Recurrent insulin-induced hypoglycemia induces AngII and COX2 leading to renal (pro)renin receptor expression and oxidative stress

Wael A. Alanazi 1, Mohammad B. Uddin 2, Selim Fakhruddin 2, Keith E. Jackson 2 *

1 Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh, 11451, Kingdom of Saudi Arabia.
2 Department of Basic Pharmaceutical Sciences, School of Pharmacy, University of Louisiana at Monroe, Monroe, LA 71201. USA
*Corresponding author E-mail: kjackson@ulm.edu

Abstract

Background: Recurrent insulin-induced hypoglycemia (RIIH) is an avoidable consequence in the therapeutic management of diabetes mellitus. RIIH has been implicated in causing hypertension through an increase in renal and systemic AngII production.

Objective: The present study was performed to assess the hypothesis that chronic insulin treatment enhances AngII and COX2 formation which in turn increases (pro) renin receptor (PRR) expression and NADPH oxidase-mediated oxidative stress, leading to renal and cardiac injury.

Methods: The present studies were conducted in Male Sprague Dawley rats treated with daily subcutaneous injections of 7u/kg insulin or saline for 14 days. On the 14th day, surgery was performed for treatment infusion (captopril 12mg/kg, NS398 0.3mg/kg or vehicle), and renal interstitial fluid sample and urine collections for biomarker measurements. At the end of the experiments, kidneys and hearts were harvested to evaluate PRR and NOX2 (NADPH oxidase subunit) expression and oxidative stress.

Results: We found that RIIH enhanced AngII and COX2 activity, leading to renal PRR expression and NADPH oxidase-induced oxidative stress in the heart and kidney. 8-isoprostanate was evaluated as a renal biomarker of oxidative stress, which was induced in insulin treated animals and modulated by captopril and NS398. In addition, there was a slight increase in NGAL, a urinary biomarker of acute kidney injury (AKI), in insulin treated animals when compared to control.

Conclusion: These results demonstrate that RIIH induces renal PRR expression and oxidative stress through increasing AngII and COX2 in the heart and kidney, leading to end-organ damage.

Keywords: Angiotensin II (AngII); Cyclooxygenase-2 (COX2); (Pro) Renin Receptor (PRR); Oxidative Stress; Recurring Insulin-Induced Hypoglycemia (RIIH)

1. Introduction

Free radicals are the byproducts of various enzymatic reactions in cellular metabolic processes. These byproducts have physiological and pathophysiological functions in several body systems (Bergendi et al. 1999, Dröge 2002). Free radical formation is associated with various cellular functions including signal transduction and gene transcription (Finkel 2011, Kunsch & Medford 1999). In 1956, Denham Harman mentioned in his “free radical theory” that free radical formation occurs mainly during enzymatic reactions involving oxygen molecules and results in cellular damage at both the DNA and protein level (Harman, 1956). However, the imbalance in free radical production leads to disturbances in many biological processes, which promotes cellular dysfunction. Reactive oxygen species such as superoxide (O2-), hydrogen peroxide, hydroxyl radical and peroxynitrite (ONOO-) have unpaired electrons in their outer orbit (Pavelescu 2015). The excess amount of these unpaired electrons destroys cellular lipids, proteins, and nucleic acids that have a role in different biological processes (Pruchnik et al. 2016). However, the overproduction of reactive oxygen species accelerates the incidence or complications of disorders for example diabetes mellitus, cardiovascular diseases, diabetic nephropathy, Alzheimer’s disease, and Parkinson’s disease (Popolo et al. 2013).

