

Increase Renal CYP2C23 Expression Using Thiazide Diuretics Attenuate the Progression of Cardiovascular Disease in Rat Model of Chronic Renal Failure

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Abstract

Background: Cardiovascular diseases considered as major cause of morbidity and mortality in patients with chronic renal failure. CYP2C23 epoxygenases is one of CYP2C subfamily enzymes that expressed in the kidney. They convert arachidonic acid (AA) to Epoxyeicosatrienoic acids (EETs) which have an important role in regulation of cardiovascular function.

Aim of Study: Present study aimed to study the effect of indapamide on the expression of CYP2C23 and its impact on cardiovascular function in rat model of CRF.

Methods: Twenty four adult male albino rats were randomly divided to a sham operated, CRF which underwent 5/6 subtotal nephrectomy and CRF treated with indapamide 1 mg/kg per day for 10 weeks.

Results: We demonstrated significant increase in systolic and diastolic blood pressure, deterioration in cardiac function (EF% and FS%) and impaired vascular reactivity in CRF group as compared to control group. Administration of indapamide significantly reverse the cardiovascular deterioration with increase in renal CYP2C23 expression and decrease cardiac NF- κ B as compared to untreated group.

Conclusion: We concluded that indapamide prevent progression of cardiovascular disease in rat model of chronic renal failure. We attributed this improvement to ability of indapamide to increase renal CYP2C23 expression which ameliorated oxidative stress and inflammation.

Key Words: Renal CYP2C23 – Vascular reactivity – Cardiac NF- κ B – Indapamide – Chronic renal failure.

Introduction

CARDIOVASCULAR complications in chronic kidney disease patients are common and have great impact on human suffering and health economics

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[1]. The exact mechanisms of the cardiovascular pathophysiology in CKD/ESRD have not been completely elucidated. The kidney appears to act as a perceiver and modulator of cardiovascular disease [2].

The CYP2C subfamily enzymes are the major P450 epoxygenases in the kidney. CYP2C23 is the main enzyme expressed in the rat kidney that converts arachidonic acid to 8,9-,11,12- and 14,15- epoxyeicosatrienoic acids (EETs) in a ratio of 1:2:1 [3]. These EETs are further metabolized to their corresponding less-active dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH) [4]. The P450 w-hydroxylases produce 20-hydroxy-eicosatetraenoic acid (20-HETE). Both EETs and 20-HETE are involved in the regulation of vascular function [5].

These EETs are involved in the maintenance of cardiovascular homeostasis [6]. They are considered vasodilatory regulators of vascular tone [7] and regulate renal sodium and water excretion [8]. Also, they have an anti-inflammatory properties [9] and thrombolytic activity [10] and can protect blood vessels from remodeling by inhibiting vascular smooth muscle cell proliferation [11]. Accumulating evidence suggested that EETs have protective effects on cardiovascular system and pharmacological up regulation of EETs signalling pathway may be useful for endothelial function [12].

The valuable functions of EETs raised them as a target for management of end organ disease accompanied CVD.

Indapamide is known as a diuretic that has a significant hypotensive effect with a lower incidence of severe hypokalemia and hyperglycemia [14], and retains value in patients with chronic kidney disease [15]. It has been reported that it reduces left ventricular hypertrophy [16,17] and reduces microalbuminuria in patients with diabetes and hypertension [18]. Ma et al., 2013, found that indapamide could affect the expression of CYP2C23 in kidney causing increase in the concentration of EETs [19].

We hypothesized that indapamide could increase kidney tissue concentrations of epoxyeicosatrienoic acids (EETs) by increasing renal CYP2C23 expression. The main idea of our work is to examine the effect of the increase in renal CYP2C23 expression on the progression of cardiovascular disease in rat model of chronic renal failure (CRF).

Material and Methods

Experimental animals:

Our study was done in the Department of Physiology, Faculty of Medicine, Cairo University. The total duration of the study was 10 weeks (from September, 2017 to December, 2017). Twenty four male albino rats, approximately 12 weeks of age, and of nearly similar weights ranging from 160 to 200 grams, were included in the study. The animals were placed under ordinary living conditions in the animal house and kept at room temperature in wire mesh cages, fed on standard rat diet (standard rat chow) and subjected to 12h light/dark cycle.

