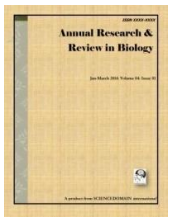

Annual Research & Review in Biology
6(5): 328-336, 2015, Article no.ARRB.2015.092
ISSN: 2347-565X

SCIENCEDOMAIN *international*
www.sciencedomain.org

Role of Interstitial Angiotensin II and ATP in Mediating Renal Injury Induced by Recurrent Insulin Induced Hypoglycemia

Priyanka Prathipati[1], Wael Alanazi[1], Fakhruddin[1], Debra W. Jackson[2] and Keith E. Jackson[1*]

[1]Department of Basic Pharmaceutical Sciences, College of Pharmacy, University of Louisiana at Monroe, Monroe, LA 71201.USA.
[2]Division of Natural Sciences and Math, School of Health Sciences, Natural Sciences and Math, Louisiana Delta Community College, Monroe, LA 71203. USA.

Authors' contributions

This work was carried out in collaboration between all authors. Author KEJ designed the study, wrote the protocol and interpreted the data. Author PP anchored the field study, gathered the initial data and performed preliminary data analysis. Authors PP, WA and Fakhruddin managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2015/16184
Editor(s):
(1) George Perry, University of Texas at San Antonio, USA.
Reviewers:
(1) Alireza Rezaeizadeh, Endocrinology Department, The University of Sydney, Australia.
(2) Anonymous, Japan.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=864&id=32&aid=8263

Original Research Article

Received 14th January 2015
Accepted 6th February 2015
Published 26th February 2015

ABSTRACT

Aim: The present study hypothesizes that recurrent insulin induced hypoglycemia (RIIH) elevates renal interstitial ATP levels which in turn enhances AngII production. This interrupts the normal tubuloglomerular feedback (TGF) mechanism by stimulating afferent arteriolar vasoconstriction resulting in hypertension, which augments oxidative stress and could promote renal damage.
Study Design: In the present study we adopted a microdialysis technique, which is a minimally invasive tool for monitoring chronic changes in renal interstitial fluid.
Place and Duration of Study: Department of Basic Pharmaceutical Sciences, University of Louisiana at Monroe between September 2012 – October 2013.
Methodology: Eight male Sprague Dawley rats (200-225 g) were anesthetized and microdialysis probes were inserted into their renal cortex. Post-surgery rats were treated with insulin (7U/kg body

**Corresponding author: E-mail: kjackson@ulm.edu;*

weight) for 2 weeks. Food and water intake were monitored daily. Physiological saline was perfused through the probe and dialysate was collected daily after insulin dosing and analyzed for ATP by luciferin-luciferase assay and AngII by EIA. At the end of the experiment, the hearts and kidneys were collected and analyzed for oxidative stress by EPR (Electron Paramagnetic Resonance) spectroscopy using CMH and CPH spin probes.

Results: ATP and AngII levels were elevated from 31.65±4.4ng/µl (day 0) to 130.96±2.9 ng/µl (day 14) and 0.1±0.01 ng/ml (day 0) to 0.247±0.02 ng/ml(day 14), respectively. Elevation of peroxynitrite and superoxide anions were observed in the hearts and kidneys of insulin treated animals when compared to the control group.

Conclusion: Thus the present study utilizes real-time chronic collections of renal interstitial samples to identify a potential mechanism where iatrogenic hypoglycemia promotes hypertension via a synergistic relationship between interstitial ATP, AngII and developed oxidative stress.

Keywords: ATP; angiotensin II; insulin; microdialysis; TGF.

1. INTRODUCTION

According to the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), 8.3% of the US population has diabetes. Among various diabetic complications, end-stage renal damage which is the result of diabetic nephropathy is increasing at an alarming rate. This is evident from the 44% increase in new kidney failure cases in 2008 as per the 2011 National Diabetes fact sheet. The present study investigates one of the possible mechanisms, whereby diabetic nephropathy could lead to kidney failure via a synergistic relationship between various biological pathways.

