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Original Article

Relationship between NOX4 level and angiotensin II signaling in Gitelman's syndrome. Implications with hypertension

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Abstract: Recent evidence showed that endogenous nicotinamide adenine dinucleotide phosphate-oxidase 4 (NOX4) may exert a protective role on the cardiovascular system inducing vasodilation, reduction of blood pressure, and anti-proliferative actions. However, the functional significance of NOX4 in the cardiovascular system in humans remains elusive. Mononuclear cell levels of NOX4 were assessed by immunoblotting in 14 Gitelman's patients (GS), a unique human model of endogenous Ang II signaling antagonism and activation of anti-atherosclerotic and anti-remodeling defenses, and compared to 11 untreated essential hypertensive patients as well as to 11 healthy normotensive subjects. The association between NOX4 and its effector heme oxygenase (HO-1) (sandwich immunoassay) was also evaluated. NOX4 protein levels were decreased in hypertensive patients as compared to both GS and healthy subjects (1.06±0.31 AU vs. 1.76±0.54, P=0.002 and vs. 1.61±0.54, P=0.018, respectively). NOX4 protein level did not differ between GS and healthy subjects. HO-1 levels were increased in GS patients as compared to both hypertensive patients and healthy subjects (8.65±3.08 ng/ml vs 3.70±1.19, P<0.0001, and vs 5.49±1.04, P=0.008, respectively. NOX4 levels correlate with HO-1 levels only in GS (r^2=0.63; P=0.001), (r^2=0.088; P=ns, in hypertensive patients and r^2=0.082; P=ns, in healthy subjects). Our findings show that NOX4 and its effector HO-1 are reduced in hypertensive patients compared to GS patients, a human model opposite to hypertension. Although the functional significance of NOX4 needs further clarification, our preliminary data in a unique human model of anti-atherosclerotic and anti-remodeling defenses activation, highlight the potentially protective role of NOX4 in the human cardiovascular system.

Keywords: Angiotensin II signaling, NOX4, Gitelman's syndrome, hypertension, cardiovascular remodeling

Introduction

Patients with hypertension have increased oxidative stress, which is involved in its pathophysiology and is a central element in the hypertension induced cardiovascular and renal target organ damage [1]. Angiotensin II (Ang II) plays major roles in hypertension as it controls the processes of both blood pressure regulation and cardiovascular remodeling. This is accomplished via different signaling systems. One, the short term signaling system, is activated by Ang II through the stimulation of the type 1 receptor (AT1R), which in turn activates calcium dependent pathways as well as RhoA/Rho kinase pathways [2, 3]. The other, the long term signaling, which is linked to cardiovascular-renal remodeling occurs mostly through the induction of oxidative stress, via upregulation of the isoforms of the NADPH oxidase, namely NOX1 and NOX2, and MAP kinases activation including MAPK/ERK [2, 4]. NOX1 and NOX2 represent the major source of superoxide anion (O_2^-) in the vasculature. Increased superoxide in turn, may reduce the nitric oxide (NO) bioavailability by NO scavenging [5].

NOX4 is another member of the NOX family of NADPH oxidases but Ang II, unlike its induction of NOX1 and NOX2, appears to inhibit NOX4 expression in experimental models [6]. NOX4, unlike other NOXs, preferentially produces hydrogen peroxide (H_2O_2) while O_2^- is produced at much lower extent [7]. This difference in

Table 1. Clinical and laboratory data of hypertensive patients, Gitelman's patients, and normotensive healthy controls included in the study

