

Klotho and catalase expression in essential hypertension

Abstract

Background: Klotho, an anti-aging gene, has emerged as novel inhibitor of oxidative stress at cellular level. Forkhead box-O (FOXO) proteins regulate crucial cellular processes, including stress-resistance, metabolism, cell-cycle arrest, and apoptosis. Klotho regulates FOXOs via inhibition of insulin/IGF-1 pathway. In rat glomerular-mesangial cells, FOXO1 has been shown to regulate Catalase expression.

Aim: This study aimed at determining Klotho and Catalase gene expressions and Catalase activity in peripheral blood mononuclear cells (PBMCs) of Indian essential hypertensive patients as compared to normotensive healthy controls.

Methods: Forty-eight hypertensives and 48 age, BMI-matched controls were recruited. Gene expression was evaluated by quantitative Real-Time PCR. Catalase enzyme activity in PBMCs and serum soluble α -Klotho levels were detected using Enzyme-Linked Immunosorbent Assay.

Results: Gene expressions for Klotho ($p < 0.001$) and FOXO1 ($p = 0.002$) were significantly low in patients as compared to controls. Catalase expression was also low but did not reach statistical significance. However, there was strong positive correlation between Δ Ct based gene expression of Klotho and Catalase in patients ($p < 0.001$) as well as controls ($p = 0.008$). Positive correlation was also observed between gene expression of FOXO1 and that of Klotho ($p = 0.006$) and catalase ($p = 0.001$) in hypertensives. Catalase activity

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in patients were significantly low ($p < 0.001$) as compared to controls and significantly correlated with soluble α -Klotho levels ($r_s = 0.32$, $p = 0.027$) which were also reduced by 30.2% ($p < 0.001$) in patient group.

Conclusion: Present study demonstrates low soluble levels and gene expression of anti-aging protein Klotho in hypertensives. Klotho may influence Catalase expression in essential hypertension through its effect on FOXO1 expression.

Keywords: α -Klotho, anti-aging gene, cardiovascular, disease, gene, expression, enzyme activity

Abbreviations: 8-iso-PGF2 α : 8-iso-prostaglandin F2 α ; FOXO: forkhead box-O; CVD: cardiovascular diseases; SOD: superoxide dismutase; JNC: joint national committee

Introduction

Essential hypertension is probably the most studied but least understood of the complex human disorders.¹ It is currently recognized as a multifactorial disease arising from the combined action of several genetic and environmental factors. Numerous physiological mechanisms are involved in the maintenance of normal blood pressure (BP), and an aberration in any one of these may play a part in the development of essential hypertension.² Recent studies have highlighted arterial aging as a risk factor for the development of hypertension.^{3,4} Klotho, an anti-aging gene identified was found to ameliorate aging phenotypes and extend lifespan. Klotho deficient transgenic mice exhibited several phenotypes resembling human aging including atherosclerosis, ectopic calcification in arterial walls and various soft tissues, pulmonary emphysema, cardiovascular diseases (CVD) and accelerated death.⁵ Subsequent studies in animal models showed that Klotho deficiency resulted in salt-sensitive hypertension.⁶ Thus, Klotho appears to be a link between aging and CVD including hypertension.

The α -Klotho gene encodes a single pass transmembrane protein—consisting of intracellular, transmembrane and extracellular domains—and a circulating α -Klotho protein. This circulating α -Klotho acts as a peptide hormone and influences various signalling pathways including p53/p21, cAMP, protein kinase C, Wnt pathways as well as the Insulin-like growth factor-1 (IGF-1) signalling cascade.⁷ The anti-aging effect of α -Klotho is partly attributed to increased resistance

to oxidative stress at the cellular level via inhibition of insulin/IGF-1 signalling pathway: an evolutionarily conserved mechanism for extending life span.⁷ Furthermore, the role of insulin/IGF1 signalling pathway in hypertension has been well documented and serum IGF-1 levels are found to be elevated in hypertensive subjects.^{8,9} Oxidative stress is one of the fundamental mechanisms responsible for the development of essential hypertension. The Superoxide Dismutases (SODs) and Catalase comprise the first line of defence system against ROS. Impaired activity of these enzymes has been associated with uncontrolled production of ROS and other free radicals.¹⁰ Klotho inhibits ROS and oxidative stress by inhibiting IGF-1/Insulin-FOXO (Forkhead box-O transcription factors) dependent deactivation of these antioxidant enzymes.^{11,12}

