfusion/Double perfusion of Microvessels and Tubules

Afferent or efferent arteriole, vas rectum, or TAL was mounted on concentric glass pipettes, cannulated and microperfused at one end, and held at the other end with a holding pipette, respectively [P1, Fig.1; P2, Fig.2]. For investigating the tubuloglomerular feedback, the afferent arteriole and its adherent distal tubule from the same glomerulus were double cannulated and perfused, respectively. The viability of afferent and efferent arteriole and descending vasa recta was tested by rapidly increasing the perfusion pressure and assessing the change in the luminal diameter. A fast and complete constriction in response to high potassium chloride was used to identify the suitability of afferent arterioles for the subsequent experiments. The behavior of microvessels was obtained by video-microscopy. Videos were stored on digital video discs. The analysis was performed off-line. A computer with a frame grabber was used to acquire images during playback. The luminal diameter was measured from the acquired image. Vasoreactivity was quantified as the percentage of change of luminal diameter from the baseline measurement.

Iodixanol Increased Microvascular Tone and Reactivity to Angiotensin II

In order to examine the effect of iodixanol on the tone and reactivity of renal vessels, the vessels were pretreated with iodixanol for 20min, and then angiotensin II (10^{-12} mol/L to 10^{-6} mol/L) was given to vessels in the bath. Iodixanol constricted mice afferent arterioles as well as rat and human descending vasa recta significantly during 20min perfusion [P1, Fig.2 and 3; P2, Fig.3]. Iodixanol further enhanced the response of afferent arteriolar and rat descending vasa recta to angiotensin II [P1, Fig.4; P2, Fig.4]. Adding L-N^G-Nitroarginine methyl ester (L-NAME), a non-selective nitric oxide synthases inhibitor, to iodixanol led to an additive increased vasotonus compared to iodixanol alone in afferent arterioles [P2, Fig.7]. Adrenomedullin (10^{-6} mol/L) blunted the constrictive effect of iodixanol in human and rat descending vasa recta [P1, Fig.2 and 3]. However, efferent arteriolar tone and response to angiotensin II were not significantly influenced by iodixanol [P2, Fig.3 and 4].

Iodixanol Modulated Tubuloglomerular Feedback

Tubuloglomerular feedback was qualified as the absolute diameter change of afferent arteriole when the tubular perfusate was switched from 10 to 80mM NaCl. The afferent arteriole was perfused with DMEM at a pressure of 60 mmHg, and the tubule was perfused with physiological saline containing 80 or 10mM NaCl with/without iodixanol. The tubule was pretreated with iodixanol for 10min firstly and then NaCl containing iodixanol was exchanged from10 to 80mM. In our experimental model, the diameter change of afferent arteriolar was stronger during a lower dose of iodixanol treatment compared to control [4.53μm (11mg iodine/ml) vs. 2.77μm (control)]. Perfusion with 23mg iodine/ml iodixanol reduced the NaCl-induced change in the diameter [2. 31μm (23mg iodine/ml) vs. 2.77μm (control)] [MS, Fig.7].

Iodixanol Decreased Nitric Oxide Bioavailability in Afferent Arteriole and TAL

To measure nitric oxide, DAF-FM was used. DAF-FM was loaded into afferent arteriole or TAL by adding it to the perfusate or/and bath. The preparations were stimulated at 490 nm, and DAF-FM fluorescence images were acquired at wavelengths from 550 to 600 nm by using a customized set of filters [P2, Fig.5]. Iodixanol significantly decreased the DAF-FM fluorescence intensities in afferent arterioles and TALs compared to the control group [P2, Fig.6; MS, Fig.4]. L-NAME inhibited the increase of DAF-FM intensity. Iodixanol and L-NAME showed similar effects on the DAF-FM intensities of afferent arterioles and TALs in our experimental model.