Gitelman's Patients	Sex	Age (yrs)	Plasma Electrolytes (mmol/L)				Urinary Electrolytes (mmol/day)				PRA (ng ANG I/ml/h)	Aldosterone (nmol/L)
			Na^+	K^+	Cl^-	Mg^{++}	Na^+	K^+	Cl^-	Ca^{++}		
1	F	27	138	2.3	96	0.69	199	38.6	195	2.4	12	0.94
2	F	30	137	2.9	99	0.63	255	38.0	200	1.9	9	0.70
3	M	44	140	2.8	98	0.60	198	30.8	220	2.0	7	0.88
4	F	31	138	2.7	98	0.60	297	42.8	289	2.1	10	0.78
5	M	32	138	3.0	99	0.56	200	81.5	239	2.0	6	0.67
6	F	58	140	3.0	100	0.55	190	42.5	220	2.0	6	0.70
7	M	29	139	3.0	100	0.57	190	42.5	218	2.1	6	0.75
8	M	31	139	3.1	100	0.58	192	42.8	210	2.2	6	0.75
9	M	43	139	3.0	98	0.58	200	45.4	197	2.0	5.8	0.80
10	M	39	139	2.8	97	0.59	195	47.4	198	2.0	5.8	0.81
11	F	30	139	2.9	100	0.60	196	48.8	199	2.0	6.1	0.77
12	F	29	138	3.0	101	0.61	200	47.6	200	2.3	6.6	0.78
13	F	34	140	2.8	98	0.65	195	43.0	195	2.0	5.9	0.75
14	F	28	139	2.7	100	0.62	199	42.0	196	2.1	6.4	0.75
Normotensive Controls (n=11)	6M/5F	46.2±10.5	140±1.0	4.1±0.2	99±0.97	0.99±0.2	180±16.5	52.8±4.8	179.8±19.6	4.5±0.5	0.73±0.13	0.18±0.02
Hypertensive patients (n=11)	6M/5F	49.6±11.2	142±0.9	4.1±0.1	99±0.94	1.1±0.2	188±19.5	56.9±6.0	178.2±16.6	4.4±0.6	0.99±0.15	0.24±0.06

The table reports single data of patients and controls. Normal values for PRA and plasma aldosterone in our laboratory are 0.2-2.8 ng ANG I/ml/h and 0.08-0.29 nmol/l respectively. Normal values for plasma Na^+, K^+, Cl^-, Mg^{++} are 136-145, 3.5-5, 96-108, 0.65-1.05 mmol/l, respectively. Normal values for urinary Na^+, K^+, Cl^- and Ca++ excretion are: 40-220, 25-125, 110-250 and 2.5-7.5 mmol/day respectively.

Table 2. SLC12A3 mutations identified in the patients with Gitelman's syndrome

Patient	Exon	Mutation at nucleotide	Homo-hete-rozygous	Predicted effect on protein
1	23	2736G---->A	homozygous	Arg904Gln
2	22	2579C---->T	heterozygous	Arg852Cys
	23	2736G---->A	heterozygous	Arg904Gln
3	15	1950G---->A	heterozygous	Arg642His or splice donor site truncated SLC12A3 protein
	18	2246G---->A	heterozygous	Gly741Arg
4	22	2579C---->T	heterozygous	Arg852Cys
	23	2736G---->A	homozygous	Arg904Gln
5	22	2579C---->T	heterozygous	Arg852Cys
	23	2736G---->A	heterozygous	Arg904Gln
6	21	2542G---->T	heterozygous	Asp848Tyr
	10	c.1196_1202dup 7bp	heterozygous	Ser402X
7	21	2542G---->T	heterozygous	Asp848Tyr
	10	c.1196_1202dup 7bp	heterozygous	Ser402X
8	17	c.2089_2095del 7bp	heterozygous	pThr697fs
	26	2985G--->A	heterozygous	Ser402X
9	17	c.2089_2095del 7bp	heterozygous	pThr697fs
	26	2985G--->A	heterozygous	Ser402X
10	10	1195C--->T	heterozygous	pArg399Cys
	15	1220C--->T	heterozygous	pArg642His
11	26	2981G--->A	heterozygous	pCys994Tyr
	26	3005G--->A	heterozygous	pTrp1002*
12	15	1948G--->A	heterozygous	pGly650Arg
	9	1180+1G--->T	heterozygous	Splicing site
13	15	1948G--->A	heterozygous	pGly650Arg
14	25	2954G--->A	homozygous	Cys985Tyr

