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ORIGINAL ARTICLE

SIRT1, heme oxygenase-1 and NO-mediated vasodilation in a human model of endogenous angiotensin II type 1 receptor antagonism: implications for hypertension

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Reduced NO availability is associated with endothelial dysfunction, hypertension, insulin resistance and cardiovascular remodeling. SIRT1 upregulates eNOS activity and inhibits endothelial cell senescence, and reduced SIRT1 is related to oxidative stress and reduced NO-dependent dilation. Bartter's/Gitelman's syndromes (BS/GS) are rare diseases that feature a picture opposite to that of hypertension in that they present with normo/hypotension, reduced oxidative stress and a lack of cardiovascular remodeling, notwithstanding high levels of angiotensin II and other vasopressors, upregulation of NO system, and increased NO-dependent vasodilation (FMD), as well as increase in both endothelial progenitor cells and insulin sensitivity. To our knowledge, in BS/GS patients SIRT1 has never been evaluated. BS/GS patients' mononuclear cell SIRT1 (western blot), FMD (B-mode scan of the right brachial artery) and heme oxygenase (HO)-1 (sandwich immunoassay), a potent antioxidant protein, were compared with the levels in untreated stage 1 essential hypertensive patients (HPs) and in healthy subjects (C). SIRT1 (1.86 ± 0.29 *vs.* 1.18 ± 0.18 (HP) *vs.* 1.45 ± 0.18 (C) densitometric units, $P<0.0001$) and HO-1 protein (9.44 ± 3.09 *vs.* 3.70 ± 1.19 (HP) *vs.* 5.49 ± 1.04 (C) ng ml^{-1}, $P<0.0001$) levels were higher in BS/GS patients than in the other groups. FMD was also higher in BS/GS patients: $10.52 \pm 2.22\%$ *vs.* $5.99 \pm 1.68\%$ (HP) *vs.* $7.99 \pm 1.13\%$ (C) (ANOVA: $P<0.0001$). A strong and significant correlation between SIRT1 and FMD was found only in BS/GS patients ($r^2 = 0.63$, $P = 0.0026$). Increased SIRT1 and its direct relationship with increased FMD in BS/GS patients, while strengthening the relationship among SIRT1, NO and vascular function in humans, point toward a role for reduced SIRT1 in the endothelial dysfunction of hypertension.

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INTRODUCTION

Endothelial dysfunction, oxidative stress and inflammation promote the development of cardiovascular disease, and they are also associated with vascular aging. Lower endothelium-dependent dilation is a feature of both hypertension and aging.[1] Patients with essential hypertension, including middle-aged and elderly patients, have, in fact, lower endothelium-dependent dilation.[2,3] In addition, age-related decreases in endothelium-dependent dilation are predictive of future cardiovascular events, even in the absence of cardiovascular disease.[4,5] An important factor affecting endothelium-dependent dilation in both hypertension and aging is the reduced availability of nitric oxide (NO).[6,7] Understanding the factors that affect the activity of the endothelial subunit of NO synthase (eNOS), a major source of NO and thus of NO bioavailability, is likely to provide insights into the mechanisms involved in both vascular physiology and pathophysiology, as well as in cardiovascular disease.

SIRT1, a member of the sirtuin family, is an NAD-dependent protein deacetylase, which has been shown to constitute a point of convergence of several signaling pathways, including those related to oxidative stress, critical for endothelial homeostasis and the cardiovascular system.[8] SIRT1 deacetylase, in fact, regulates the activity of eNOS via the deacetylation of eNOS on lysines 496 and 506, resulting in higher eNOS activity.[9] In addition, SIRT1 has been shown to protect against oxidative stress through the regulation of lipid oxidation,[10] and reduced SIRT1 expression was linked with premature endothelial cell senescence.[11]

Heme oxygenase (HO)-1 is a potent antioxidant protein[12] that acts on heme, producing CO and biliverdin, the latter of which is further