Iodixanol Increased Superoxide Concentration in Afferent Arteriole and TAL

To measure superoxide, DHE was used. DHE was loaded into afferent arteriole or TAL by adding it to the bath. The preparations were stimulated at 380 and 490 nm, and DHE fluorescence intensities were acquired between 400-450 nm and 520-600 nm, respectively. Compared to the control group, iodixanol (23mg iodine/ml) significantly enhanced the DHE intensity ratio in afferent arterioles and TALs [P2, Fig.9; MS, Fig.3]. Tempol, a superoxide dismutase mimetic, significantly blunted the increase of DHE intensity ratio of iodixanol. However, a lower dose of iodixanol (11mg iodine/ml) did not change the DHE intensity ratio in TAL.

Iodixanol Accelerated the Tubular Cell Death Rate in TAL

To estimate the cell death rate, propidium iodide was used. Propidium iodide (5×10^{-3} mol/L) was added to the perfusate and the bath to load TAL and was always present during the experiment. The preparations were stimulated at 490 nm, and propidium iodide fluorescence intensities were acquired between 520 and 600 nm by using a customized set of filters [MS, Fig.1]. At the end of experiment, trypan blue (50% V/V) was added into the bath to stain dead cells [MS, Fig.2]. During four-hour recording, the intensity of propidium iodide was significantly increased by iodixanol. This significance happened already after 20min perfusion with iodixanol [MS, Fig.5]. After staining by trypan blue, the fraction of blue colour was increased in iodixanol group [MS, Fig.6].

Iodixanol Impaired Endothelial Cells of Renal Interlobar Arteries

To study the influence of iodixanol on the morphology of endothelial cells, scanning electron microscopy was used. Isolated segments of rat interlobar arteries were perfused with vehicle, iodixanol and/or adrenomedullin for 20min. The vessels were then prepared for investigation by using scanning electron microscopy. The scanning electron microscopy images showed a smooth vessel surface, and endothelia with a slight, physiologic bulging of the perinuclear area in the control condition. Following the iodixanol perfusion, the vessel surface was irregular and ragged. Endothelial cells acquired a spindle-like shape and bulged into the vessel lumen. Adrenomedullin attenuated endothelial cell bulging and blebbing in iodixanol-treated vessels compared to iodixanol alone [P1, Fig.5].

Iodixanol Increased the Expression of Oxidative-Stress-Related Genes but not Superoxide Dismutase (SOD) Activity in Medullary Tubular Structures

Rats were anaesthetized and instrumented. They received PBS or iodixanol intra-arterially as bolus (1.5 ml, about 23mg iodine/ml). Two hours later, the circulatory bed of the kidneys was isolated. The aorta was opened and both kidneys were perfused with PBS containing iron oxide (1%). The kidneys were removed and medullary regions of the kidneys were dissected and smashed. Tubular structures were then separated from vasculatures using sterilized magnets. The total RNA from tubules was extracted using RNA-Bee Isolation Reagent. Reverse transcription was followed by the measurement of relative gene expression of 84 genes involved in oxidative stress. Relative changes in gene expression were calculated using the comparative threshold cycle (ddCT) method with five housekeeping genes (Rplp1, Hprt1, Rpl13a, Ldha, Actb) for normalization. The results showed there were only two genes differently downregulated in the tubules: thioredoxin interacting protein (TXNIP) and superoxide dismutase 3 (SOD3) [MS, Fig.8].

To determine the SOD activity, the commercial kit OxiSelect O_2^--Dismutase Activity Assay was used. The assay principle is based on the ability of a Xanthine/Xanthine Oxidase system to generate O_2^- anions which are detected with a Chromagen solution. In the presence of SOD, these O_2^- anion concentrations are reduced, yielding less colorimetric signal. The results showed perfusion with iodixanol did not change SOD activity in tubules [MS, Fig.8].

Statistical Analysis

Results of vessel diameter measurements were compared using Brunner's test (a non-parametric ANOVA-like test for repeated measurements and multiple comparisons). For testing differences between groups in RNA microarray and SOD activity assay measurements the Student's t-test was performed. $P<0.05$ was used to reject the null hypothesis.

B. Gender Differences in the Function of Renal Interlobar Arteries

Perfusion of Mice Interlobar Arteries

After sacrificing the adult male and female C57BL/6 mice, interlobar arteries were dissected and mounted on a 40μm wire in a small vessel myograph. The preparation was supplied with a O_2 (95%)/CO_2(5%). Arterial force was recorded using a data-acquisition system. Maximal constriction to 100mmol/L KCl was used as a criterion for comparing the effects of vasoconstrictive substances. For investigation of the influence of vasodilator substances, vessels were constricted to 50% of maximal KCl-induced contraction with phenylephrine. Relaxation was expressed as percentage of phenylephrine-induced constriction.