Klotho initiates a signalling cascade that begins with its binding to a yet unidentified Klotho receptor on the cell membrane, inhibiting the tyrosine kinase activity of the insulin/IGF-1 receptor,¹³ and subsequently the insulin receptor substrates. FOXOs are the downstream targets of insulin-like signalling that regulate organismal aging.¹⁴⁻¹⁷ FOXO proteins mediate the inhibitory action of insulin /IGF-1 on key functions in diverse pathways including cell metabolism, proliferation, differentiation, oxidative stress, cell survival and senescence, autophagy and aging in mammals.¹⁸ The phosphatidylinositol 3-kinase (PI3K)-Akt serine-threonine kinase (AKT) signalling pathway signalling cascade, are activated by this Klotho mediated inhibition. A recent study in cell lines demonstrated that Klotho inhibited PI3K/AKT-mediated phosphorylation of FOXO3a and subsequently enhanced FOXO3a binding to the Mn-SOD promoter, thereby leading to Klotho increased Mn-SOD mRNA and protein expression in mitochondria.¹⁹

Yamamoto,¹¹ found the effect of Klotho protein was more pronounced on the FOXO1 than on the FOXO3a or FOXO4 proteins in The La cells as well as murine models.¹¹ Further, Venkatesan et al have reported the presence of FOXO1 DNA binding element in the promoter region of Catalase gene.²⁰ This study therefore aimed at analysing the expressions of Klotho and Catalase as well as the activity of Catalase in PBMCs of hypertensive patients as compared to normotensive controls. The soluble levels of α -Klotho were also analysed in both study groups. F2-isoprostanes, which are relatively stable and ubiquitous in human plasma and urine, have proven to be among the most sensitive and reliable biomarkers for the investigation of lipid peroxidation and hence oxidative stress in vivo.^{21,22} Therefore, 8-iso-prostaglandin 2 α (8-iso-PGF 2 α) levels were measured in serum of all subjects to quantitate the oxidative stress in the two study groups.

Materials and methods

Study subjects

Forty-eight patients diagnosed with essential hypertension (SBP/DBP > 140/90 mmHg) and 48 age and Body Mass Index (BMI) matched healthy normotensive (SBP/DBP < 120/80 mmHg) controls were recruited for the study. Recruitment of hypertensive individuals was in accordance with the Seventh Joint National Committee (JNC7)²³ report guidelines. These patients were monitored for BP and referred to a consultant immediately if SBP/DBP was more than 160/100; and after monitoring for 6-8 days if SBP/DBP < 160/100-prior to recruitment. On confirmation of essential hypertension, these subjects were recruited under hypertension group after obtaining written informed consent. Detailed information regarding demographic status, clinical history, family history and medication was obtained. Recruited subjects were not on any medication. The study protocol was approved by the Institutional Ethics Committee, which follows the ethical standards laid down by the Indian Council of Medical Research's (ICMR) Ethical Guidelines for biomedical research on human participants.

Biochemical analysis

Fasting venous blood samples were collected in plain and K2-EDTA vacutainers at the time of enrolment (for subjects identified with high BP, this sample was collected prior to starting hypertensive medication). Serum samples were used to perform routine biochemical investigations such as lipid profile, renal profile and liver profile tests. Those healthy individuals with levels beyond normal range were excluded from control group. Remaining serum was aliquoted and stored at -80°C until further use.

Oxidative stress levels (8-iso-PGF 2 α)

8-iso-PGF 2 α levels were detected in serum and urine samples of patients and controls using Enzyme-Linked Immunosorbent Assay (ELISA) method (Immuno-Biological Laboratories Co., Ltd., Hamburg, Germany). Total 8-iso-PGF 2 α -both free and esterified -was measured by this method. Lipoprotein or phospholipid coupled 8-iso-PGF 2 α were hydrolysed as per manufacturer's instructions prior to the assay.

Soluble α -Klotho levels

Soluble α -Klotho levels in serum samples were detected using Enzyme-Linked Immunosorbent Assay (ELISA) method (Immuno-Biological Laboratories Co., Ltd., Hamburg, Germany).

Catalase activity assay

Enzyme activity of Catalase was measured in PBMC lysates using commercially available ELISA. PBMCs were isolated from K2-EDTA anti-coagulated blood. Ficoll-Histopaque®-1077 (Sigma-Aldrich) gradient separation method. PBMCs were lysed according to manufacturer's instructions and the cell lysate was stored at -80°C until further analysis. Catalase enzyme activities were measured using Oxiselect Activity assay kits (STA-341, Cell Biolabs, Inc, USA) as per manufacturer's instructions.