We randomly divided the animals into the following:

- *Group 1 : Sham operated (Sh) group (n= 8) :*

Eight rats were included in this group and underwent sham operation. Animals belonging to this group were kept under the same experimental conditions as the rest of the groups for 10 weeks.

- *Group 2: Chronic renal failure model (CRF) group (n=8):*

Eight rats were assigned in this group and underwent 5/6 subtotal nephrectomy and then housed for 10 weeks after operation.

- *Group 3: Chronic renal failure model treated with indapamide (CRF+ IDP) group (n=8):*

Eight rats were assigned and underwent the same operation as group 2 and were also housed for 10 weeks. This group received indapamide at dose 1mg/kg per day in drinking water [20] throughout the study till the 10 week.

Experimental protocol:

Nephrectomy procedure:

We induce CRF in rats surgically through one-step 5/6th nephrectomy [20]. Briefly, Rats were anesthetized with 0.15ml/100gm body weight of prepared solution formed of ketamine 100mg/kg and xylazine 10mg/kg cocktail injected intraperitoneal [21]. A left kidney was exposed via midline abdominal incision and decapsulated. We burnt Two-thirds of the kidney using electro-cautery and replaced the remnant kidney. Afterthat, the right kidney was located and decapsulated. The renal vessels were ligated and the whole kidney removed. Antibiotics were given after surgery to avoid post-operative infection.

A sham procedure was done in control animals where we decapsulated both kidneys solely and replaced them intact.

Data collection:

1- Biochemical measurements:

Ten weeks later, we were with drawn blood samples via retro orbital route by using capillary tubes and then we collected them in Eppendorf tubes.

We were separated Plasma by centrifugation and then stored at $\leq -20^{\circ}\text{C}$ for biochemical estimation of the following parameters:

- Serum urea was measured using Quanti-ChromTM Urea Assay kit (DIUR-500) [22].
- Serum creatinine was estimated by Quanti-ChromTM creatinine Assay Kit [23].
- TNF- α in serum was measured using quantitative sandwich enzyme immunoassay technique [24].
- Malondialdehyde (MDA) was estimated through thiobarbituric acid (TBA) diluted in 2 Mol. sodium sulfate, 1ml of 0.67% TBA and 2.5ml of 20% trichloroacetic acid.

2- In vivo studies:

Ten weeks later, at the end of the experiment, blood pressure and echocardiography were done to all groups:

• Blood pressure measurements:

After warming rats till 32C, the arterial blood pressure was measured using the noninvasive tail-cuff method (ML 125NIBP, AD Instruments, Australia).

• *Echocardiography:*

Echocardiography was done with a system that is equipped with a 12-MHz phased-array transducer (SONOS 5500; Philips Medical System, Best). We assessed LV systolic function through measuring of ejection fraction (EF) and LV fractional shortening (FS) which calculated from LV M mode by the following equations:

$$FS\% = [(LVEDD - LVESD) / LVEDD] \times 100$$

$$EF\% = [(LVEDV - LVESV) / LVEDV] \times 100$$

3- *In vitro studies:*

At the end of the study, rats were sacrificed using high dose of phenobarbitone, and then the thoracic aorta, renal tissue and heart tissue were excised and prepared as follows:

A- *Isolated aorta preparation:*

The chest cavity was exposed and the thoracic aorta excised. Then, it was divided into rings of 3mm length and kept in organ chambers that are filled with 10ml of Krebs-Heinseleit solution (mM: NaCl 118, CaCl₂ 2.5, NaHCO₃ 25, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, glucose 10) maintained at 37°C, pH 7.4 and gassed with 95% O₂ and 5% CO₂. After that, ring was carried between 2 hooks and attached to an isometric force transducer which is then connected to a data acquisition system (Power lab 8SP, AD Instruments) to allow continuous monitoring of tension of 1 gram for one hour. Concentration-response curves were generated to phenylephrine (264 µg) and were compared only at 2, 4, 8 and 16 µg concentrations. After contraction with phenylephrine reached a plateau, acetylcholine was added at cumulative concentrations (2–16 µg) without wash. The value of phenylephrine precontraction was measured, and then the rates of relaxation were expressed as percentages to this phenylephrine contraction [26].