Gender Differences Presented in Response to Vasoconstrictors

Angiotensin II and phenylephrine (10^{-10} mol/L to 3×10^{-7} mol/L) concentration response curves were performed in interlobar arteries from female and male mice. Vessels from male mice constricted more to angiotensin II than that from female mice [P3, Fig.1]. The concentration response to phenylephrine was similar in both groups [P3, Fig.2]. In order to investigate the contribution of nitric oxide and AT_2R, four manipulations were performed: (a) mechanical removing of the endothelium; (b) simultaneous blockade of nitric oxide synthases and AT_2R; (c) blockade of AT_2R; and (d) inhibition of nitric oxide synthases. If (a), (b), (c) or (d) was administered, respectively, the responses to angiotensin II were significantly enhanced in the female group. In the male group, only manipulations (a) or (d) increased angiotensin II responses. There were no gender differences in the angiotensin II response when comparing vessels from (a), (b) and (d) [P3, Fig.3].

Gender Differences Presented in Response to Vasodilators

In order to test the endothelium dependent relaxation, acetylcholine (10^{-9} mol/L to 10^{-6} mol/L) concentration response curve was performed. To test the endothelium-independent relaxation, sodium nitroprusside (10^{-9} mol/L to 10^{-4} mol/L) was used. Acetylcholine induced relaxation was more pronounced in the male group than in the female group and L-NAME decreased this relaxation in both groups. The decrease of relaxation by L-NAME was stronger in the female group [P3, Fig.4]. The relaxation induced by sodium nitroprusside was similar in male and female mice [P3, Fig.5].

Statistical Analysis

Results of vessel diameter measurements were compared using Brunner's test (a non-parametric ANOVA-like test for repeated measurements and multiple comparisons). $P<0.05$ was used to reject the null hypothesis.

Discussion

A. The Effects of Contrast Media on Renal Arterioles and Tubules

Our main findings are as following: 1. Contrast media increase afferent arteriolar tone and reactivity to angiotensin II, while not significantly influencing efferent arterioles. 2. The degree of constriction by contrast media in human descending vasa recta is similar to the constriction observed in rat descending vasa recta. 3. Decreased nitric oxide bioavailability and increased superoxide concentration may be important mechanism for increased tone and vasoreactivity. 4. The toxicity of contrast media leads to TAL cell death and endothelial cells damage.

The resistances from afferent and efferent arterioles are determinants for the effective glomerular filtration pressure. Increasing resistance of afferent arterioles and/or decreasing resistance of efferent arterioles would reduce the effective glomerular filtration pressure. A single vessel resistance is determined by three primary factors: vessel radius (r), vessel length (L), and viscosity of the blood (η). According to Hagen–Poiseuille equation, the resistance can be expressed as following:

$$R \propto \frac{\eta \cdot L}{r^4}$$

Of these three factors, the most important quantitatively and physiologically is vessel diameter. Our results show that contrast media led to a significant constriction of afferent arterioles. Contrast media decreased the afferent arteriolar diameter by 7%, which corresponds to a physiological increase of the resistance of the blood vessel of about 20%. Furthermore, contrast media enhanced afferent arteriolar response to angiotensin II. This peptide plays a vital role in interaction with nitic oxide and in regulating renal blood flow (Patzak et al. 2001). Thus, contrast media significantly decrease the blood flow in afferent arterioles in two routes: by directly constricting afferent arterioles and by increasing the arteriolar responses to angiotensin II. However, efferent arteriolar tone and reactivity to angiotensin II did not change by contrast media. The different responses to contrast media comparing afferent and effect arterioles may contribute to the reduced blood flow and reduced effective glomerular filtration pressure. These data are in agreement with a decreased glomerular filtration rate after contrast media administration in clinical and experimental studies (Tumlin et al. 2006).