ATP, the energy currency of the cell, regulates various hemodynamic functions in cardiovascular tissues and organs like the kidney [1]. The sensing components of the tubuloglomerular mechanism are present in macula densa cells which respond to changes in sodium composition of the tubular fluid. These signals are transmitted to the afferent arterioles to regulate glomerular filtration rate through alterations in preglomerular vascular resistance. Emerging evidence supports a major role for ATP as a signaling component between macula densa cells and the afferent arteriolar during TGF [2]. It was proposed that, renal interstitial concentration of ATP regulates TGF dependent adjustments in renal vascular resistance [3,4]. Additionally, it was also reported that P2 receptor activation by ATP acts as a prerequisite for TGF dependent auto regulatory afferent arteriolar vasoconstriction [5]. Various *in vitro* studies have also demonstrated the role of ATP in promoting oxidative stress [6-10].

AngII, one of the key components of the renin angiotensin-aldosterone system (RAS) plays an important role in maintaining blood pressure through increases in vascular resistance. When there is an increase in blood pressure, several negative feedback mechanisms control renin release from the juxtaglomerular cells and in turn regulate the production of AngII [11]. We have recently reported that hypertension could be produced by HO-I (Hemeoxygenase I) induction and CO elevation during acute AngII infusions [12]. We have also recently reported that recurrent insulin induced hypoglycemia could promote hypertension through a mechanism that remains to be elucidated [13]. In addition, AngII was reported to play a key role in the development of diabetic nephropathy by accelerating the decline in renal function [14]. Taken together, AngII has been demonstrated to play an important role in the development of hypertension and the progression to renal injury.

Given the previously demonstrated independent roles of ATP and AngII on the renal vasculature, the current study was performed to evaluate the hypothesis that recurrent insulin injections elevate ATP levels, which induces an increase in renal AngII production resulting in inhibition of the negative feedback loop, alteration in TGF and development of diabetes associated hypertension. This persistent hypertension in turn accelerates oxidative stress by elevating levels of reactive oxygen species (ROS) thus exacerbating diabetic end-organ renal damage.

2. MATERIALS AND METHODS

2.1 Materials

CMA30 linear microdialysis probes were purchased from CMA microdialysis (Harvard Apparatus, Holliston, MA). ATP bioluminescent assay kits and deferoxaminemesylate were purchased from Sigma-Aldrich (St.Louis, MO).

AngII EIA kits were purchased from Phoenix Pharmaceuticals, Inc., (Burlingame, CA). CMH, CPH and diethyldithiocarbamic acid were purchased from Enzo Life Sciences (Farmingdale, NY). Isoflurane was purchased from Piramal Critical Care (Bethlehem, PA). Development of oxidative stress was determined by Bruker EMX EPR spectrometer with Q microwave cavity.

2.2 Animals

Seven-week-old Male Sprague-Dawley rats (200-250 g, Harlan, Indianapolis, IN; n = 8) were housed at room temperature with 12/12-hours light/dark cycle. They had free access to food and water throughout the experiment. All animal experiments were approved by the University of Louisiana at Monroe Institutional Animal Care and Use Committee (IUCAC).

2.3 Experimental Procedure

Animal surgeries were performed as previously detailed, briefly animals were anesthetized throughout the surgical procedure using an isoflourane anesthesia setup (Ez-anesthesia system) [11]. A small midsagittal incision was made to excise the kidney. Microdialysis probes were inserted into the cortex of the kidneys which were exited from the nape of the neck. The inserted probes were attached to the microdialysis setup for physiological saline [15] infusion (3 µl/min) and sample collection. The incisions were sutured and animals were given 3 days to recover. A recovery period of 3 days and the currently used probe insertion technique were previously shown to be sufficient to minimize damage to the renal tissue and to provide meaningful recovery of the substance of interest [1]. Following the experiments, kidneys were collected and the location of the microdialysis membrane was confirmed by surgical exposure of the probe.

2.4 Treatment and Sample Collection

After the recovery period, animals were treated with a daily 7Units/kg subcutaneous dose of insulin for 14 days. This insulin dose was determined in previous studies to promote sustained hypoglycemia [13]. Renal interstitial fluid samples were collected 1 hour post insulin injection for a period of 9 hours. Collected samples were stored at -80ºC until analyzed.