Several studies have shown the correlation between oxidative stress and the pathogenesis of both major types of diabetes as well as the development of diabetic complications (Yang et al. 2011, Giacco & Brownlee 2010). In diabetic patients, free radicals are formed by different metabolic pathways such as glucose oxidation, degradation of glycated proteins, nonenzymatic glycation of proteins, and/or a decline of antioxidant defenses (Fiorentino et al. 2013). All these mechanisms participate in the elevation of oxidative stress, which in turn causes insulin resistance, and persist in the development of diabetic complications. During the management of diabetes, hypoglycemia is the most common adverse complication which arises as one tries to obtain a state of euglycemia. In type1 diabetes (T1D), studies have shown that the progressive glycemic control caused up to 10 hypoglycemic episodes per week and produced mortality in 2-4% of patients with T1D (The DCCT Research Group 1993, The DCCT Research Group 1997, Cryer 2004). In addition, it has been documented that the frequency of hypoglycemic episodes in patients with type2 diabetes (T2D) is more common in insulin and sulphonylurea-dependent T2D (Kalra et al. 2013, Shafiee et al. 2012). Severe hypoglyce-
mia activates several physiological actions, causing autonomic activation which in turn induces release of adrenaline and other stress hormones. However, these stressors cause hemodynamic changes including an increase in the heart rate, stroke volume and systolic blood pressure as the body aims to maintain a continuous glucose supply to the brain (Wright & Frier 2008). Diabetic patients with insulin therapy often have impaired awareness of hypoglycemia leading to disturbances in various organs such as the brain, heart, and kidney (Kalra et al. 2013). Previous studies have found a positive correlation between hypoglycemia and tachycardia, myocardial contractility, cytokine secretion and endothelial dysfunction (Fisher et al. 1990, Desouza et al. 2010). All of these are factors, which are known to contribute to the incidence and development of cardiovascular diseases.

Hypoglycemia which is associated with intensive insulin therapy or starvation has been indicated in the elevation of oxidative stress in different hypoglycemic models (Razavi Nematollahi et al. 2009, Bharadwaj et al. 1998). A previous study has found that the depletion of scavenging agents for instance glutathione S-transferase (GST), glutathione peroxidase (GPx), and glutathione reductase (GR) promote oxidative stress in the brain, liver and kidney during RIH or starvation (Bharadwaj et al. 1998). On the other hand, NADPH oxidase was recently proposed as a source of hypoglycemia-induced oxidative stress during glucose reperfusion, which was blocked by apocynin treatment in in-vitro and in-vivo studies (Suh et al. 2007). Taken together, the literature does support activation of NADPH oxidase and/or inhibition of scavengers has a cause of oxidative stress development during hypoglycemic conditions.

The renin angiotensin aldosterone system (RAAS) has an essential role in maintaining blood pressure and body fluid hemostasis through interactions with various cellular pathways. Angiotensin II (AngII) is the major effector peptide in the RAAS, which is well known as a potent vasoconstrictor. As previously described, chronic AngII treatment caused an elevation in the mean arterial pressure, prostatesglin induction which lead to endothelial dysfunction and oxidative stress (King & Fink 2006, Schrader et al. 2007, Hernández et al. 2002, Touyz & Schiffrin 1999). We have previously shown that in addition to AngII mediated vasoconstriction, it also activates other pathways including enhancing carbon monoxide release (CO) by increasing HO-I (Heme oxygenase I), which leads to persistent hypertension (Quadri et al. 2013). Moreover, it has been documented that AngII plays a critical role in the development of hypertension and renal dysfunction through afferent arteriole vascular constriction and sodium/water reabsorption (Baltatzi et al. 2011).

In the renal vasculature, AngII via type 1 receptors (AT1) increases prostaglandin production by inducing PLA2 and COX2, which provides a counteracting mechanism to AngII-induced vasoconstriction (Hu et al. 2002). PGE2 and PGJ2 modulate AngII-induced renal vasoconstriction, which would lead to a reduction in the renal blood flow (RBF) and glomerular filtration rate (GFR) (Imig 2006). Therefore, renal COX2-derived prostanooids, specifically PGE2 and PGJ2 act as physiological buffers by maintaining normal blood pressure and renal excretory function.