Percentage of acetylcholine relaxation = (Value of phenylephrine precontraction - value of phenylephrine after relaxation) / value of phenylephrine precontraction x 100).

B- *Detection of relative gene expression by real time PCR:*

Renal and heart tissue were extracted and prepared for Biochemical detection of relative gene expression levels of CYP2C23 and Nuclear factor-κB (NF-κB) respectively according to the following steps.

Total RNA extraction:

Total RNA were isolated by Trizol reagent (Invitrogen, Carlsbad, CA) and then purified by

using an RNeasy system (Qiagen, Valencia, CA). The yield of the extracted RNA was assessed spectrophotometrically via measuring the optical density at 260nm.

Complementary DNA (cDNA) synthesis:

Complementary DNA was synthesized from 1 mg RNA by using SuperScript III First-Strand Synthesis System as described in the manufacturer's protocol (#K 1621, Fermentas, Waltham, MA, USA). In brief, 1 microgram of total RNA mixed with 50 micro M oligo (dT) 20, 50 ng/microLitre random primers, and 10 mM dNTP mixed in a volume of 10 mL. The mixture will be incubated at 56°C for 5 min, and then placed on ice for another 3 min. The reverse transcriptase master mix containing 2 µL of 10× RT buffer, 4 µL of 25 mM MgCl₂, 2 µL of 0.1 M DTT, and 1 µL of SuperScript® III RT (200 U/µL) was added to the mixture and was incubated at 25°C for 10 min followed by 50 min at 50°C.

Real-time quantitative PCR:

Real-time PCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The reaction contained SYBR Green Master Mix (Applied Biosystems), gene-specific primer pairs were designed with Gene Runner Software (Hasting Software, Inc., Hasting, NY) from RNA sequences from the gene bank. All primer sets had a calculated annealing temperature of 60°. Quantitative RT-PCR was performed in a 25-µL reaction volume consisting of 2X SYBR Green PCR Master Mix (Applied Biosystems), 900 nM of each primer and 2 µL of cDNA.

Conditions of amplification were as follows:

- 2 min at temperature of 50°.
- 10 min at temperature of 95°.
- 40 denaturation cycles for 15s.
- Annealing/extension at temperature of 60° for 10 min.

We calculated data from real-time assays by using the v1.7 sequence detection software from PE Biosystems (Foster City, CA). Messenger RNA Relative expression was measured by using comparative Ct method. All values were then normalized to the control housekeeping gene, beta actin, which was reported as fold change over background levels detected in the diseased groups.

• *The primer sequence for CYP2C23 gene:*

Forward Primer: 5'- GATGCTGTCTTCCGT-CATGC-3'.

Reverse primer: 5'- GCTCTGGCCACAGG-TACCAT-3'.

• The primer sequence for NF- κ B gene

Forward primer: 5'-CATTGAGGTGTATTTCA CGG-3.

Reverse primer: 5'-GGCAAGTGGCCATTGTG TTC-3.

• Specific primer sequence for b-actin (used as housekeeping gene):

Forward primer: 5'-GGTCGGTGTGAACGG ATTTGG -3

Reverse primer: 5'-ATGTAGGCCATGAGGT CCACC-3

Statistical methods:

Data were collected and evaluated using the statistical package SPSS version 24. Then, it was summarized by using mean and standard deviation. Comparison between groups was done using analysis of variance (ANOVA) with multiple comparisons post hoc test [27]. It considered as statistically significant when p -values is less than 0.05.

Results

As shown in Table (1), there was a significant change in mean values of serum urea, serum creatinine, TNFalpha, and MDA in CRF group as compared to control group and administration of indapamide in group 3 causes significant improvement in these values when compared to CRF group.

Table (1): Comparison of the mean values of serum urea (mg/dl), serum creatinine (mg/dl), TNFalpha (ng/ml) and MDA (nmol/l) in all groups.

	Group 1 sham operated (Sh)	Group 2 chronic renal failure (CRF)	Group 3 chronic renal failure+Indapamide (CRF+IDP)
Serum urea mg/dl	46.25±4.11	96.22±5.24*	64.72±4.35 *#
Serum creatinine mg/dl	0.38±0.05	1.18±0.11*	0.65±0.04*#
TNFalpha ng/ml	33.23±3.06	128.36±4.86*	85.57±3.46*#
MDA nmol/l	1.22±0.06	20.1±3.06*	10.04±1.79*#

Values are presented as mean \pm SD.