2.5 Analysis of the Samples

2.5.1 ATP analysis

ATP levels in the interstitial samples were quantified using luciferin-luciferase bioluminescent assay. ATP present in the sample reacts with luciferin and upon the action of firefly luciferase and oxygen, bioluminescent light is produced. The light emitted is proportional to the concentration of ATP in the sample.

2.5.2 Angiotensin II analysis

AngII was measured using a commercially available AngII EIA kit based on the principle of competitive enzyme immunoassay. The intensity of the yellow color produced due to the interaction of streptavidin-horse radish peroxidase (SA-HRP) is inversely proportional to the amount of peptide in the samples. The unknown concentration of AngII in the samples was determined from the standard curve.

2.5.3 EPR spectrometer

Oxidative stress was analyzed using a versatile, non-destructive analytical technique, EPR – Electron Paramagnetic Resonance Spectroscopy. EPR spectrometer consists of a microwave radiation source, a resonator cavity, external electrical magnets and a detector (Fig. 1). EPR is based on the principle of absorption of microwave radiation by unpaired electrons thus generating a spectrum obtained due to the transition of these electrons in the presence of an external magnetic field. Initially, the resonator cavity is tuned to absorb all the energy delivered to the cavity from the microwave generator. Upon the application of an external magnetic field, the paramagnetic unpaired electrons act like tiny independent compasses thus orienting themselves in a direction either parallel or anti-parallel to the magnetic field, creating two different energy levels for the unpaired electrons. It was observed that the number of electrons occupying the lower energy state is greater than the number at the higher energy state. This results in a larger number of transitions to the upper energy level upon absorption of microwave energy. This gives a continuous wave of EPR spectra which is the most widely used experimental approach [16]. The absorption spectra are converted to first-derivative spectra for better apparent resolutions.

EPR consists of a microwave source, circulator, resonator cavity for sample placement, external

electromagnets which provide a magnet field and the detector diode. The EPR signal is obtained as spectra, where the amplitude of the signal is proportional to the free radicals present in the sample.

ROS and peroxynitrite (ONOO-) in the samples were evaluated using EPR spectrometer. Spin probes CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine HCl) and CPH (1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine HCl) were used for superoxide radical and ONOO- determination, respectively. Briefly, kidneys and hearts were harvested at the end of the experiment, flash frozen with liquid nitrogen and stored at -80ºC until analyzed. At the time of the experiment, tissues were cut into 2mm similarly sized pieces and incubated for 1 hour at 37ºC in 0.5 ml of Krebs/HEPES buffer(pH 7.4) containing 25 µM DF and 5 µM DETC along with 5 mM of CMH or CPH [17]. The following settings were used to record EPR spectra: field sweep, 80G; microwave frequency, 9.39 GHz; microwave power, 2 mW; modulation amplitude, 5G; conversion time, 327.68 ms; time constant, 5242.88 ms; 512 points resolution; and receiver gain, 1×10^4 [18]. The amplitude of the signal was measured and quantitated to determine the amount of ROS, superoxide and ONOO- present in the sample.

2.5.4 Statistics

Data were expressed as mean ± SE and analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test when appropriate (INSTAT 3). ($P<0.05$) was accepted as statistically significant.

3. RESULTS

3.1 ATP Measurement

ATP levels in the dialysate were analyzed using luciferin-luciferase bioluminescent assay. During the 2 week period, ATP levels increased. There was a significant increase in ATP levels from day 8 (77.76±2.8 ng/µl) through day 14 (130.96±2.9 ng/µl) when compared to day 0 (31.65±4.4 ng/µl) (Fig. 2A).

3.2 Angiotensin II Measurement by EIA

Enzyme Immuno Assay (EIA) was performed on the dialysate to determine the renal production of AngII. During the recurrent insulin treatments, AngII levels increased from day 0 to day 14. There was an increase in AngII levels from day 11 (0.207±0.02 ng/ml) today 13 (0.213±0.02 ng/ml), however the largest increase was observed on day 14 (0.247±0.02 ng/ml) as compared to day 0 (0.1±0.01 ng/ml) (Fig. 2B).