In the kidney, the most vulnerable part to contrast media is the inner part of the outer medulla because of its low oxygen tension and high metabolic activity (Persson et al. 2005). Descending vasa recta are the microvessels supplying the medulla with blood. It is very likely that contrast media induce constriction of vasa recta and then generate medullary hypoxia. We found that contrast media constricted rat descending vasa recta by roughly 50% and even enhanced their constriction to angiotensin II in our model, which could be one cause of medullary hypoxia in CI-AKI. The rat descending vasa recta lumen is 12-18μm in diameter, compared with 8μm diameter of red blood cells. If such constriction occurs in vivo, it might halt the flow of red blood cells. Evidence of red blood cell stasis in the medullary vasculature has been described in animal models of CI-AKI (Heyman et al.

1991). In our studies, we investigated the influence of contrast media on human descending vasa recta and found that during 20min perfusion contrast media reduced its diameter similarly to rat vasa recta, indicating that conclusions drawn from contrast media based on animals can be extended to humans.

Contrast media constrict afferent arterioles and descending vasa recta. This results in a decrease in the blood supply and the oxygen level in the kidney. The cytotoxicity of contrast media, independent on hypoperfusion and hypoxia, has been demonstrated in several *in vitro* models, where the oxygen levels during experiments were actually higher than *in vivo* (Sendeski 2011). Combined with the direct cytotoxicity, contrast media might damage medullary TAL. We used propidium iodide, a red-fluorescent dye and only permeable to dead cells, to estimate the TAL cell death rate by contrast media. Our findings reported propidium iodide intensity significantly increased in the contrast media perfused TAL, indicating an accelerated death rate of cells. The trypan blue experiments also indicated that TALs were damaged by contrast media treatment. Trypan blue is used to color dead cells blue while healthy viable cells do not absorb trypan blue. Further, we used a model of isolated rat interlobar arteries to study how contrast media damage the endothelial cells of renal vessels. We did not choose afferent or efferent arteriole, vas rectum or TAL, because they are all too small to be opened lengthwise for observation. We found that endothelial lining showed a ragged, irregular picture, with sharply protruding intimal folds in the scanning electron microscopy. This pattern of damage was severe, including endothelial cell lesion and desquamation, 'bleb' formation and the extension of intercellular spaces. This strongly suggests a cytotoxic effect of contrast media on the endothelial cells.

As mentioned, contrast media affect endothelial cell structure and function. Adrenomedullin has been shown to protect against loss of endothelial function (Temmesfeld-Wollbruck et al. 2007). It is a widely expressed peptide, and stabilizes endothelial barrier function directly. To test the protective effect, adrenomedullin was applied during contrast media perfusion in human and rat descending vasa recta. A less pronounced constriction and response to angiotensin II were observed by adding adrenomedullin to contrast media. Adrenomedullin was also administered in contrast perfused interlobar arteries and it prevented the anatomical damage of endothelial cells seen in scanning electron microscopy. These results support the idea of contrast media-induced endothelial damage and the usefulness of adrenomedullin for the prevention of such effects.

Our results of increased tubuloglomerular feedback function at 11mg iodine/ml, and slightly less than normal at 23mg iodine/ml may reflect the important role of superoxide in the physiology of the macula densa (Liu et al. 2004). Since superoxide enhances the signaling in tubular sodium chloride, slight initial increases in superoxide due to contrast media may have marked functional repercussions. In the light of our experiments with isolated TAL which showed signs of cell death already taking place at the concentration of 23mg iodine/ml, it is reasonable to suppose that the apparently 'normalization' of the tubuloglomerular feedback apparent on our series may in fact represent loss of function due to cell damage.

Impaired nitric oxide bioavailability seems to be the key to understand the dysfunction of afferent arterioles and TALs in response to contrast media. Our study showed a significant decrease of DAF-FM intensity along with contrast media administration in afferent arterioles and TALs. The evolution of DAF-FM fluorescence with time by contrast media was similar to that of L-NAME, demonstrating a serious harm of nitric oxide bioavailability. Nitric oxide is known to exert a wide range of effects on renal blood flow and affect tubule function (Bachmann et al. 1995). It also balances constrictive actions of angiotensin II (Patzak et al. 2001).The loss of nitric oxide bioavailability contributes to reduced glomerular filtration rate and tubular damage. Moreover, together with L-NAME, contrast media led to an additive vasoconstriction compared to contrast media alone. It suggested the constriction caused by contrast media is not only from a lower nitric oxide bioavailability, but also from other impacts on vessels.