*: Significant change as compared to corresponding value in group 1 ($p < 0.05$).

#: Significant change as compared to corresponding value in group 2 ($p < 0.05$).

Present work reported significant increase in relative gene expression of renal CYP2C23 in group 3 compared to CRF groups (Fig. 1). There was also significant deterioration in systolic and diastolic blood pressure and echocardiographic parameters (EF% and FS%) in group 2 (CRF) compared to control group and these values improved significantly in the group subjected to indapamide (Figs. 2,3). Significant changes recorded also in relative gene expression of NF- κ B in heart tissue among studied groups (Fig. 4).

Assessment of vascular function in studied groups revealed significant changes in aortic contraction response to the phenylephrine and aortic relaxation response to acetylcholine (Figs. 5,6).

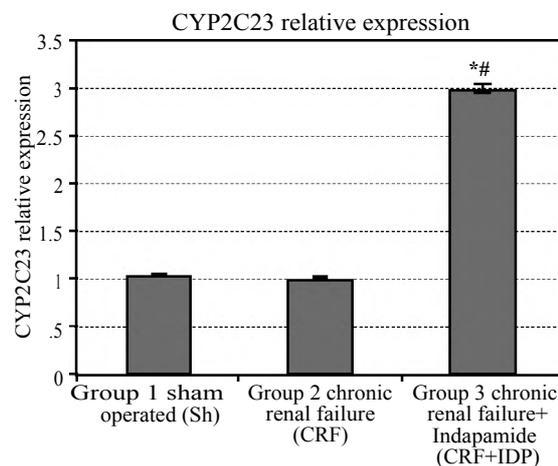


Fig. (1): Comparison of the mean values of relative gene expression of CYP2C23 in all groups.

*: Significant change as compared to corresponding value in group 1 ($p < 0.05$).

#: Significant change as compared to corresponding value in group 2 ($p < 0.05$).

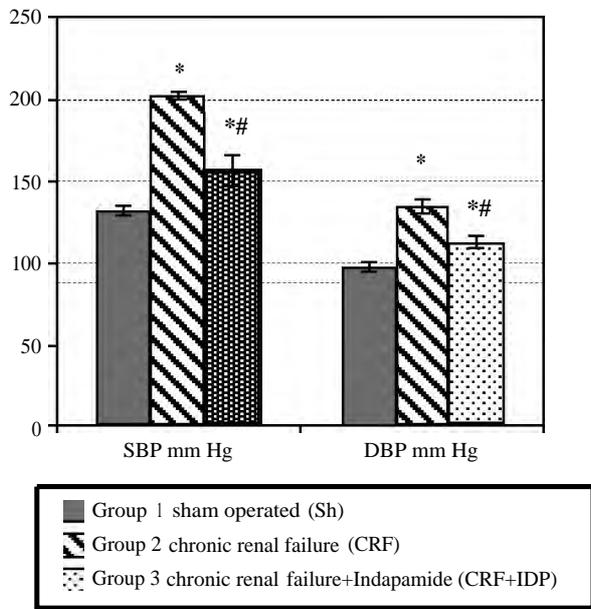


Fig. (2): Comparison of the mean values of systolic and diastolic blood pressure in all groups.

*: Significant change as compared to corresponding value in group 1 ($p < 0.05$).
#: Significant change as compared to corresponding value in group 2 ($p < 0.05$).

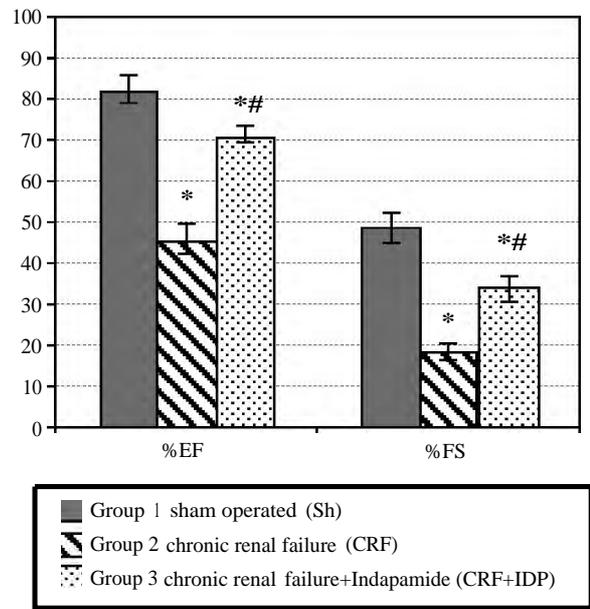


Fig. (3): Comparison of the mean values of echocardiographic parameters (Ejection fraction EF and Fractional shortening FS) in all groups.