In recent reports, AngII and COX2 have been targeted to investigate the mechanism for enhanced expression of (pro) renin receptor (PRR) during AngII-dependent hypertension. PRR has been identified recently as a receptor for renin and its precursor prorenin, where it induces local production of AngII (Nguyen et al. 2002). PRR expression has been shown to induce local RAS activation in the kidney, heart, brain and pancreas (Li et al. 2012). In the kidney, the PRR is localized in mesangial cells, intercalated type-A cells and podocytes (Nguyen et al. 1996, Ichihara et al. 2008, Advani et al. 2009). Intensive investigations have been undertaken to understand the role of AngII in the regulation of PRR expression in the kidney. In AngII-induced hypertension, studies have discovered that COX2-derived PGE2 play an essential role in enhancing PRR expression through binding to EP4 receptors in the renal medulla (Green et al. 2012, Wang et al. 2014, Yang 2015). However, cross-talk between renal medullary PRR and COX2-derived PGE2 has been documented during AngII-induced hypertension.

Several studies support the affirmation that AngII plays an essential role in superoxide production and nitric oxide (NO) reduction via induction of several pathophysiological pathways which in turn cause oxidative stress. In diabetic patients, RAAS activation promotes renal oxidative stress through NADPH oxidase-induced superoxide, and it also regulates nitric oxide synthase (NOS) in afferent arterioles through AT1 and AT2 receptors (Ratliff et al. 2010). Emerging evidence has found a circuitous relationship between cyclooxygenases (COX1 and COX2) and NADPH oxidase (Hernanz et al. 2010). Namely, ROS enhance cyclooxygenase production, which in turn induce ROS formation through prostanoides formation and other ROS generating pathways (Hernanz et al. 2010).

In the current study, we hypothesize that RIIH augments PRR expression and oxidative stress through AngII and COX2 induction leading to cardiac and renal injury.

2. Methods

2.1. Materials

CMA 30 linear microdialysis probes were obtained from CMA/Microdialysis (Harvard Apparatus, Holliston, MA, USA). Humulin was purchased from Eli Lilly and Company (Indianapolis, IN, USA). 4-20% gradient SDS-PAGE was obtained from Life Technologies Collaborations (Grand Island, NY, USA). Primary antibodies for PRR and NOX2 were purchased from Abcam (Cambridge, MA, USA). ELISA kits (8-iso-PGF2alpha and NGAL), spin trapping reagents (CMH and CPH) and diethylthiocarbamic acid were obtained from Enzo Life Sciences (Farmingdale, NY, USA). Chemicals such as inactin (thiobutabarbital sodium), captopril, Ns398 and 2-Methylbutane (isopentane) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other materials were obtained from Fisher Scientific (Houston, TX, USA).

2.2. Animals

Male Sprague-Dawley rats (200 - 250g, n= 20, Harlan, Indianapolis, IN, USA) were housed in a controlled environment and had free access to food and water throughout the study. All experiments were approved by the University of Louisiana at Monroe Institutional Animal Care and Use Committee (IUCAC). In the current study, we followed a previously described hypoglycemia model protocol, which was used to investigate the roles of hypoglycemia in neurological functions and hemodynamic changes (Paranjape & Briski 2005, Quadri et al. 2014, Prathipati et al. 2015). Briefly, two groups of animals were treated subcutaneously with 7units/kg of humulin insulin (n=15) or saline (n=5) for two weeks.

2.3. Experimental procedure

After two weeks of insulin treatment, rats were anaesthetized by inactin (thiobutabarbital sodium, 120mg/kg ip). Surgical procedures were performed as previously described (Quadri et al. 2013, Alanazi et al. 2016). Briefly, the trachea was cannulated to allow an open airway throughout the experiment. The carotid artery, jugular vein and bladder were cannulated for hemodynamic evaluation, treatment infusion and urine sampling, respectively. After the cannulation procedures, rats were placed on their right side, and a small midsgatinal incision was made to expose the left kidney. CMA 30 linear microdialysis probes were inserted into the renal cortex for renal interstitial sample collection.

2.4. Treatment and samples collection

After surgery, a 45min recovery period was observed followed by a 4 hour control period in both normal and insulin treated groups. After the control period, the hypoglycemic animals were treated with a bolus dose of vehicle (7U+saline), captopril, 12mg/kg (7U+captopril) or NS398, 0.5mg/kg (selective COX2 inhibitor).
(7U+NS398). Throughout the experiments, urine and renal interstitial samples were collected and stored at -80°C until analyzed for neutrophil gelatinase-associated lipocalin (NGAL) and 8-isoprostane (8-iso-PGF2α) analysis, respectively. At the end of the experiments, hearts and right kidneys were harvested and rapidly immersed in isopentane for snap freezing and stored at -80°C until analyzed.