3.3 Electron Paramagnetic Resonance Spectroscopy (EPR)

Chronic treatment with 7U/Kg insulin produced a significant increase in ROS, superoxide and peroxynitrite levels when compared to the control. Kidney and hearts were incubated with CMH (Fig. 3) and CPH (Fig. 4) which were analyzed using EPR spectroscopy. Resultant EPR spectra (Fig. 5) indicated the presence of ROS, superoxide and peroxynitrite, respectively. There was a significant increase in ROS and peroxynitrite levels during the 14 day experimental period.

3.4 Food and Water Intake

There were no significant differences in food and water intake during the study period (Table 1). Therefore, the observed production of hypertension during RIIH was not due to changes in food and water intake.

Table 1. Effects of 2 week administration of 7U/Kg insulin and probe insertion on food intake (gm) and water intake (ml)

	Day 0	Day 8	Day 14
Food (grams)	20±1.06	24±0.59	24±1.1
Water (ml)	30±2.9	32±4	34±3.8

There were no significant differences observed in food and water intake (Values are Mean ± S.E.M = Mean values ± Standard error of means of eight animals)

4. DISCUSSION

The present study adopts a 14 day chronic renal microdialysis technique for accurate assessment of renal interstitial AngII and ATP levels in a conscious rat model. Microdialysis provides direct sampling of renal interstitial fluid thus allowing accurate measurement of the analytes in the target renal tissue. This technique is preferable over the traditional method of blood collection as it avoids unwanted hemodynamic changes due to repeated blood sampling. Microdialysis probes also exclude unwanted substances, thus allowing only the desirable molecules into the analyte. It was previously proposed that the intra renal concentration of AngII was 1000 fold higher than that of circulating plasma levels. Thus, indicating that

there is a difference in the interstitial and circulating concentrations of substances [14].

TGF helps in maintaining the glomerular filtration rate as macula densa cells detect changes in sodium composition of tubular fluid and transmit these signals to the afferent arterioles. Depending on the concentration of NaCl in tubular fluid, afferent arterioles either dilate or constrict to maintain consistent glomerular filtration rates and renal blood flow. Previous studies have identified ATP as the mediator of macula densa cell signaling [3-5,19-23]. It was also proposed that macula densa cells express mitochondria at higher levels and basolateral Na+-K+-ATPase at lower levels. This suggests that ATP is synthesized in mitochondria and is later released into the interstitial fluid, where it acts as an extracellular signaling molecule and thus modulates renal microvascular function [3,4]. Hence renal interstitial fluid was sampled using microdialysis and an elevation in ATP levels was observed during hypoglycemia. The observed increase in ATP levels has been reported to stimulate afferent arteriolar vasoconstriction which could promote hypertension and the development of renal injury. Our data is consistent with previous studies that were performed in anesthetized dogs, where ATP was demonstrated to play a significant role in regulating TGF [1].