*: Significant change as compared to corresponding value in group 1 ($p < 0.05$).
#: Significant change as compared to corresponding value in group 2 ($p < 0.05$).

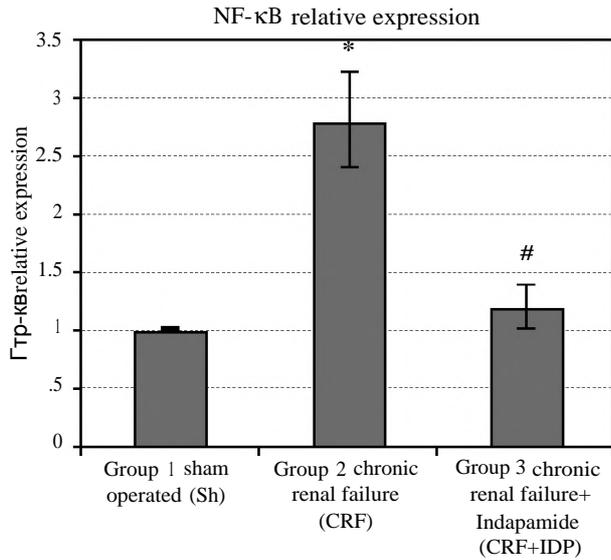


Fig. (4): Comparison of the mean values of relative gene expression of NF-κB in all groups.

*: Significant change as compared to corresponding value in group 1 ($p < 0.05$).
#: Significant change as compared to corresponding value in group 2 ($p < 0.05$).

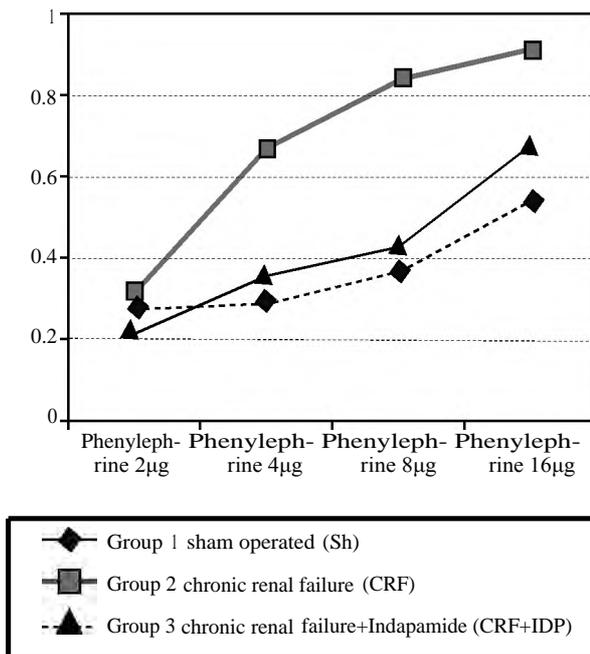


Fig. (5): The aortic ring response to phenylephrine among studied groups.

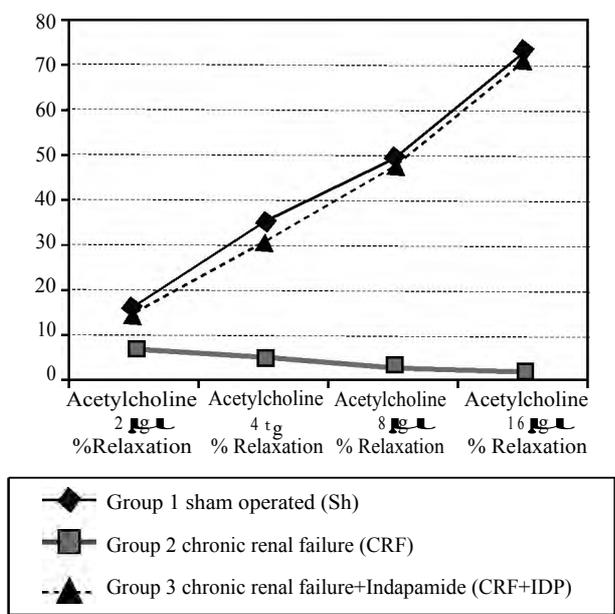


Fig. (6): Cumulative concentration response curves of variable concentration of Acetylcholine.