According to other studies, increased oxidative stress has long been recognized as a major factor in the pathophysiology of CI-AKI (Heyman et al. 2010). Therefore, we investigated whether contrast media generate oxidative stress in afferent arterioles and TALs. DHE, a specific fluorescent dye, was used to estimate superoxide concentration in our experiments. We found that contrast media increased the DHE fluorescence intensity ratio in afferent arterioles and TALs, respectively, indicating an increased concentration of superoxide in vessels and tubules. Tempol, a superoxide dismutase mimetic, inhibits the increase of superoxide by contrast media. This proved that the changes we measured in the DHE fluorescence intensity ratio indeed resulted from the increase in the concentration of superoxide. This enhanced reactive oxygen species has deleterious effects, especially when nitric oxide is limited. Reactive oxygen species may further scavenge nitric oxide which leads to vasoconstriction. The results of our gene expression array which measured the expression of several genes linked to oxidative stress did not show any significant upregulation of the 84 genes investigated. Significant changes in expression were found only as downregulation of SOD3 and TXNIP (which may be involved in the initiation of local inflammation). Interestingly, our SOD activity assays showed that the lower SOD3 expression did not go along with decreases in SOD activity. It should be noted that our rats were neither dehydrated nor presented any of the risk factors that increase the risk for CIAKI in humans. It is possible that the depression of SOD activity requires circulating vasoactive substances in the microenvironment of the medulla.

In conclusion, our studies add to our knowledge of CI-AKI by showing that a type of contrast media significantly affects the renal blood vessels. In contrast to efferent arterioles, the diameter of afferent arterioles significantly decreased by contrast media, which could decrease glomerular filtration rate consequently. Contrast media induce hypoxia in the medulla when descending vasa recta are significantly constricted by contrast media. Both TALs and interlobar endothelium are damaged by contrast media. This goes along with decreased nitric oxide bioavailability and increased superoxide concentration, which contributes to the contrast media induced reduction in blood flow.

B. Gender Differences in the Function of Renal Interlobar Arteries

This study shows that male interlobar arteries constricted stronger to angiotensin II compared to that of female. Blockade of AT_2R increased the angiotensin II-induced constriction only in the female group.

Compared to angiotensin II, phenylephrine and KCl effects were similar on female and male interlobar arteries. This suggests the gender differences presenting in our contractile experiments are restricted to angiotensin II. In the angiotensin II signalling pathways, nitric oxide plays an important role (Walsh et al. 2009), so we wanted to test whether a greater nitric oxide bioavailability in females might contribute to the differences. Preincubated with L-NAME, both female and male interlobar arteries constricted stronger than their control conditions, but L-NAME abolished the difference between arteries from male and female mice in their response to angiotensin II. This indicates that these differences are due to distinct level of nitric oxide bioavailability in vessels from female and male. While estrogen deficiency is related to augmented constriction to angiotensin II, it is very possible that the estrogen takes effects on activating the endothelial nitric oxide synthase as well as increasing its expression and nitric oxide production (Orshal & Khalil 2004).

Angiotensin II exerts its effects via angiotensin II type 1(AT_1R) receptor and AT_2R. Both can induce nitric oxide release in endothelial cells. An activation of the AT_1R mainly induces vasoconstriction, whereas AT_2R induces dilation (Mehta & Griendling 2007). In our model, the blockade of AT_2R increased the angiotensin II-induced vessel constriction in female, but not in male. L-NAME abolished the gender differences during angiotensin II treatment. So it is likely that AT_2R could increase nitric oxide bioavailability during angiotensin II stimulation in interlobar arteries from female. This conclusion is supported by recent studies *in vivo* (Brown et al. 2012; Hilliard et al. 2011).