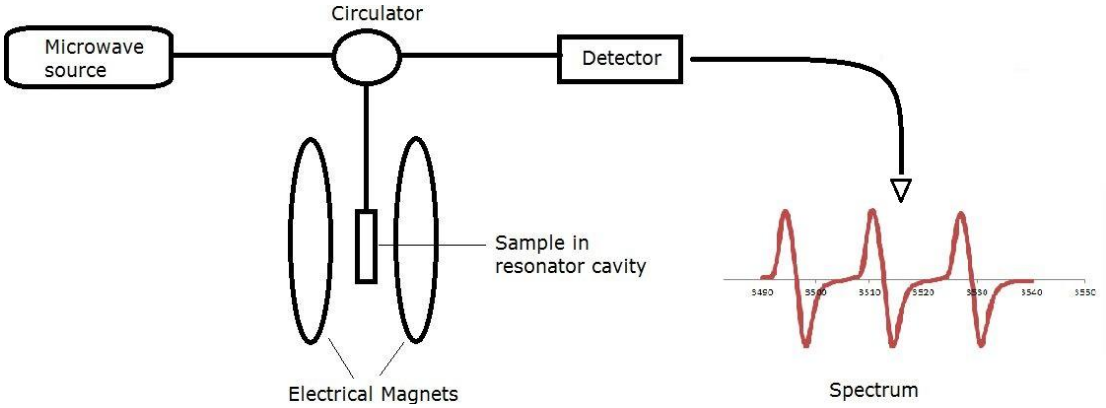

Fig. 1. EPR spectrometer

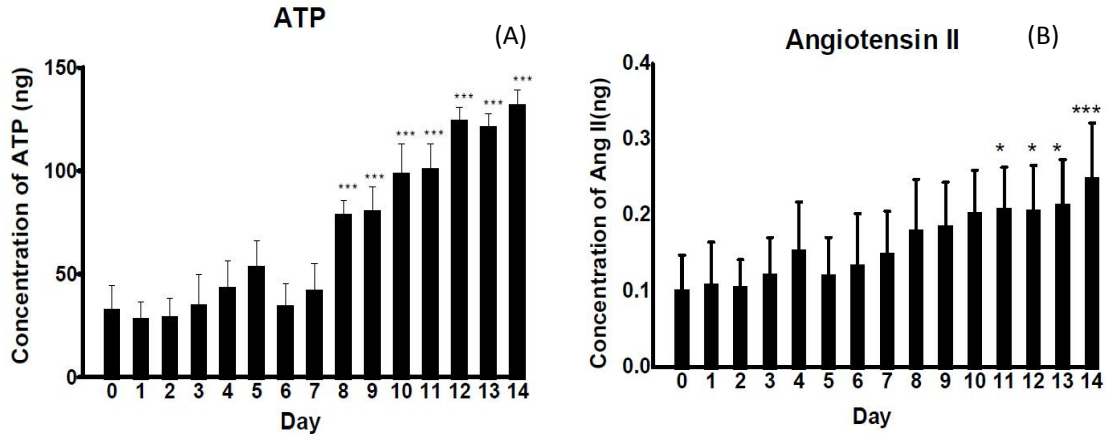

Fig. 2. (A) Analysis of ATP in awake rats, ATP levels increased from day 8 to day 14. Data were analyzed using one-way ANOVA followed by Tukey-Kramer test. (Adjusted P value<0.0001 for days 8 to 14 when compared to day 0; n = 8). (B) Analysis of AngII. AngII levels were elevated from day 11 to day 14. Data were analyzed using one-way ANOVA followed by Tukey-Kramer test. (Adjusted P value for days 11, 12, 13 and 14 – 0.0349, 0.0426, 0.0186 and <0.001 respectively when compared to day 0; n = 8)

Our lab has recently reported that there was an elevation in AngII levels during RIIH [13]. Given the observed systemic increase in AngII, we employed renal microdialysis to determine the source of the increase in circulating AngII levels. It was observed that AngII levels were elevated during the 2 weeks of insulin treatment. These elevated levels of interstitial AngII were believed to be involved in stimulating renal injury by acting on proximal tubules of the kidney ultimately resulting in increased blood volume and blood pressure. We observed that ATP levels were significantly elevated by day 8 followed by an increase in AngII levels beginning on day 10. Taken together the current study provides strong evidence for the ability of a local renal elevation in ATP to enhance renal production of AngII, where a consistent elevation in ATP disrupts the TGF mechanism. This disturbed TGF mechanism promotes an elevation in renin release and an increase in AngII production. This enhanced level of AngII further facilitates higher sodium reabsorption from the tubules disrupting both the negative feedback mechanism of RAS and TGF. Thus the synergistic effect of both ATP and AngII interrupts both RAS negative feedback and TGF ultimately resulting in persistent hypertension during RIIH.