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metabolized into bilirubin, itself a potent antioxidant. Upregulation of HO-1 protects against vascular diseases, including atherosclerosis, by inducing anti-inflammatory activity, inhibiting smooth-muscle-cell proliferation and regulating vascular tone by increasing cellular antioxidant activities.[13] HO-1's oxidative stress-protective effects are associated with SIRT1 upregulation.[14] Moreover, SIRT1 and HO-1 might be linked via the transcriptional coactivator PGC-1α. PGC-1α was shown to have an important role in SIRT1's lipid oxidation-regulatory activity,[10] and to upregulate HO-1 in HO-1-related endothelium-cytoprotective, anti-inflammatory and antiproliferative effects.[15]

Patients with Bartter's/Gitelman's syndromes (BS/GS), which are rare diseases caused by genetic defects in specific kidney transporters and ion channels, present a puzzling clinical, biochemical and molecular picture, characterized by hypokalemia, sodium depletion and activation of the renin–angiotensin–aldosterone system, with increased plasma levels of angiotensin (ang) II and aldosterone and yet normo/hypotension, reduced peripheral resistance and hyporesponsiveness to pressor agents.[16] Importantly, in contrast to hypertension and/or age-related oxidative stress, endothelial dysfunction, insulin resistance, decline in NO and NO-related enzyme activity, such as NO-mediated vasodilation, and reduced endothelial progenitor cells,[6,17,18] BS/GS patients demonstrate upregulation of the NO system,[19–21] reduced oxidative stress,[22,23] increased endothelium-dependent vasodilation[24] and insulin sensitivity,[25] increased number of circulating endothelial progenitor cells,[26] lack of cardiovascular remodeling[24,27] and normo/hypotension, despite high levels of ang II.[19,28–30] These aspects of BS/GS and, in particular, the syndromes' upregulation of the NO system, increased endothelium-dependent vasodilation and reduced oxidative stress make these patients a mirror image of hypertension; thus, these patients are potentially useful as subjects in exploring the linkage of SIRT1 and NO levels, and in doing so using a clinically relevant system.

The current study compared BS/GS patients' mononuclear cell (peripheral blood mononuclear cell, PBMC) protein levels of SIRT1 and HO-1 to the levels found in the PBMCs of either hypertensive patients or healthy normotensive subjects. In addition, endothelium-dependent vasodilation (FMD) was measured in all of the study groups.

METHODS

Patients

Eleven Bartter's/Gitelman's (BS/GS) patients (six male patients and five female patients, age range 27–58 years old), with either BS ($n=1$) or GS ($n=10$), were recruited from our cohort of BS/GS patients; all of the patients had a full Bartter's/Gitelman's biochemical characterization, with the ten patients with GS having undergone full genetic analyses and the one with BS awaiting the results of genetic screening (Table 1 and Table 2). Their blood pressure (BP) ranged from 100 to 120 mm Hg for systolic and from 68 to 82 mm Hg for diastolic.

Healthy normotensive subjects (six male patients and five female patients, aged 44.6 ± 10.5 years), from the staff of the Department of Medicine of the University of Padova, were used as the control group. Their BP ranged from 122 to 136 mm Hg for systolic and 72 to 86 mm Hg for diastolic.

Eleven uncomplicated, nonsmoking and untreated essential hypertensive patients (six male patients and five female patients, aged 39–60 years) were selected from the cohort of patients of the Padova Hypertension Unit in the Department of Medicine, Clinica Medica 4, when identified, and were enrolled for participation in the study. Their BP ranged from 142 to 156 mm Hg for systolic and 94 to 98 mm Hg for diastolic.

Automated office BP was measured in all of the study participants using an Omron blood pressure monitor with a 705 IT interface (Omron Health Care, Ukyo-Kyoto, Japan) after the subjects had been seated quietly for at least 10 min. The average of three readings, obtained at 1-min intervals, was considered for the study.

Table 1 Clinical and laboratory data of Bartter's/Gitelman's patients (BS/GS), hypertensive patients and healthy controls included in the study