RNA extraction from PBMCs and cDNA synthesis

PBMCs were isolated from K2-EDTA anti-coagulated blood. Ficoll-Histopaque®-1077 (Sigma-Aldrich) gradient separation method was used for isolation. RNA was extracted from PBMCs by NucleoSpin RNA isolation kit (Machery-Nagel). The quality and quantity of RNA was assessed using agarose gel electrophoresis and Qubit 2.0 Fluor meter respectively. One μ g RNA was reverse transcribed to cDNA for each sample using The Prime Script 1st strand cDNA Synthesis Kit (Takara Bio USA, Inc.) as per the manufacturer's instructions.

Gene expression studies

The gene expression was evaluated using quantitative Real time Polymerase Chain reaction (qRT-PCR) in Step OnePlus™ Real-Time PCR System (Applied Biosystems) using TaqMan gene expression assays for Klotho (Hs00183100_m1), FOXO1 (Hs00231106_m1) and Catalase (Hs00156308_m1). The housekeeping gene GAPDH (Hs03929097_g1) was used as internal control. Relative gene expression was calculated according to the comparative Ct method (2- $\Delta\Delta$ Ct) described by Livak and Schmittgen.²⁴ All experiments were performed in duplicates according to the manufacturer's protocol.

Statistical analysis

Results are expressed as frequency and percentage, mean \pm standard deviation (SD) for parametric variables and median with inter quartile (25th/75th) ranges for non-parametric variables. Student's unpaired t test and Mann-Whitney U test were used to determine the significance of differences between the two study groups for parametric and non-parametric variables respectively. Correlations were evaluated by Spearman's rank correlation test. For all tests p value < 0.05 was considered statistically significant. Analyses were performed using statistical software SPSS (version 21.0, Chicago, IL). For expression studies, patients were grouped according to their age, BMI and gender, and compared against a matched healthy control. Therefore, all correlations between the groups are for a total of 48 patients and corresponding 17 controls.

Results

Demographic and clinical characteristics

The baseline demographic characteristics of patients and controls are depicted in Table 1. The comparison of lipid profile of patients and controls is given in Table 2, which did not differ significantly between the two groups.

8-iso-Prostaglandin F2 α Levels

In hypertensive patients, the levels of 8-iso-PGF2 α were significantly high (p < 0.001) in serum (11.3 fold/91.1%, p < 0.001) as compared to controls (Figure 1). In control group, 8-iso-PGF2 α levels

negatively correlated with both soluble α -Klotho levels ($r_s=-0.413$, $p=0.004$) and catalase activity ($r_s=-0.344$, $p=0.017$). There was no correlation observed between this oxidative stress marker and lipid parameters.

Table 1 Comparison of demographic parameters between patient and control groups

Parameters	Controls (N=48)	Patients (N=48)
Age (years)	45.8±6.7	48.8±5.7
Male/Female	43/5	45/3
BMI (kg/m ²)	26.3±3.9	27.±5.77
SBP (mmHg)	124.6±7.7	164.3±14.8***
DBP (mmHg)	79.58 ±6.0	102.08±6.78***

*** $p<0.001$ compared to respective control groups. Values are expressed as mean \pm SD. BMI, Body Mass Index; SBP, Systolic Blood Pressure, DBP, Diastolic Blood Pressure.

Table 2 Comparison of lipid profile between patient and control groups

Parameters	Controls (N=48)	Patients (N=48)
TC (mg/dl)	169.6±34.4	172.6±31.6
HDL-C (mg/dl)	38.8±11.6	43.0±11.6
TC / HDL-C	4.6±1.3	4.2±1.0
Triglycerides (mg/dl)	110.9±43.7	126.5±69.7
VLDL-C (mg/dl)	22.2±8.7	25.3±13.9
LDL-C (mg/dl)	108.6±30.2	104.3±29.0
LDL-C/ HDL-C	2.9±1.1	2.6±0.9

Values are expressed as mean \pm SD. TC, Total cholesterol; HDL-C, High Density Lipoprotein Cholesterol; VLDL-C, Very Low Density Lipoprotein Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol.