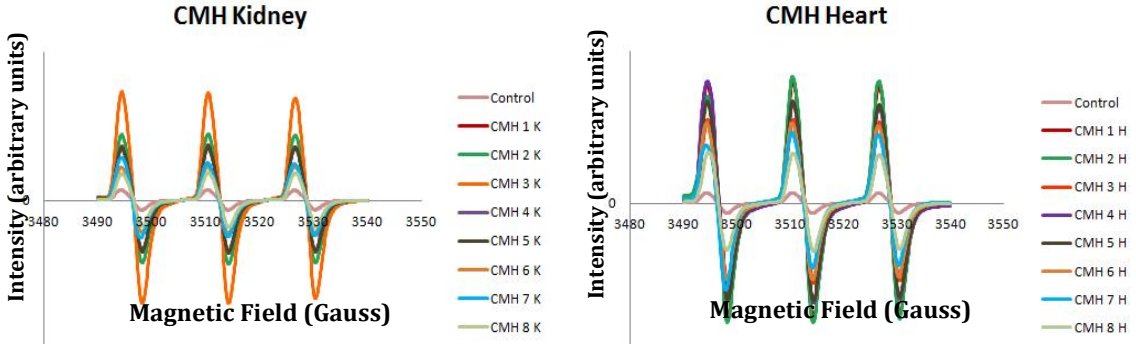

Fig. 3. Detection of ROS and superoxide. Spectra of (A) kidneys and (B) hearts obtained from EPR Sprectroscopy after incubating for 60 minutes with CMH. Insulin treatment significantly increased ROS production, which is evident from the peaks as compared to the control

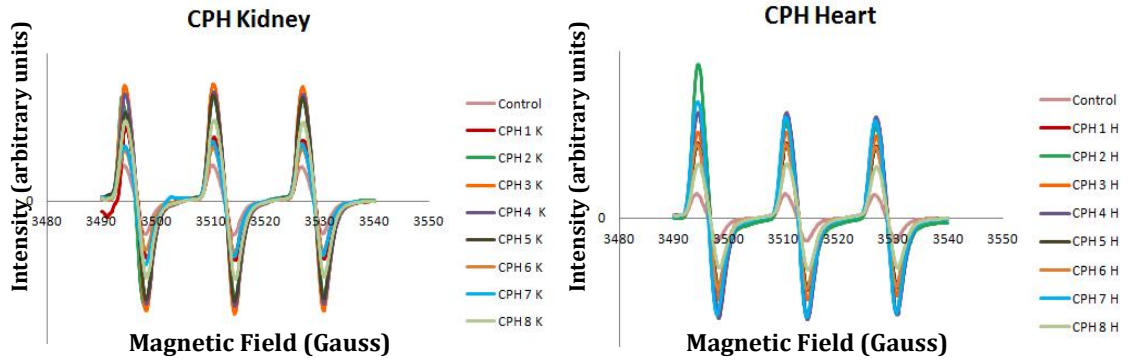

Fig. 4. Detection of Peroxynitrite. Spectra of (A) kidneys and (B) hearts obtained from EPR Sprectroscopy after incubating for 60 minutes with CPH. Insulin treatment significantly increased peroxynitrite production, which is evident from the peaks as compared to the control

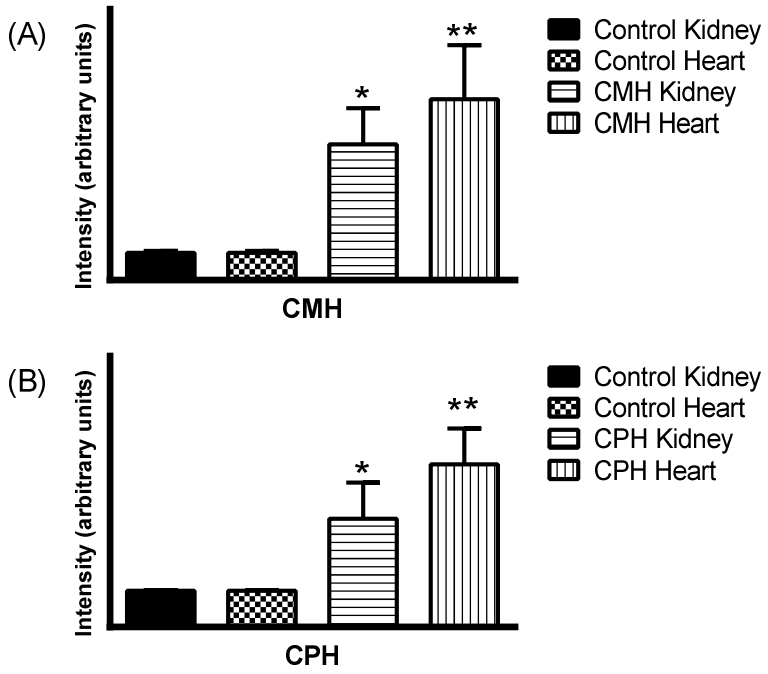

Fig. 5. Oxidative stress in the kidney and the heart Derived bar graphs from the EPR spectra peaks to compare oxidative stress produced in kidney and heart when treated with CMH (A) to detect ROS and superoxide and CPH (B) to detect peroxynitrite respectively. Data were analyzed using one-way ANOVA followed by Tukey-Kramer test. (Adjusted P value <0.0001 for CPH and CMH heart, Adjusted P value= 0.0082 for CMH Kidney and 0.0032 for CPH Kidney respectively when compared to control; n = 8)