In the response to one vasodilator - acetylcholine, application of L-NAME led to a more pronounced change of vasodilation in female than that of male, indicating the nitric oxide mediated effects of acetylcholine in renal interlobar artery tone are more important in female. The similarity in sodium nitroprusside induced dilation in female and male suggests the vasodilation of our vessels is dependent on differences in the endothelial nitric oxide production, and not due to the response of vascular smooth muscle cells to nitric oxide.

In conclusion, this study demonstrates that gender differences in the angiotensin II response of renal interlobar arteries are mainly due to AT_2R mediated nitric oxide release in females.

References

Bachmann, S., Bosse, H. M. & Mundel, P. 1995. Topography of Nitric-Oxide Synthesis by Localizing Constitutive No Synthases in Mammalian Kidney. *American Journal of Physiology-Renal Fluid and Electrolyte Physiology* 268: F885-F898.

Briguori, C., Tavano, D. & Colombo, A. 2003. Contrast agent--associated nephrotoxicity. *Prog.Cardiovasc.Dis.* 45: 493-503.

Brown, R. D., Hilliard, L. M., Head, G. A., Jones, E. S., Widdop, R. E. & Denton, K. M. 2012. Sex Differences in the Pressor and Tubuloglomerular Feedback Response to Angiotensin II. *Hypertension* 59: 129-U309.

Heyman, S. N., Brezis, M., Epstein, F. H., Spokes, K., Silva, P. & Rosen, S. 1991. Early renal medullary hypoxic injury from radiocontrast and indomethacin. *Kidney Int* 40: 632-642.

Heyman, S. N., Rosen, S., Khamaisi, M., Idee, J. M. & Rosenberger, C. 2010. Reactive Oxygen Species and the Pathogenesis of Radiocontrast-Induced Nephropathy. *Investigative Radiology* 45: 188-195.

Hilliard, L. M., Nematbakhsh, M., Kett, M. M., Teichman, E., Sampson, A. K., Widdop, R. E., Evans, R. G. & Denton, K. M. 2011. Gender Differences in Pressure-Natriuresis and Renal Autoregulation Role of the Angiotensin Type 2 Receptor. *Hypertension* 57: 275-282.

Liu, R. S., Ren, Y. L., Garvin, J. L. & Carretero, O. A. 2004. Superoxide enhances tubuloglomerular feedback by constricting the afferent arteriole. *Kidney International* 66: 268-274.

Mehta, P. K. & Griendling, K. K. 2007. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *American Journal of Physiology-Cell Physiology* 292: C82-C97.

Orshal, J. M. & Khalil, R. A. 2004. Gender, sex hormones, and vascular tone. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* 286: R233-R249.

Patzak, A., Mrowka, R., Storch, E., Hocher, B. & Persson, P. B. 2001. Interaction of angiotensin II and nitric oxide in isolated perfused afferent arterioles of mice. *J.Am.Soc.Nephrol.* 12: 1122-1127.

Persson, P. B., Hansell, P. & Liss, P. 2005. Pathophysiology of contrast medium-induced nephropathy. *Kidney Int.* 68: 14-22.

Sendeski, M. M. 2011. Pathophysiology of renal tissue damage by iodinated contrast media. *Clinical and Experimental Pharmacology and Physiology* 38: 292-299.

Temmesfeld-Wollbruck, B., Hocke, A. C., Suttorp, N. & Hippenstiel, S. 2007. Adrenomedullin and endothelial barrier function. *Thrombosis and Haemostasis* 98: 944-951.

Tumlin, J., Stacul, F., Adam, A., Becker, C. R., Davidson, C., Lameire, N. & McCullough, P. A. 2006. Pathophysiology of contrast-induced nephropathy. *American Journal of Cardiology* 98: 14K-20K.

Walsh, T., Donnelly, T. & Lyons, D. 2009. Impaired Endothelial Nitric Oxide Bioavailability: A Common Link Between Aging, Hypertension, and Atherogenesis? *Journal of the American Geriatrics Society* 57: 140-145.

Publication 1

Sendeski MM, Bondke Persson A, Liu ZZ, Busch JF, Weikert S, Persson PB, Hippenstiel S, Patzak A.

Iodinated contrast media cause endothelial damage leading to vasoconstriction of human and rat vasa recta.