			Plasma electrolytes (mmol l^{-1})	*Urinary electrolytes (mmol per day)*		
	Sex	*Age (years)*	*Na^+, K^+, Cl^-, Mg^{2+}*	*Na^+, K^+, Cl^-, Ca^{2+}*	*PRA (ng ang $l\,ml^{-1}\,h^{-1}$)*	*Aldosterone (nmol l^{-1})*
Bartter's Patient						
1	F	27	138, 2.3, 96, 0.93	160, 38.6, 190, 3.8	12	0.94
Gitelman's Patients						
1	F	30	137, 2.9, 99, 0.63	255, 38.0, 200, 1.9	9	0.70
2	M	44	140, 2.8, 98, 0.60	198, 30.8, 220, 2.0	7	0.88
3	F	31	138, 2.7, 98, 0.60	297, 42.8, 289, 2.1	10	0.78
4	M	32	138, 3.0, 99, 0.56	200, 81.5, 239, 2.0	6	0.67
5	F	58	140, 3.0, 100, 0.55	190, 42.5, 220, 2.0	6	0.70
6	M	29	139, 3.0, 100, 0.57	190, 42.5, 218, 2.1	6	0.75
7	M	31	139, 3.1, 100, 0.58	192, 42.8, 210, 2.2	6	0.75
8	M	43	139, 3.0, 98, 0.58	200, 45.4, 197, 2.0	5.8	0.80
9	M	39	139, 2.8, 97, 0.59	195, 47.4, 198, 2.0	5.8	0.81
10	F	30	139, 2.9, 100, 0.60	196, 48.8, 199, 2.0	6.1	0.77
Controls	6M/5F	46.2	140, 4.1, 99, 0.99	180, 52.8, 179.8, 4.5	0.73	0.18
($n=11$)		± 10.5	± 1.0, 0.2, 0.97, 0.2	± 16.5, 4.8, 1, 9.6, 0.5	± 0.13	± 0.02
Hypertensives	6M/5F	39.6	142, 4.1, 99, 1.1	188, 56.9, 178.2, 4.4	0.99	0.24
($n=11$)		± 11.2	± 0.9, 0.1, 0.94, 0.2	± 19.5, 6.0, 16.6, 0.6	± 0.15	± 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 $ml^{-1}\,h^{-1}$ and 0.08–0.29 nmol l^{-1}, respectively. Normal values for plasma Na^+, K^+, Cl^-, Mg^{++} are 136–145, 3.5–5, 96–108, 0.65–1.05 mmol l^{-1}, respectively. Normal values for urinary Na^+, K^+, Cl^- and Ca^{2+} excretion are: 40–220, 25–125, 110–250 and 2.5–7.5 mmol per day, respectively.

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

Patient	Exon	Mutation at nucleotide	Homo-heterozygous	Predicted effect on protein
1	23	2736 G——>A	homozygous	Arg904Gln
2	22	2579C——>T	heterozygous	Arg852Cys
	23	2736 G——>A	heterozygous	Arg904Gln
3	15	1950 G——>A	heterozygous	Arg642His or splice donor site truncated SLC12A3 protein
	18	2246 G——>A	heterozygous	Gly741Arg
4	22	2579C——>T	heterozygous	Arg852Cys
	23	2736 G——>A	homozygous	Arg904Gln
5	22	2579C——>T	heterozygous	Arg852Cys
	23	2736 G——>A	heterozygous	Arg904Gln
6	21	2542 G——>T	heterozygous	Asp848Tyr
	10	c.1196_1202dup 7 bp	heterozygous	Ser402X
7	21	2542 G——>T	heterozygous	Asp848Tyr
	10	c.1196_1202dup 7 bp	heterozygous	Ser402X
8	17	c.2089_2095del 7 bp	heterozygous	pThr697fs
	26	2985 G—>A	heterozygous	Ser402X
9	17	c.2089_2095del 7 bp	heterozygous	pThr697fs
	26	2985 G—>A	heterozygous	Ser402X
10	10	1195C—>T	heterozygous	pArg399Cys
	15	1220C—>T	heterozygous	pArg642His

None of the study patients had cardiac failure or evidence of coronary heart disease; left ventricular hypertrophy was ruled out by conventional M-mode echocardiography. The study participants had a normal BMI ($<25\,kg\,m^{-2}$), fasting serum glucose $<126\,mg\,dl^{-1}$, normal renal function by serum creatinine $<1\,mg\,dl^{-1}$ and urinary albumin excretion $<30\,mg\,g^{-1}$ of urinary creatinine. Plasma renin activity (PRA) and aldosterone levels before and after 50 mg of captopril (captopril test) were used to rule out secondary hypertension. Lipid profiles were normal, and the patients were not taking lipid-lowering drugs or aspirin. All of the subjects reported consuming a normal Italian diet, which consists of ~150 mmol of sodium per day. The BS/GS patients were taking potassium and magnesium supplements. All of the subjects abstained from food, alcohol and caffeine-containing drinks for at least 12 h prior to the study.