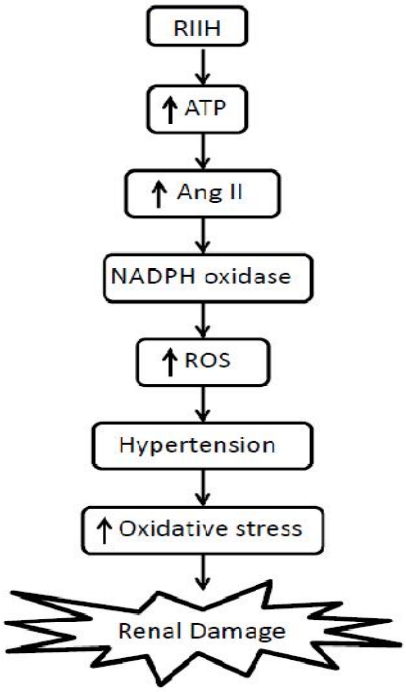

Fig. 6. Effects of RIIH

When the fine balance between the advantageous and adverse actions of various reactive oxygen species (ROS) is lost, it results in their uncontrolled generation leading to various pathological conditions. ROS such as superoxide(O^{2-}.), peroxynitrite (ONOO-), and the hydroxyl radical were found to be elevated in conditions such as hypertension, diabetes, atherosclerosis, hypercholesterolemia and various neurodegenerative diseases [24]. Thus an increase in AngII stimulates ROS generation by NADPH oxidase activation. ROS reacts with nitric oxide resulting in the production of ONOO-. A significant increase in ROS was observed in the current study during an elevation in renal ATP and AngII levels, therefore suggesting a mechanism in which hypoglycemia may promote end organ renal damage.

5. CONCLUSION

In summary the present study demonstrates that RIIH promotes an increase in renal ATP levels, which stimulates a sustained production of AngII. ATP and AngII were observed to act synergistically to promote an increase in ROS,

which has been demonstrated to produce renal injury (Fig. 6 above). This study is first of its kind to establish a correlation between renal ATP, AngII and the development of hypertension during RIIH.