Am J Physiol Renal Physiol. 2012 Dec;303(12):F1592-8. doi: 10.1152/ajprenal.00471.2012.

Publication 2

Liu ZZ, Viegas VU, Perlewitz A, Lai EY, Persson PB, Patzak A, Sendeski MM.

Iodinated contrast media differentially affect afferent and efferent arteriolar tone and reactivity in mice: a possible explanation for reduced glomerular filtration rate.

Radiology 2012 Dec;265(3):762-71. doi: 10.1148/radiol.12120044.

Publication 3

Viegas VU, Liu ZZ, Nikitina T, Perlewitz A, Zavaritskaya O, Schlichting J, Persson PB, Regitz-Zagrosek V, Patzak A, Sendeski MM.

Angiotensin II type 2 receptor mediates sex differences in mice renal interlobar arteries response to angiotensin II.

J Hypertens. 2012 Sep;30(9):1791-8. doi: 10.1097/HJH.0b013e32835731dd.

Manuscript

Liu ZZ, Schmerbach K., Lu Y, Perlewitz. A., Persson PB, Seeliger E, Cantow K, Patzak A, Liu R., Sendeski MM.

Cytotoxicity of iodinated contrast media causes tubular cell damage and dysfunction, with impairment of tubulo-glomerular feedback.

Curriculum Vitae

My resume is not published for privacy reasons in the electronic version of dissertation.

List of Publications

- Sendeski MM, Liu ZZ, Busch JF, Ikromov O, Weikert S, Persson PB, Patzak A.
  Functional characterization of isolated, perfused outer medullary descending human vasa recta
  Acta Physiol (Oxf). 2013 May;208(1):50-6.
- Sendeski MM, Persson AB, Liu ZZ, Busch JF, Weikert S, Persson PB, Hippenstiel S, Patzak A.
  Iodinated contrast media cause endothelial damage leading to vasoconstriction of human and rat vasa recta
  American Journal of Physiology Renal Physiology, 2012 Dec;303(12):F1592-8.
- Liu ZZ, Viegas VU, Perlewitz A, Lai EY, Persson PB, Patzak A, Sendeski MM.
  Iodinated contrast media differentially affect afferent and efferent arteriolar tone and reactivity in mice: a possible explanation for reduced GFR
  Radiology, 2012 Dec, 265(3):762-71.
- Viegas VU, Liu ZZ, Nikitina T, Perlewitz A, Zavaritskaya O, Schlichting J, Persson PB, Regitz-Zagrosek V, Patzak A, Sendeski MM.
  Angiotensin II type 2 receptor mediates gender differences in mice renal interlobar arteries response to angiotensin
  Journal of Hypotension, 2012, Sep; 30(09): 1791-8.
- Hu Z, Liu ZZ, Deng L, Pan Y, Chen G.
  GST-Melittin Expression in Schizosaccharomyces Pombe
  Chinese Journal of Pharmaceutical Biotechnology, 2010, Jan. 33-38.
- Yang Y, Hu Z, Liu ZZ, Wang Y, Chen X, Chen G.
  High human GLUT1, GLUT2 and GLUT3 expression in *Schizosaccharomyces pombe*
  Biochemistry (Moscow), 2009 Jan;74(1):75-80.

List of Patents

- Hu Z, Liu ZZ, Chen G, Deng L, Pan Y, Zhang T, Tu Y.
  Gene cloning, heterologous over-expression and purification of Melittin in *Pichia pastoris*
  Patent Number: ZL 2009 0104221.2
  Application Number: 200910104221.2, Publication Number: 101591622
  Application Date: 2009-06-30, Publication Date: 2009-12-02
- Chen G, Hu Z, Den X, Liu ZZ, Gu F.
  Human RANTES heterologous expression and purification
  Application Number: 200810069952.3, Publication Number: 101407812
  Application Date: 2008-07-09, Publication Date: 2009-04-15
- Hu Z, Yang Y, Chen G, Chen X, Wang Y and Liu ZZ.
  Methods of heterologous expression and purification of High human GLUT1, GLUT2 and GLUT3