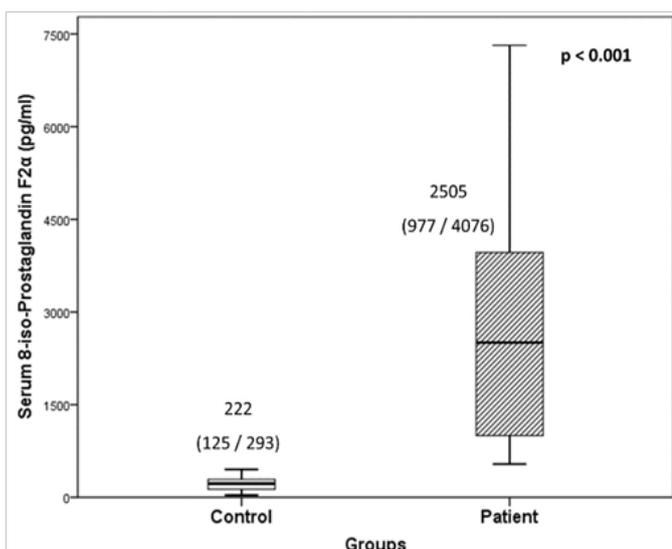


Figure 1 Serum levels of 8-iso-Prostaglandin F₂ α in hypertensive patients and controls. Values are expressed as median with interquartile (25th /75th) range.

Soluble α -Klotho levels

The levels of soluble α -Klotho in serum of hypertensive patients was significantly low (30.2%, $p=0.001$) as compared to controls (Figure 2).

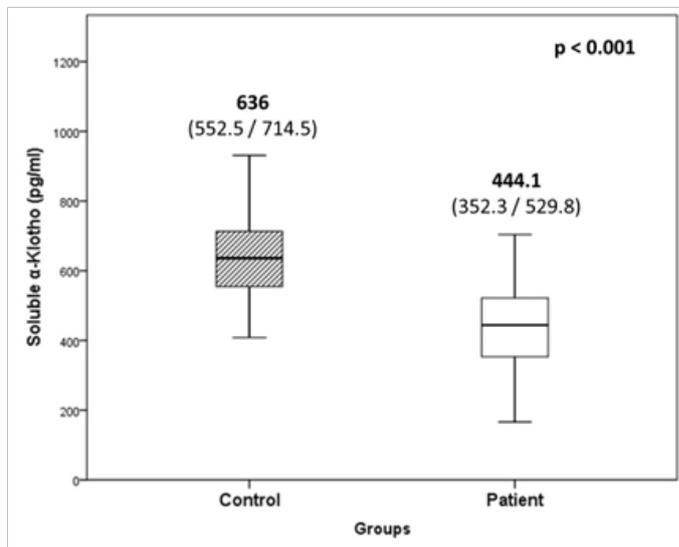


Figure 2 Soluble α -Klotho levels in hypertensive patients and controls. Values are expressed as median with interquartile (25th /75th) range.

Catalase Activity Assay

In hypertensive patients, the enzyme activity of Catalase was found to be significantly low (80.3%, $p<0.001$) as compared to controls (Figure 3) and showed positive correlation with soluble α -Klotho levels ($r_s=0.32$, $p=0.027$).

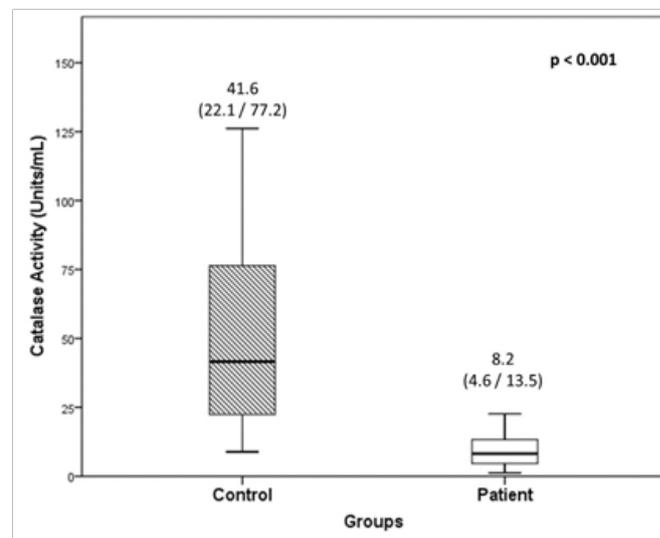


Figure 3 Catalase activity in PBMcs of hypertensive patients and controls. Values are expressed as median with interquartile (25th /75th) range.