2.5. Samples analysis

2.5.1. Western blot analysis

As previously described, renal and cardiac homogenates were assayed for PRR and NOX2 proteins by western blotting (Gupta et al. 2012). Briefly, tissue lysates were prepared by using NP40 cell lysis buffer for protein extraction. Protein concentrations were determined via a bichinonic acid (BCA) protein assay kit. The obtained proteins were resolved by using 4-20% gradient SDS-PAGE. After separation, proteins were transferred to a nitrocellulose membrane and blocked in 5% fat-free milk in 0.05% Tween-20, 2 mM phosphate buffered saline, pH 7.4 (PBST). The blots were incubated with anti-PRR or anti-NOX2 antibodies at 4°C overnight and then washed with PBS and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a protein loading control.

2.5.2. EPR spectrometer

Electron Paramagnetic Resonance (EPR) spectrometer targets reactive oxygen species that have paramagnetic characteristics namely, O2- and ONOO-. The unpaired electrons of the O2- and ONOO- absorb the applied microwave radiation in a resonator cavity of EPR spectrometer within a static paramagnetic field that is produced by external magnets. A spectrum is generated due to the transition of the unpaired electrons to either parallel or anti-parallel direction to the magnetic field.

In the current study, O2- and ONOO- were measured in hearts and kidneys by using EPR spectrometer. CMH (1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine.HCl) and CPH (1-hydroxy-3-carboxyl-2, 2, 5, 5-tetramethylpyrrolidine.HCl) were used as spin trapping reagents to trap O2- and ONOO- for measurement by EPR spectrometer. On the day of analysis, the stored hearts and kidneys were thawed and cut into 2 mm slices. The cardiac and renal pieces were incubated for 1 hour at 37°C in 0.5 ml of D/F/DETC/HEPES buffer containing 5 mM of CMH or CPH (Dikalov et al. 2007). After incubation, a micropipette was filled with the sample solution to be ready for EPR analysis. EPR spectrometer was adjusted to the following settings: field sweep, 80 G; microwave frequency, 9.64 GHz; microwave power, 1.34 mW; modulation amplitude, 5 G; conversion time, 327.68 ms; time constant, 10.24 ms; 512 points resolution; and receiver gain, 1×104.

2.5.3. 8-isoprostane analysis

8-iso-PGF2α or 8-isoprostane is known as a biomarker of oxidative stress (Cracowski et al. 2002). The collected renal interstitial samples were analyzed for the presence of 8-isoprostane to confirm the induction of oxidative stress during RIIH. The collected renal interstitial samples were analyzed by commercially available ELISA kits.

2.5.4. Neutrophil gelatinase-associated lipocalin (NGAL) analysis

In the present study, the urinary NGAL was measured as a biomarker of acute kidney injury (AKI) (Devarajan 2010). Urine samples were analyzed by commercially available ELISA kits.

2.6. 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 considered significant.

3. Results

3.1. PRR expression

Western blot analysis of PRR showed a significant increase in PRR protein abundance in the kidney during RIIH (7U+vehicle) as compared with control. Captopril (7U+captopril) and NS398 (7U+NS398) treatment reduced RIIH-induced renal PRR protein (Fig.1). In the heart, RIIH enhanced the abundance of PRR protein but captopril and NS398 administration did not return PRR protein to control levels (Fig.2).

Fig. 1: PRR Expression in the Kidney. A Significant Increase in PRR Expression during RIIH as Compared with Control (**P<0.01). Captopril and NS398 Significantly Decreased PRR Expression during RIIH. Values are expressed as Mean ± SE.

Fig. 2: PRR Expression in the Heart. A Significant Increase in PRR Expression was Observed during RIIH as Compared with Control (**P<0.01). Captopril and NS398 Didn’t Change PRR Expression Significantly during RIIH. Values are expressed as Mean ± SE.