products may be significant as unlike O_2^-, H_2O_2 does not react with NO and may induce vasodilation [7-9]. This difference might explain why recent experimental studies showed that endogenous NOX4 may exert a protective role on cardiovascular system by inducing vasodilation and reduction of blood pressure [8] as well as by inducing anti-proliferative actions on cells from the vasculature [10]. However, despite these reported favorable effects, the functional significance of NOX4, particularly in the human cardiovascular system, remains undetermined [6].

Gitelman's syndrome (GS) is a rare disease caused by mutations in the gene coding for the thiazide sensitive Na-K cotransporter (NCC/SLC12A3), which induce hypokalemia, salt wasting, hypomagnesemia, hypocalciuria and increased Ang II and aldosterone levels yet normal or even low blood pressure [11]. These patients show blunted short and long term Ang II signaling via AT1R [12-14], reduced oxidative stress [14, 15], lack of cardiovascular remodeling [14, 16, 17], upregulation of NO system [12, 14], increased NO mediated vasodilation [14, 16] and activation of Ang II signaling via AT2R [14, 18]. In addition BG/GS patients have also increased expression of heme oxygenase (HO)-1 [14, 15], which protects from oxidative stress and tissue inflammation [19, 20]. These patients thus likely represent a human model of endogenous Ang II signaling antagonism and activation of anti-atherosclerotic and anti-remodeling defenses [14]. Interestingly, increased expression of HO-1 has been related to NOX4 activation [6]. All the above mentioned characteristics make these patients a unique human model in which to study the links between Ang II signaling, NOX4 and NO bioavailability in a clinical setting.

This preliminary study was specifically done to provide an exploratory assessment of the lev-