Discussion

CKD (chronic kidney disease) is a highly prevalent Condition and is considered as severe disease, mostly due to the high prevalence of CV (cardiovascular) complications [28]. Renal Epoxyeicosatrienoic acids (EETs), cytochrome P450 (P450) epoxygenase metabolites of arachidonic acid, are involved in cardiovascular homeostasis. The inability of the kidney to increase epoxyeicosatrienoic acid levels considered a hallmark of renal and cardiovascular diseases [29]. So, we studied the impact of increasing the levels of these (EET) by indapamide on progression of cardiovascular diseases in rat model of chronic renal failure.

We have observed that chronic renal failure induced by 5/6 surgical nephrectomy caused deterioration in systolic and diastolic blood pressure and cardiac function (EF% and FS%) compared to control group. Impaired vascular reactivity was also noticed in this study as we reported increase in aortic contraction response to the phenylephrine and decrease in aortic relaxation response to acetylcholine in CRF group compared to the control group.

Standing with our data, J. Švíglerová & colleagues in 2010 showed that CRF that induced by 5/6 surgical nephrectomy has caused worsening of contraction force and prolongation of contraction and relaxation time in the left ventricle papillary muscle 10 weeks after subtotal nephrectomy [30]. They also reported increase in blood pressure, left ventricular hypertrophy and resting tachycardia.

Impairment of endothelial function and defective endothelium-dependent vasodilatation have been reported also in CKD [31]. Study done by Kujal et al., on Ren-2 transgenic rats (TGR) after 5/6 renal mass reduction (5/6 NX) elicited increase in blood pressure with the decrease in the EETs: DHETEs ratio in the early and late phase after 5/6 NX [32]. They suggested that increased conversion of EETs to DHETEs, as indicated by the increased renal expression of sEH protein in 5/6 nephrectomy model is likely the cause of reduced intrarenal availability of biologically active epoxygenase metabolites with no change in protein expression of the CYP2C23 enzyme. In accordance with our study as we didn't report change in the CYP2C23 expression in our model of CRF.

Study done by Ma et al., reported that administration of indapamide for 8 weeks in spontaneous hypertensive rats (SHRs) could reduce arterial blood pressure, improve vascular function and attenuate myocardial hypertrophy [19]. They concluded that indapamide caused increase in EET production through stimulation of CYP2C23 and the inhibition of sEH. They suggested that EET ameliorates the hypertension observed in SHRs by increasing cAMP and PKA expression in the renal microvessels and decreasing expression of the NADPH oxidase subunits, NF- κ B, and TGF- β 1 in the renal cortex.

In this context, we found that administration of indapamide for 10 weeks in CRF rat model resulted in significant increase in relative gene expression of renal CYP2C23 and this improvement was associated with improvement in renal function (serum urea and creatinine). We also reported that indapamide has cardiovascular protective effect in CRF model as indicated by improvement in blood pressure, cardiac function and vascular function in group receiving indapamide.

Accumulating evidence have revealed that 11,12-EET analogs cause an increase in cAMP [33] and activate renal smooth muscle cell Ca²⁺-activated K⁺ channels causing dilation of renal arteries [34], which are dependent on PKA activation [35] and these changes eventually lead to a decrease in blood pressure. Previous studies has shown also that CYP metabolites including renal EETs have major impact on renal blood flow regulation and long-term arterial blood pressure control [36,37]. In addition, many reports have found that CYP2C has a significant role in long-term regulation of endothelial function and arterial blood pressure [38,39].