3.2. Superoxide and peroxynitrite measurement in the heart

Two weeks of 7U/Kg insulin treatment induced ROS (O2- and ONOO-) formation significantly in the heart when compared to saline treated animals (Fig.3) (Fig.4). The incubated cardiac tissues with CMH and CPH showed a massive induction of O2- and ONOO- in 7U+vehicle treated animals as compared to the control. Captopril treatment decreased O2- and ONOO- formation in the 7U+captopril group and returned them to the normal levels when compared to 7U+vehicle and control groups. NS398 treatment reduced RIIH-induced O2- and ONOO- significantly in 7U+NS398 group as compared to 7U+vehicle group (Fig.3) (Fig.4).
3.3. Superoxide and peroxynitrite measurement in the kidney

O2- and ONOO- levels were significantly elevated in the kidney during two weeks of 7U insulin treatment as compared to saline treated animals (Fig.5) (Fig.6). Incubation of renal slices with CMH and CPH allowed EPR spectrometer to record significant increases in O2- and ONOO- levels in 7U insulin treated rats when compared to the control group. In 7U+captopril group, captopril blocked O2- and ONOO- formation and returned them to the normal levels during RIH as compared to 7U+vehicle and control groups. NS398 reduced O2- and ONOO- induction in 7U+NS398 treated animals when compared to 7U+vehicle group (Fig.5) (Fig.6).

Fig. 3: Superoxide Measurement in the Heart. (A) Spectra were obtained from EPR Spectrometer where Cardiac Samples were incubated with CMH. (B) Bar Graphs were derived from the Peaks of EPR Spectra to Compare Superoxide Levels in the Hearts of All Groups. (***P <0.001) Values are expressed as Mean ± SE.

Fig. 4: Peroxynitrite Measurement in the Heart. (A) Spectra were obtained from EPR Spectrometer Where Cardiac Samples were incubated with CPH. (B) Bar Graphs were derived from the Peaks of EPR Spectra to Compare Peroxynitrite Levels in the Hearts of All Groups. (***P <0.001) Values are expressed as Mean ± SE.

Fig. 5: Superoxide Measurement in the Kidney. (A) Spectra were obtained from EPR Spectrometer Where Renal Samples were Incubated with CMH. (B) Bar Graphs were derived from the Peaks of EPR Spectra to Compare Superoxide Levels in the Kidneys of All Groups. (***P <0.001) Values are expressed as Mean ± SE.
3.4. NOX2 expression

Western blot analysis of NOX2 showed a significant increase in NOX2 protein abundance in the kidney and heart during RIIH (7U+vehicle) as compared with control. Captopril (7U+captopril) and NS398 (7U+NS398) treatment reduced RIIH-induced NOX2 protein in the kidney and heart (Fig.7) (Fig.8).

\textbf{Discussion}

The present study investigates the role of AngII and COX2 during RIIH in inducing PRR expression and development of oxidative stress, which promotes organ damage. The current results discovered for the first time that RIIH increases PRR expression in the heart. In the heart, we found that RIIH also increased 8-isoprostanate (8-iso-PGF2α) levels in the renal interstitial fluid during RIIH (Fig.10).