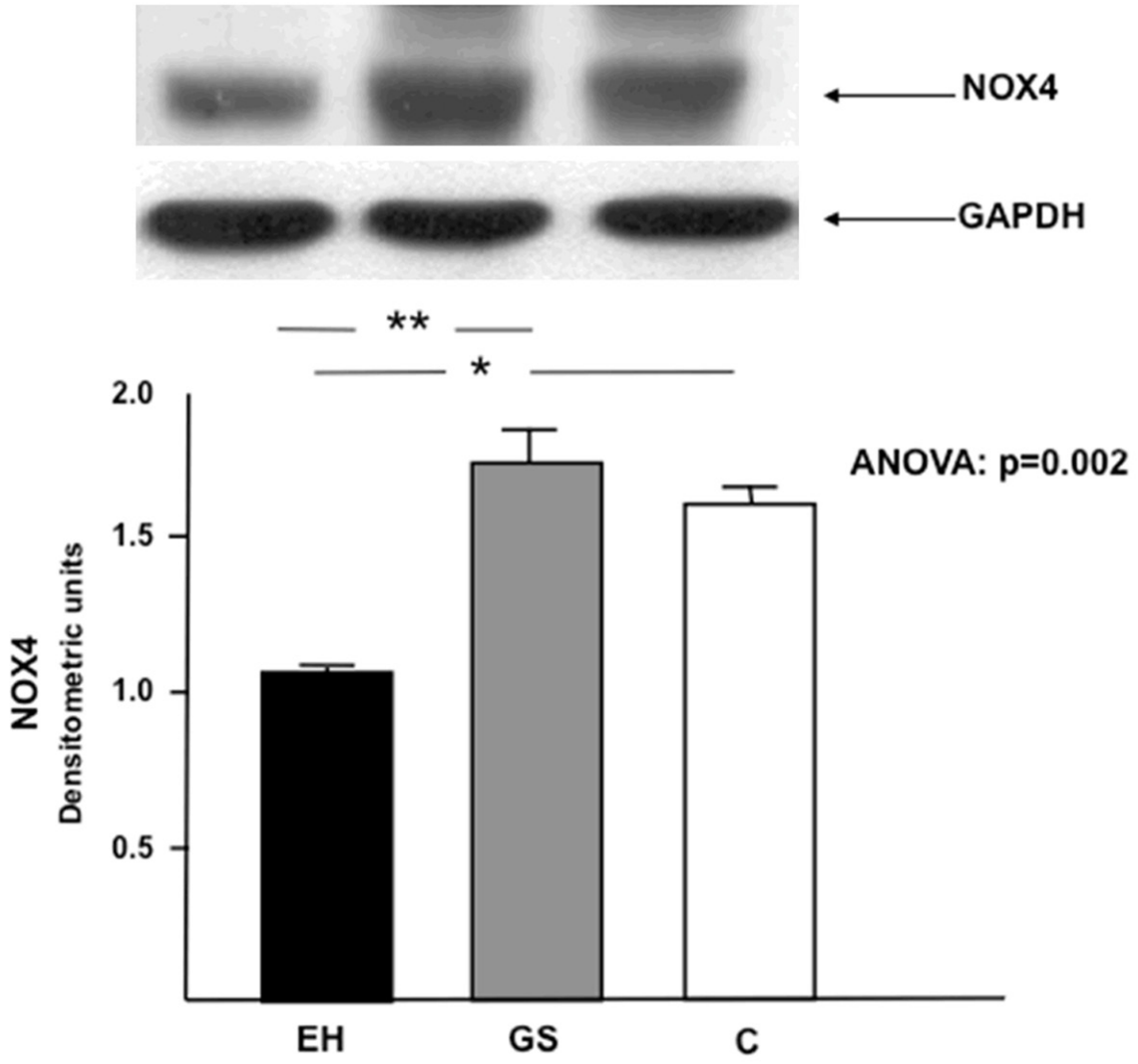

Figure 1. Mononuclear cell NOX4 protein level in essential hypertensive patients (EH), Gitelman's patients (GS) and normotensive healthy subjects (C). **P=0.002; *P=0.018.

els of NOX4 and HO-1 in mononuclear cells from our cohort of GS patients compared with those in hypertensive patients and healthy normotensive subjects using the unique human model of GS, opposite to hypertension, to obtain information on NOX4 level, its relationship with Ang II signaling, its possible implications in hypertension and biological role in the cardiovascular system.

Material and methods

Patients

Fourteen patients (6 males and 8 women, age 35.8±9.3 years) from our cohort of GS patients were included in the present study. All patients had a full biochemical characterization (**Table 1**) and full genetic analysis (**Table 2**).

Eleven uncomplicated, nonsmoking and never treated essential hypertensive patients (6 males, 5 females, age 49.6±11.2 years) were selected from the patient population seen at the Padova Hypertension Clinic, Department of Medicine, and enrolled in the study.

A total of 11 normotensive healthy individuals, (6 males, 5 females, age 46.2±10.5 years) from the staff of the Department of Medicine, University of Padova, were studied as control group.