Gene expression studies

Relative gene expression levels of Klotho, FOXO1 and Catalase in hypertensive patients are depicted in Figure 4. The relative gene expression of Klotho, FOXO1 and Catalase were found to be 3.02-

fold, 1.55-fold and 1.23-fold low respectively in hypertensive patients as compared to controls. The ΔCt values for the genes studied are given in Table 3. Based on ΔCt values, hypertensive patients demonstrated significantly low Klotho ($p=0.001$) and Catalase ($p=0.001$) gene expression as compared to control group. Based on ΔCt values, there

was positive correlation observed between expressions of Klotho and Catalase in hypertensive ($r_s=0.753$, $p<0.001$) as well as in control ($r_s=0.618$, $p=0.008$) groups. Furthermore, gene expression of FOXO1 positively correlated with that of Klotho ($r_s=0.388$, $p=0.006$) and Catalase ($r_s=0.471$, $p=0.001$) in the patient group (Table 4).

Table 3 Gene expression based on ΔCt values of patients and controls

GENE	ΔCt values Median (25th / 75th Percentile)		Significance level (p value)
	Controls (N=17)	Patients (N=48)	
Klotho	14.938 (14.278 / 16.244)	16.936 (15.943 / 18.556)	0.001
FOXO-1	5.945 (5.202 / 6.806)	7.039 (6.018 / 7.623)	0.002
Catalase	4.76 (3.755 / 5.481)	5.305 (4.630 / 5.999)	0.076

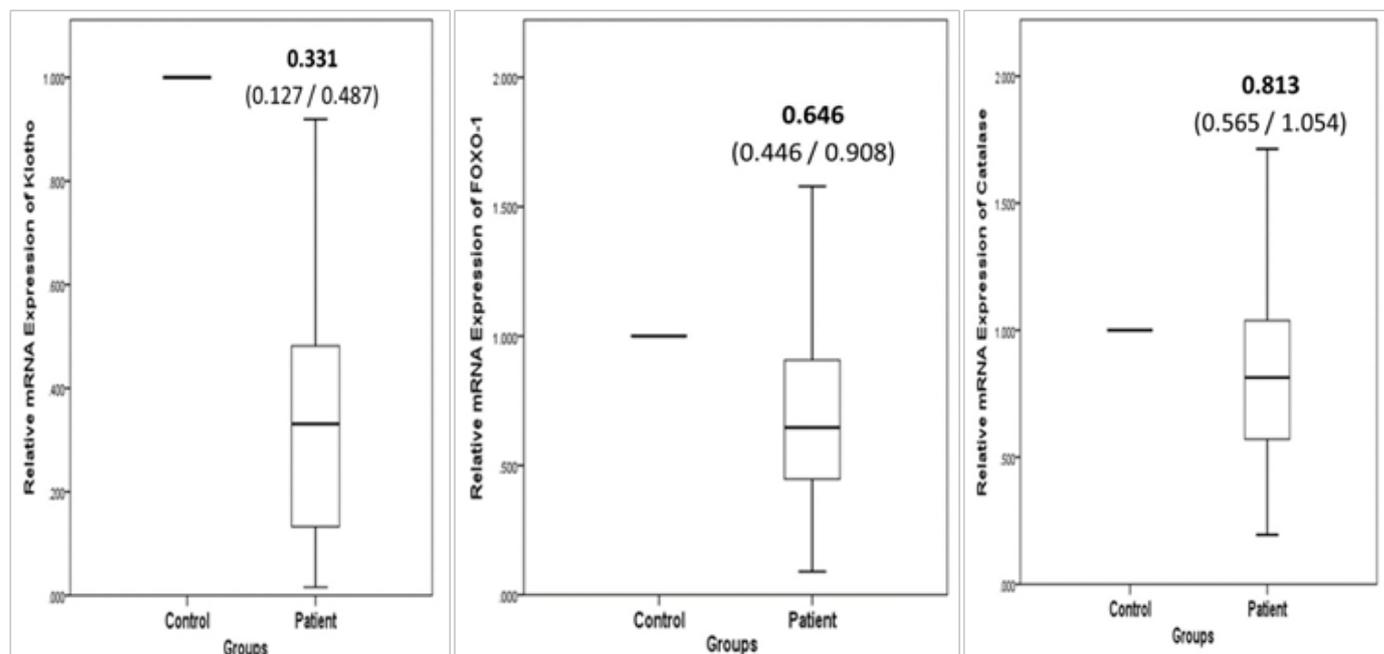


Figure 4 Relative gene expression levels for (a) Klotho, (b) FOXO1 and (c) Catalase in hypertensive patients compared to normotensive controls.

The gene expression of patients relative to that of controls are shown. Values are expressed as median with interquartile (25th / 75th) range.