3.5. 8-isoprostanate measurement in the renal interstitial fluid

In 7U+vehicle treated group, 8-isoprostanate was elevated significantly (834.55±28.42pg/ml) as compared with the control group (559.92±30.46pg/ml). Captopril and NS398 blocked the RIIH-induced oxidative stress in 7U+captopril (530.34±16.83pg/ml) and 7U+NS398 (531.53±24.01pg/ml) treated groups respectively when compared to 7U+vehicle and control groups (Fig.9).
Previous reports have demonstrated that hypoglycemia increases production of reactive oxygen species, however the underlying mechanism is still unclear (Haces et al. 2010, McGowan et al. 2006, Páramo et al. 2010). Our previous results have shown that AngII induced hypertension during RIIH promotes an increase in CO levels through the inducible HO enzyme, HO-1 (Quadri et al. 2014). Through daily measurements of renal interstitial ATP and AngII, we found that RIIH augmented ATP and AngII leading to attenuation in tubuloglomerular feedback (TGF) which might increase oxidative stress in kidneys (Prathipati et al. 2015). However, in the current study, we found that AngII inhibition reduced RIIH-induced reactive oxygen species formation (O2- and ONOO-) in renal and cardiac tissues. These results validated that RIIH-induced AngII is involved in causing oxidative stress. In recent reports of our lab, RIIH was shown to activate renal COX2-derived prostanoids, which are known to enhance superoxide formation (Wong et al. 2010, Tian et al. 2012, Alanazi et al. 2016 in press). Hence, we targeted COX2 to investigate its role in the development of renal and cardiac oxidative stress during RIIH. As anticipated, COX2 inhibition showed a reduction in O2- and ONOO- in the hypoglycemic animals, thus demonstrating that COX2 plays a role in RIIH-induced oxidative stress. Taken together, RIIH augments reactive oxygen species formation via activation of AngII and COX2-derived prostanoids. In addition, RIIH might also enhance superoxide formation via PRR activation, which was frequently implicated in arteriosclerotic smooth muscle cell induction of superoxide release (Liu et al. 2014).

For further investigation, renal interstitial samples were evaluated for the presence of oxidative stress through measurement of 8-isoprostane, which is a biomarker of oxidative stress. Renal 8-isoprostane levels were elevated during RIIH demonstrating the presence of oxidative stress in the kidney. AngII and COX2 inhibition attenuated the elevation in renal 8-isoprostane, thus demonstrating the underlying mechanism for oxidative stress formation in the kidney. Moreover, RIIH-induced AKI was evaluated through measurement of urinary NGAL, which showed a slight increase in NGAL levels that might indicate mild AKI. Hence, these results suggest that long term hypoglycemic episodes might be associated with progression to chronic kidney diseases (CKD) in insulin-dependent diabetes mellitus.

AngII has been implicated in causing oxidative stress in numerous diseases including hypertension and diabetes via enhanced superoxide formation mainly through activation of NADPH oxidase (Dikalov & Nazarwcz 2013). AngII-bound AT1 receptors mediate the translocation of cytoplasmic subunits of NADPH oxidase to attach to the cell membrane subunits for activation (Wei et al. 2006). ACE inhibition (captopril) and AT1 receptor blockade (losartan) modulated AngII-induced superoxide formation through reduced NADPH oxidase activation and an increase in NO bioavailability (Rajagopalan et al. 1996, van der Giet et al. 2002, Deng et al. 2009). It is suggested that AngII impairs production of endothelial NO through a reduction in eNOS activity, during chronic disease states including diabetes, diabetic nephropathy and hypertension (Zhang et al. 2003, Toda et al. 2007). The literature supports the potential for crosstalk between COX2 and NADPH oxidase (Cheng et al. 2013, Hernanz et al. 2014). A previous report showed that COX2 plays a significant role in AngII-induced NADPH oxidase-derived 02- production (Wu et al. 2005). Rofecoxib (Selective COX2 inhibitors) blocked superoxide formation and reduced cardiac hypertrophy, which provided antioxidative properties during enhanced AngII activity (Wu et al. 2005). Collectively, these results demonstrate that AngII induces oxidative stress either directly through activation of reactive oxygen species sources or via an enhancement in COX2 activity. This synergistic action of AngII might be an amplifying mechanism, which leads to organ damage that is commonly associated with serious disease stages like hypertension. However, further investigation is required to clarify the particular role of each prostanoid in the activation of AngII/COX2/NADPH oxidase pathway that is correlated with oxidative stress during hypoglycemic conditions.

5. Conclusion

The current results demonstrated that AngII and COX2 play an essential role in promoting renal PRR expression and oxidative stress during RIIH (Fig. 11). Hence, AngII blockade and COX2 inhibition maybe critical therapeutic targets for the prevention of RIIH-mediated renal and cardiovascular injury.

References


[54] Touyz RM & Schiffrin EL (1999) Ang II-stimulated superoxide production is mediated via phospholipase D in human vascular smooth muscle cells. Hypertension. 34, 976-82. https://doi.org/10.1161/01.HYP.34.4.976.