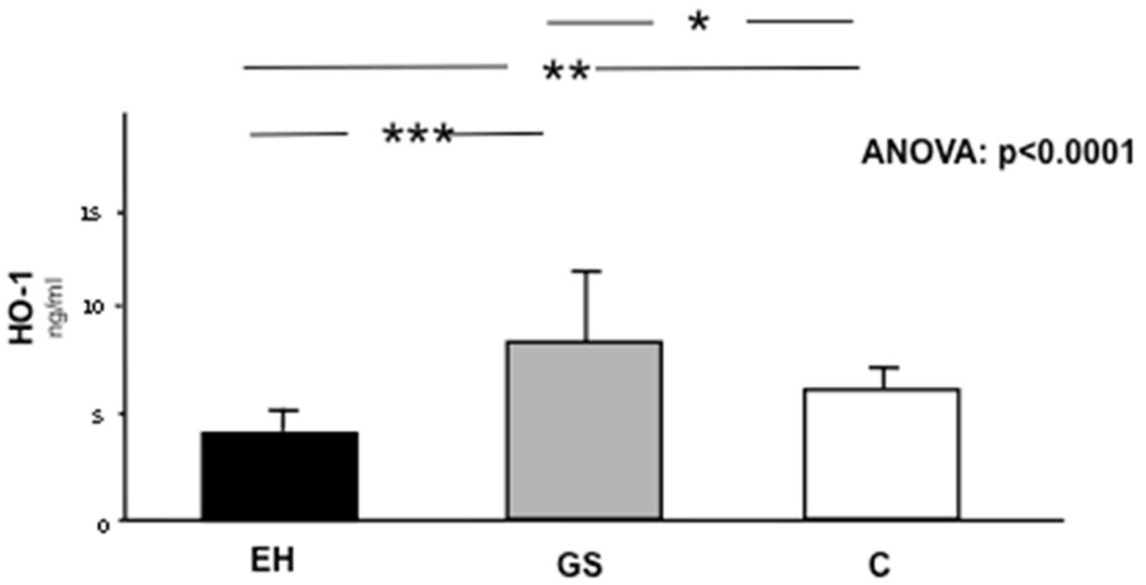

Figure 2. Mononuclear cell Heme Oxygenase (HO)-1 protein level in essential hypertensive patients (EH), Gitelman's patients (GS) and normotensive healthy subjects (C). ***P=0.0001; **P=0.008; *P=0.009.

All the subjects included in the study had the following parameters in the normal range: BMI (< 25 kg/m^2), fasting serum glucose (< 126 mg/dl), serum creatinine (< 1.0 mg/dl) and urinary albumin excretion (< 30 mg/g of urinary creatinine). Lipid profiles were normal, and the patients were not taking lipid-lowering drugs or aspirin. All the subjects were on regular Mediterranean diet, the salt consumption was approximately 150 mmol of sodium/day. The GS patients were taking potassium and magnesium supplements.

None of the patients had cardiac failure or evidence of coronary heart disease; left ventricular hypertrophy was ruled out by conventional M-mode echocardiography.

Informed consent was obtained from all the study participants and the study protocol was approved by our institutional authorities.

All of the subjects abstained from food, alcohol and caffeine-containing drinks for at least 12 h before blood withdrawal.

Mononuclear cell NOX4 protein expression

Peripheral blood mononuclear cells were isolated by Ficoll Paque Plus gradient (GE Healthcare, Uppsala, Sweden) from 35 ml of EDTA anticoagulated blood. Mononuclear cell NOX4 protein expression was performed using western blot analysis. Total protein extracts were obtained by cells lysis with a ice-cold buffer (Tris HCl 20 mM, NaCl 150 mM, EDTA 5.0 mM, Niaproof 1.5%, Na_3VO_4 1.0 mM, SDS 0.1%, PMSF 0.5 mM) added with protease inhibitors (Complete Protease Inhibitor Cocktail, Roche Diagnostics, Mannheim, Germany). Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes (Hybond ECL, Amersham, Uppsala, Sweden) and blocked overnight with no-fat milk (5% in Tween-PBS). Membranes were probed with primary polyclonal antibody anti-Nox4 (SantaCruz Biotechnology, SantaCruz, CA, USA). After incubation with proper secondary antibodies HRP-conjugated (Amersham Biosciences, Uppsala, Sweden), immunoreactive proteins have been visualized with chemiluminescence using SuperSignal WestPico Chemiluminescent Substrate (Pierce, Rockford, USA). GAPDH was used as loading control (Millipore, Billerica, MA, USA). Nox-4 protein expression was quantified using a densitometric semiquantitative analysis using NIH image software, and was normalized to GAPDH used as loading control.