Application Number: 200810069723.1, Publication Number: 101285066
Application Date: 2008-05-22, Publication Date: 2008-10-15

Own Work Declaration/Affidavit

I, Zhi Zhao Liu, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic *Renal Vascular Function and Contrast Media Induced Acute Kidney Injury*. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

_____

Date Signature

Declaration of any eventual publications

Zhi Zhao Liu had the following contribution in the publications:

[P1] Sendeski MM, Bondke Persson A, Liu ZZ, Busch JF, Weikert S, Persson PB, Hippenstiel S, Patzak A. Iodinated contrast media cause endothelial damage leading to vasoconstriction of human and rat vasa recta. Am J Physiol Renal Physiol. 2012 Dec;303(12):F1592-8. (IF_{2012}=3.68)

The doctoral candidate performed rat vasa recta perfusion experiments, analyzed them and approved the manuscript's final version.
[Total contribution of the doctoral candidate: 20%]

[P2] Liu ZZ, Viegas VU, Perlewitz A, Lai EY, Persson PB, Patzak A, Sendeski MM. Iodinated contrast media differentially affect afferent and efferent arteriolar tone and reactivity in mice: a possible explanation for reduced glomerular filtration rate. Radiology. 2012 Dec;265(3):762-71. (IF_{2012}=5.73)

The doctoral candidate carried out all experiments. This included the investigation of the vasotonus and vasoreactivity of afferent and efferent arterioles and the performance of fluorescence experiments for detection of nitric oxide and reactive oxygen species. The candidate analyzed data, prepared figures, drafted manuscript and approved the final version. [Total contribution of the doctoral candidate: 80% (100% of the experimental work)]

[P3] Viegas VU, Liu ZZ, Nikitina T, Perlewitz A, Zavaritskaya O, Schlichting J, Persson PB, Regitz-Zagrosek V, Patzak A, Sendeski MM. Angiotensin II type 2 receptor mediates sex differences in mice renal interlobar arteries response to angiotensin II. J Hypertens. 2012 Sep;30(9):1791-8. (IF_{2012}=4.02)

The doctoral candidate contributed in several ways to this publication, including performing experiments, analyzing them, discussing the results and approving the manuscript's final version.
[Total contribution of the doctoral candidate: 20%]

Signature, date and stamp of the supervising University teacher

_____

Signature of the doctoral candidate

_____

Acknowledgement

Firstly, I would like to express my sincere gratitude to my professor - Andreas Patzak. It is you gave me the chance to study abroad. It is also you took me to be a member of studying 'renal vessel physiology'. Studying how to dissect and perfuse microvessels is an extremely hard work. It is a long period and full of insipidities. During nearly one year of practice, I was doubt about my ability many times and thought about giving up. But you always knew what on my mind was and tried to encourage me in kinds of ways. Without your help, I can not experience the happiness of our experiments and even can not finish my doctor study at all. Except the knowledge from you, the most valuable thing is your attitude to treat man and people. Your hard working, carefulness, modesty and helpfulness are my examples to be a good man the whole life.

And my colleagues: Mauricio Sendeski gave me a lot of advises on my experiments and my scientific writing in recent years. Kristin Schmerbach gave me wonderful ideas about the organization of the thesis. And my lovely classmates, Andrea Perlewitz, Vinicius Viegas, Tatiana Nikitia, Nanmei Liu, Anica Högner, Amira Al Masri… It is you that make my academic career full of fun and I enjoy the time with you so much. Of course, the technical assistances: Ulrike Neumann, Andrea Gerhardt, Jeannette Werne…you made my study more easy going and I can be concentrated on my project.

At last, also comes the most important part in Chinese way. I really want to thank my husband - Ran Shu and my families. In my opinion, families are the most precious treasures in one's life. There would be nothing left without families. Ran, I am so glad to meet you and marry you in Berlin. Berlin is really a magic place in my life. Ran, you always take care of me for everything and do your best to make me smile everyday. I love you. And my families, you are delighted for every achievement I got. Even something from my study which you can not understand, you are happy for me.

Thanks for everyone. Thanks for you joining in my life. I am proud of it!

Zhizhao Liu
29. Jan. 2013