Discussion

The present study is the first to report a direct correlation between Catalase and Klotho gene expression in hypertensive patients as well as normotensive controls. Further, we also report lower expression of Klotho along with FOXO1 transcription factor in the patient group. Both Catalase activities analysed in PBMCs, and the soluble α -Klotho levels in serum were significantly low in patients as compared to controls and a significant positive correlation was observed between these two parameters in patient group. The lower soluble Klotho levels in patients as compared to controls observed

in this study is in accordance with several previous reports. In Chinese population, Su & Yang have attributed, albeit partially, the occurrence of systolic hypertension to reduction in serum α -Klotho levels.²⁵ The association of reduced circulating levels of α -Klotho with the occurrence and severity of coronary artery disease,²⁶ CVD,²⁷ and as an independent marker of arterial stiffness in patients with chronic kidney disease,²⁸ have been reported in Spanish, Italian and Japanese cohorts respectively. The present study suggests that α -Klotho levels are depleted in essential hypertension patients as well, and that this may be an important feature of the disease.

Evidence strongly suggests that oxidative stress, due to enhanced production of ROS and/or a decrease in the antioxidant reserve, is an important contributor to the development and persistence of hypertension. Previous studies in humans and animal models have established the state of increased oxidative stress in hypertension.^{29–31} The present study also found elevated 8-iso-PGF2 in hypertensive patients accompanied by a significant decrease in Catalase activity. The role of Catalase in hypertension has been previously studied and its over expression has been reported to prevent hypertension in animal models.³¹ Gomes et al showed that Catalase activity was markedly down regulated in spontaneously hypertensive rat (SHR) cells.³² In a previous study by the same group, they have also reported down regulation of the protein expression level of Catalase in SHR cells.³³ Thus, the low activity and expression of Catalase in SHR cells could be increasing their vulnerability to oxidant-induced cell death.³² Furthermore, the enhanced sensitivity to oxidative stress in SHR cells as compared to the control normotensive Wistar Kyoto (WKY) rat cells could be attributed to the impaired cell protection by antioxidant enzyme systems.^{32,34} It has been documented that providing antioxidant therapy to SHRs resulted in a significant increase in the Catalase enzymatic activity in their cortex and medulla.³⁴ Over expression of Catalase in transgenic mice helped removal of ROS in mitochondria and also extended their lifespan.³⁵ Thus, Catalase plays an important role in the prevention of hypertension. Several clinical studies are dedicated towards increasing the antioxidant activity.

A number of studies have explored the role of Klotho in antioxidant systems and suggested that its anti-aging properties are a result of its ability to combat oxidative stress. Previous studies have reported the regulation of Catalase expression by FOXO3a.³⁶ However, Awad³⁶ have demonstrated that activation of AMP-activated protein kinase (AMPK), one of the kinases known to regulate FOXO1, led to increased FOXO1 (total and nuclear) protein as well as Catalase mRNA and protein content. Further, inhibition of AMPK inhibited FOXO1 and Catalase, but not FOXO3a.³⁷ The significance of FOXO1 is further demonstrated by the presence of FOXO1 DNA binding element in the Catalase promoter region.²⁰ Venkatesan et al demonstrated that hydrogen peroxide (H₂O₂) negatively regulated FOXO1 by PI3K/Akt-dependent phosphorylation, eventually inhibiting Catalase expression.²⁰ The link between Klotho and FOXO1 activation via the insulin/IGF-1/PI3K/Akt pathway has been already proposed by Yamamoto.¹¹ We further postulate that Klotho may regulate Catalase expression in a similar manner, via FOXO1. The direct correlation observed between Klotho and Catalase expression in hypertensive patients as well as controls and lower PBMC Catalase activity and expression of Klotho along with FOXO1 transcription factor in the patient group, in the present study supports this theory. However, studying the exact mechanism at the cellular levels is required to validate this postulation. In conclusion, the present study demonstrated increased oxidative stress, diminished antioxidant capacity in terms of Catalase, decreased soluble Klotho levels, and decreased Klotho expression in PBMCs to be associated with essential hypertension. Additionally, a direct correlation between Klotho and Catalase expression as well as lower expression of FOXO1 transcription factor was also observed in hypertensives. Considering the regulatory role of Klotho in insulin/IGF-1 signalling, the application of Klotho against oxidative stress related diseases such as hypertension assumes great significance. This prospective application needs to be explored further.

Acknowledgments

None.

Conflicts of interest

Authors declare that there is no conflict of interest.

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