Heme oxygenase-1 protein quantification

Total protein extracts from peripheral blood mononuclear cells were obtained by cell lysis

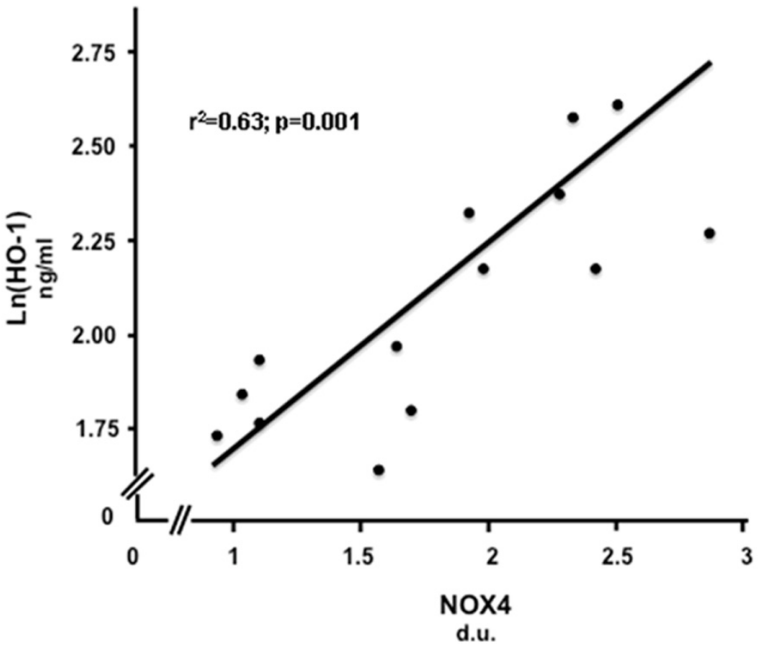

Figure 3. Correlation analysis between NOX4 protein level and Heme Oxygenase (HO)-1 in GS patients.

with an ice-cold buffer (Tris HCl 20 mM, NaCl 150 mM, EDTA 5.0 mM, Niaproof 1.5%, Na3VO4 1.0 mM, SDS 0.1%), with protease inhibitors added (Complete Protease Inhibitor Cocktail, Roche Diagnostics, Mannheim, Germany). Protein concentration was evaluated by bicinchoninic acid assay (BCA Protein Assay, Pierce, Rockford, IL, USA).

An equal amount of total protein was used for the determination of HO-1, using a sandwich immunoassay for the detection and quantitation of human HO-1 protein in cell lysates, according to the manufacturer's specifications (Stressgen Bioreagents, Ann Arbor, MI, USA). After the test, absorbance was measured at 450 nm. The resulting readings were plotted against a standard curve to determine the concentration of HO-1 in each sample (ng/ml). The intra-assay and inter-assay coefficients of variation were both <10%.

Statistical analysis

Heme oxygenase-1 protein Gaussian distribution was achieved by log transformation and confirmed at Kolmogorov-Smirnov test. Comparison of quantitative variables across groups was carried out by ANOVA followed by Bonferroni's *post hoc* test. The association between NOX4 and HO-1 was completed at linear regression analysis.

Data were expressed as means±SDs and were analyzed using the JMP (ver. 9.0) (SAS, Cary, NC, USA) statistical package running on a Mac Pro (Apple, Cupertino, CA). Values at less than 5% level ($P<0.05$) were considered significant.

Results

All the GS patients of our cohort, as shown in **Table 1**, exhibited the biochemical characteristics of the syndrome: low plasma K, salt wasting, hypomagnesemia, hypocalciuria and activation of the renin-angiotensin-aldosterone system (RAAS) yet normo-hypotension [11]. In addition the diagnosis had been confirmed by the identification of the mutations on the SLC12A3 gene coding for the thiazide sensitive Na-K cotransporter responsible of the syndrome [11] (**Table 2**).