Our findings support the notion that increase in the endogenous levels of EETs by inhibiting the enzyme sEH exerts a beneficial effect by preventing the development of cardiac hypertrophy which preserve the cardiac chamber, with no deterioration of cardiac contractility. It has previously been shown that EETs have an anti-inflammatory effect as they can inhibit NF- κ B-mediated gene transcription [40]. NF- κ B is one of the transcription factors that has a role in regulation of the expression of genes which are involved in the stress response following different physiologic or pathologic stimuli [41]. NF- κ B is inactive when bound to I κ B, an inhibitory protein that is phosphorylated by I κ B kinase (IKK). Phosphorylation of I κ B triggers its degradation, which allows translocation of NF- κ B to the nucleus, where it can activate the transcription of inflammatory and immune response target genes [42]. EETs inhibit IKK, so they can prevent degradation of I κ B and keep NF- κ B in its inactive form [40]. Tumor necrosis factor (TNF α) is a relevant mediator of cardiovascular diseases and NF- κ B is known to be regulated by this cytokine. Patients with heart failure have presented with elevated levels of circulating TNF- α and have shown myocardial NF- κ B activation [43].

In our study, we noticed significant elevation of TNF- α together with increase NF- κ B expression in heart in the CRF rat model and these levels decreased significantly in rats receiving indapamide. We suggested that increase expression of CYP2C23 with use of indapamide improved cardiac function mainly through increase production of EETs that subsequently affect the levels of TNF- α and NF- κ B expression in heart.

One of nontraditional risk factors for developing cardiovascular disease in CKD is oxidative stress [44]. It is known to induce endothelial dysfunction and progression of atherosclerosis by reducing nitric oxide availability [45].

CYP2C23 causes increase in the EETs levels and also, it can upregulate the expression of endogenous PPAR α activators, HEETs, which have antioxidant and anti-inflammatory properties [46]. Ma et al., [19] have examined the effect of indapamide on oxidative stress in spontaneous hypertensive rats (SHRs), they reported decreased levels of MDA and ameliorated oxidative stress in the renal cortex of SHRs, potentially by decreasing p47phox and p67phox expression and increasing SOD expression.

In accordance, our study showed significant elevation in levels of MDA (oxidative stress mark-

er) in CRF group, and these levels improved significantly in group receiving indapamide.

In conclusion, we found in this study that indapamide increased expression of CYP2C23 and ameliorated cardiovascular function in model of chronic renal failure. We provided evidence that increased expression of CYP2C23 by use of indapamide has antioxidant and anti inflammatory effect through which the progression of renal and cardiovascular disease in CRF could be attenuated.

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زيادة التعبير الجيني CYCP23 في الكلى باستخدام مدرات البول ثيازاييد تقلل من مضاعفات أمراض القلب والأوعية في نموذج الفئران المصابة بفشل كلوي مزمن

الخلفية البحثية: مضاعفات الإصابة بأمراض القلب والأوعية من أهم أسباب تدهور حالة مريض الفشل الكلوي المزمن. CYCP23 له تعبير جيني في الكلى وينتج أحماض EETs أبوكسيساتريونيك التي لها القدرة علي تنظيم وظائف القلب والأوعية.

أهداف البحث: صمم هذا البحث لدراسة تأثير مدرات البول ثيازاييد على مضاعفات أمراض القلب والأوعية في الفئران المصابة بفشل كلوي مزمن من خلال تأثير الثيازاييد على زيادة التعبير الجيني CYCP23 في الكلى.

طرق البحث: تمت الدراسة على ثلاث مجموعات من الجرذان البيضاء (١٠ جرذان لكل مجموعة) المجموعة الضابطة، المجموعة المصابة بالفشل الكلوي وأخيراً المجموعة المصابة ومعالجة بالإنداباميد (ثيازاييد) تم قياس ضغط الدم، وظائف القلب والأوعية و التعبير الجيني CYCP23 في الكلى.

النتائج: أظهرت المجموعة بالفشل الكلوي زيادة في ضغط الدم وتدهور وظائف القلب والأوعية، أظهرت المجموعة المعالجة بالإنداباميد (ثيازاييد) تحسن في هذه النتائج وزيادة في التعبير الجيني CYCP23 في الكلى.

الخلاصة: استخدام مدرات البول إنداباميد قلل من تدهور وظائف القلب والأوعية الفئران المصابة بفشل كلوي مزمن من خلال تأثير الثيازاييد على زيادة التعبير الجيني CYCP23 في الكلى.