Fig. 6 summarizes the study. Recurrent insulin injections promote hypoglycemia which elevates renal interstitial ATP levels. This stimulates an increase in AngII production leading to an elevation in NADPH oxidase and an induction of oxidative stress. An increase in superoxide and ATP production produces a positive feedback, which promotes the development of AngII induced hypertension. This ultimately results in diabetes associated renal damage.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Nishiyama A, Jackson KE, Majid DS, Rahman M, Navar LG. Renal interstitial fluid ATP responses to arterial pressure and tubuloglomerular feedback activation during calcium channel blockade. Am J Physiol Heart Circ Physiol. 2006;290(2):H772-7.
2. Komlosi P, Fintha A, Bell PD. Renal cell-to-cell communication via extracellular ATP. Physiology (Bethesda). 2005;20:86-90.
3. Nishiyama A, Majid DS, Taher KA, Miyatake A, Navar LG. Relation between renal interstitial ATP concentrations and autoregulation-mediated changes in renal vascular resistance. Circ Res. 2000;86(6):656-62.
4. Nishiyama A, Majid DS, Walker M, Miyatake A, Navar LG. Renal interstitial ATP responses to changes in arterial pressure during alterations in tubuloglomerular feedback activity. Hypertension. 2001;37(2 Pt 2):753-9.
5. Inscho EW, Cook AK, Imig JD, Vial C, Evans RJ. Physiological role for P2X1 receptors in renal microvascular autoregulatory behavior. J Clin Invest. 2003;112(12):1895-905.
6. Nishiyama A, Rahman M, Inscho EW. Role of interstitial ATP and adenosine in the regulation of renal hemodynamics and microvascular function. Hypertens Res. 2004;27(11):791-804.
7. Chan CM, Unwin RJ, Burnstock G. Potential functional roles of extracellular ATP in kidney and urinary tract. Exp Nephrol. 1998;6:200-7.
8. Abbracchio MP, Burnstock G. Purinergic signalling: Pathophysiological roles. Jpn J Pharmacol. 1998;78(2):113-45.
9. Sauer H, Klimm B, Hescheler J, Wartenberg M. Activation of p90RSK and growth stimulation of multicellular tumor spheroids are dependant on reactive oxygen species generated after purinergic receptor stimulation by ATP. Faseb J. 2001;15:2539-41.
10. Poelstra K, Heynen ER, Baller JF, Hardonk MJ, Bakker WW. Modulation of anti-Thy 1 nephritis in the rat by adenine nucleotides: evidence for an anti-inflammatory role for nucleotidases. Lab Invest. 1992;66:555-63.
11. Prathipati P, Quadri S, Jackson DW, Jackson KE. Adoptation of renal microdialysis for chronic interstitial collection of angiotensin II and ATP. International Journal of Biology. 2012;4(4):127-35.
12. Quadri S, Prathipati P, Jackson DW, Jackson KE. Augmentation of heme oxygenase promotes acute angiotensin II induced hypertenion. Clinical and Experimental Medical Sciences. 2013;1(1):21-43.
13. Quadri S, Prathipati P, Jackson DW, Jackson KE. Hemodynamic consequences of recurrent insulin-induced hypoglycemia. Clin Exp Pharmacol Physiol. 2014;41:81-8.
14. Siragy HM, Awad A, Abadir P, Webb R. The angiotensin II type 1 receptor mediates renal interstitial content of tumor necrosis factor-alpha in diabetic rats. Endocrinology. 2003;144(6):2229-33.
15. Hoar WS, Hickman CP. A laboratory companion for general and comparative physiology: Englewood Cliffs, N.J: Prentice- Hall; 1975.
16. Dikalov S, Griendling KK, Harrison DG. Measurement of reactive oxygen species in cardiovascular studies. Hypertension. 2007;49(4):717-27.
17. Elks CM, Mariappan N, Haque M, Guggilam A, Majid DS, Francis J. Chronic NF-{kappa}B blockade reduces cytosolic and mitochondrial oxidative stress and attenuates renal injury and hypertension in SHR. Am J Physiol Renal Physiol. 2009;296(2):F298-305.
18. Dikalova A, Clempus R, Lassegue B, Cheng G, McCoy J, Dikalov S, et al. Nox1

overexpression potentiates angiotensin II-induced hypertension and vascular smooth muscle hypertrophy in transgenic mice. Circulation. 2005;112(17):2668-76.
19. Incho EW, Carmines PK, Navar LG. Juxtamedullary afferent arteriolar respones to P1 and P2 purinergic stimulation Hypertension 1991;17:1033-7.
20. Incho EW, Cook AK, Mui V, Miller J. Direct assessment of renal microvascular responses to P2- purinoceptor agonists. Am j physiol Renal Fluid Electrolyte Physiol. 1998;274:F718-F27.
21. Inscho EW, Cook AK, Navar LG. Pressure-mediated vasoconstriction of juxtamedullary afferent arterioles involves P2-purinoceptor activation. Am J Physiol Renal Fluid Electrolyte Physiol. 1996;271:F1077-F85.
22. Majid DS, Inscho EW, Navar LG. P2 purinoceptor saturation by adenosine triphosphate impairs renal autoregulation in dogs. J Am Soc Nephrol. 1999;10:492-8.
23. Mitchell KD, Navar LG. Modulation of tubuloglomerular feedback responsiveness by extracellular ATP. Am J Physiol Renal Fluid Electrolyte Physiol. 1993;264:F458-F66.
24. Spasojevic I, Mojovic M, Ignjatovic A, Bacic G. The role of EPR sepctroscopy in studies of the oxidative status of biological systems and the antioxidative propertie of various compounds. Journal of the Serbian Chemical Society. 2011:647-77.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=864&id=32&aid=8263