GS patients' systolic blood pressure ranged from 105 to 120 mmHg and the diastolic blood pressure ranged from 60 to 72 mmHg.

In hypertensive patients the systolic blood pressure ranged from 142 to 156 mmHg, the diastolic blood pressure ranged from 94 to 98 mmHg. In these patients the diagnosis of essential hypertension was confirmed by the exclusion of secondary hypertension via the evaluation of plasma renin activity and plasma aldosterone before and after 50 mg of captopril (captopril test).

Systolic blood pressure in normotensive healthy subjects ranged from 124 to 134 mmHg and the diastolic blood pressure from 79 to 83 mmHg.

The levels of NOX4 in the different groups of subjects studied are presented in **Figure 1**.

NOX4 protein levels were decreased in hypertensive patients as compared to both GS and

healthy subjects (1.06±0.31 densitometric units (d.u.) vs 1.76±0.54, P=0.002 and vs 1.61±0.54 P=0.018, respectively).

NOX4 protein level did not differ between GS and healthy subjects (1.76±0.54 d.u.) vs 1.61±0.54, P=ns).

HO-1 levels were increased in GS patients as compared to both hypertensive patients and healthy subjects (8.65±3.08 ng/ml vs 3.70±1.19, p=0.0001, and vs 5.49±1.04, P=0.008, respectively, **Figure 2**).

The protein levels of HO-1 in mononuclear cell were significantly reduced in hypertensive patients compared to healthy normotensive control subjects (3.70±1.19 ng/ml vs 5.49±1.04 ng/ml, P=0.009, **Figure 2**).

As presented in **Figure 3** NOX4 levels were positively correlated with HO-1 levels (r^2=0.63; P=0.001) in GS patients.

In healthy subjects or in the essential hypertensive patients no correlation was observed between NOX4 and HO-1 (r^2=0.082; p:ns, r^2=0.088; p:ns, respectively).

Discussion

Major findings of the present study are that: 1) GS patients characterized by endogenous antagonism of Ang II signaling [14] presented with NOX4 levels indistinguishable from normal subjects, whereas hypertensive patients had reduced NOX4 levels; 2) the protective HO-1 protein was increased only in GS patients and positively correlated with NOX4 levels.

Ang II production is enhanced in hypertension and, through activation of its signaling via AT1R stimulation, plays a central role in the development of target organ damage by inducing oxidative stress upregulating the isoforms NOX1 and NOX2 of the NADPH oxidase, the major O_2^- generating enzyme in the cardiovascular system [21]. ROS increase leads to the uncoupling of endothelial NO synthase, and to the reduction of NO synthesis as well as to the loss of NO associated antioxidant and vasodilating actions. These effects are in part due to the reduction of NO bioavailability and the increased production of the highly reactive oxidant specie $ONOO^-$ as a result of the reaction between O_2^- and NO [22].

NOX4 is the NOX homolog that induces preferentially the production of H_2O_2 [7]. The functional significance of NOX4 is still not fully elucidated [6]. In order to study the linkage between Ang II and NOX4 in humans we studied the GS patients, which present increased Ang II levels, blunted Ang II signaling effects, normotension or hypotension. Previously we have extensively studied these patients in order to better understand the mechanistic details of the cellular, biochemical and molecular events involved in Ang II signaling in humans [12-14] independently of high blood pressure, since GS do not develop hypertension and cardiovascular remodeling in spite of high Ang II levels and activation of the renin-angiotensin-aldosterone system (RAAS) [14]. The findings of the present study contribute to further extend the knowledge on the linkage of Ang II with the NOX family member NOX4.

NOX4 levels were higher in GS patients than hypertensive patients, and similar to the levels found in normotensive subjects. All this suggests that, in humans, Ang II may contribute to the reduction of NOX4 levels, particularly in hypertension. The increased level of HO-1 in GS patients in contrast to the reduced levels found in hypertensive patients might be related to the increased NOX4 expression in these patients as maintenance/increase of HO-1 expression has been shown to be a downstream effect of NOX4 activity [9]. In this regard, the existence of a relationship between HO-1 and NOX4 is further bolstered by the statistically significant linear correlation between HO-1 and NOX4 found in GS patients. Interestingly, we previously found an upregulation of nitric oxide system and increased flow mediated dilation (FMD), a measure of endothelium dependent and nitric oxide-mediated response in the vasculature in our GS patients [14, 16]. Moreover GS patients also exhibited a significant correlation between HO-1 protein levels and FMD [23]. Taken altogether, these findings in GS support a protective role of NOX4 activity in the human cardiovascular system. Opposite this is a case for reduced NOX4 levels being harmful as lower levels were found in our hypertensive patients. Hypertensive patients are characterized by RAAS activation, downregulation of NO system, reduced FMD [24], and activation of proinflammatory pathways of vascular remodeling [2]. These lower levels also suggest that NOX4

might be inhibited by Ang II particularly in hypertension. However, the nature of the relationship between NOX4 levels and its cardiovascular effects is complicated by studies reporting negative effects for elevated levels. These studies found that excessive production of H_2O_2 induced by increased levels of NOX4 was proinflammatory and proliferative [25] and contributed to the increased oxidative stress related to stroke as well as the induction of neurodegeneration [26]. These negative effects have in part been related to elevated NOX4 in the presence of growth factors such as TGFβ [5]. Of note, while GS patients presented with similar NOX4 levels as normotensive subjects, they have reduced TGFβ gene expression both at baseline and after Ang II challenge [14,15]. Thus it is conceivable that NOX4 might be involved in vascular damage at very low or very high expression levels. It has been recently underlined the possible "multifarious" nature of NOX4 at vascular level and suggested that the divergent effects, vasodilation and cardiovascular protective effects or vasoconstriction, reduced NO bioavailability, endothelial dysfunction and remodeling, might relate to the relative levels of NOX4 generated H_2O_2 production compared with the NOX4 mediated O_2^- production [6].

In conclusion, our preliminary findings show that NOX4 protein expression and HO-1 level are reduced in hypertensive patients compared to GS patients. One limitation of this study is the small number of hypertensive and control patients enrolled but those numbers were inherent in the preliminary nature of this study. Another potential limitation is the use of circulating cells rather than vascular cells, the major target of this system. However, circulating blood cells are widely used in vascular biology to study "*ex vivo*" the pathophysiological mechanisms of hypertension and remodeling in humans [12-14, 27]. In addition, the role of inflammation and the mononuclear leucocyte infiltration in the development of hypertensive target organ damage has been increasingly recognized in the last few years [27]. Furthermore, a correlation between hypertension and intracellular oxidative stress in leucocyte (polymorphonuclear and mononuclear cells) has recently been demonstrated [28].

The factors determining NOX4 activation and its ultimate effects still need a characterization, but the involvement of Ang II signaling via AT2R, and/or the ACE2-Ang 1-7-Mas axis and/or the Ang 1-9 system might appear likely. Indeed, these systems may counteract the vasoconstriction, the inflammatory process, and cardiovascular remodeling through multiple mechanisms including NO production, the negative regulation of the RhoA/Rho kinase pathway and reduction of oxidative stress [14, 29-32]. This would then make studies in GS patients even more informative given the activation of AT2R signaling and the changes in ACE2-Ang 1-7 system found in these patients [14]. Thus, our preliminary findings support further studies including a larger number of hypertensive patients in order to more clearly connect NOX4 and HO-1 axis with Ang II signaling and its long term effects and how they are related to help in assessing the functional significance of NOX4 in cardiovascular system in humans.

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Disclosure of conflict of interest

None.

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