Informed consent was obtained from all of the study participants.

Methods

Preparation of mononuclear cells. PBMCs were isolated using Ficoll Paque Plus gradient (GE Healthcare, Uppsala, Sweden) from 35 ml of EDTA-anticoagulated blood.

SIRT1 protein expression. SIRT1 protein expression was assessed using western blot analysis. Total protein extracts were obtained by cell lysis using an 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%,), with protease inhibitor added (Complete Protease Inhibitor Cocktail, Roche Diagnostics, Mannheim, Germany). Protein concentrations were evaluated by bicinchoninic acid assay (BCA Protein Assay, Pierce). Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes (Hybond ECL, GE Healthcare) and blocked overnight with non-fat milk (5% in Tween-PBS). The membranes were probed with a primary polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), and then HRP-conjugated secondary antibodies (GE Healthcare) were added, and immunoreactive proteins were visualized with chemiluminescence using SuperSignal WestPico Chemiluminescent Substrate (Pierce).

Protein expression on western blots was quantified using a PC-based, densitometric, semiquantitative analysis with ImageJ software (NIH, USA, rsb.info.nih.gov/ij), and the results were normalized to β-actin, a housekeeping gene.

Heme oxygenase-1 protein quantification. Total protein extracts from peripheral blood mononuclear cells were obtained by cell lysis with an 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%), with protease inhibitors added (Complete Protease Inhibitor Cocktail, Roche Diagnostics). 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 ($ng\,ml^{-1}$). The intraassay and interassay coefficients of variation were both <10%.

Nitric oxide-dependent vasodilation (FMD). NO-dependent vasodilation was determined by a B-mode scan of the right brachial artery in longitudinal section above the elbow, using a 7–10-MHz linear array transducer and a standard Aspen Advanced Ultrasound System (Acuson, Mountain View, CA, USA), as previously detailed.[24] Briefly, measurements were obtained using an automatic system for computing the brachial artery diameter in real time, by analyzing B-mode ultrasound images. Endothelium-dependent response was assessed as dilation of the brachial artery for increased flow (FMD). After 1 min of acquisition for measuring the basal diameter, a cuff, placed around the forearm just below the elbow, was inflated for 5 min at 250 mm Hg and then was deflated to induce reactive hyperemia. FMD was calculated as the maximal percentage increase in the diameter of the brachial artery above the baseline. FMD measurements were performed by a single well-trained operator (physician).

Statistical analysis. The data expressed as means ± s.d.'s, and they were analyzed using the JMP (ver. 9.0) (SAS, Cary, NC, USA) statistical package running on a Mac Pro (Apple, Cupertino, CA, USA) and were evaluated using ANOVA for unpaired data. Values at less than the 5% level ($P<0.05$) were considered significant.

RESULTS

Figure 1 shows that mononuclear cell SIRT1 protein levels were higher in the BS/GS patients than in either the healthy subjects (C) or hypertensive patients (HPs), (ANOVA: $P<0.0001$): 1.86 ± 0.29 in BS/GS patients *vs.* 1.18 ± 0.18 in HPs ($P=0.001$) *vs.* 1.45 ± 0.18 densitometric units in C ($P=0.013$). The SIRT1 protein levels in hypertensive patients were also significantly reduced compared with those in the healthy subjects ($P=0.025$).

Figure 2 shows that mononuclear cell HO-1 protein levels were increased in BS/GS patients, compared with both hypertensive patients and healthy subjects (ANOVA: $P<0.0001$): 9.44 ± 3.09 in BS/GS patients *vs.* 3.70 ± 1.19 in HPs ($P=0.001$) *vs.* 5.49 ± 1.04 $ng\,ml^{-1}$ in C ($P=0.003$). HO-1 protein levels in the hypertensive patients were also significantly reduced compared with those in healthy subjects ($P=0.003$).

Confirming our previous reports,[24,26,31] FMD was higher in BS/GS patients compared with both hypertensive patients and healthy subjects (ANOVA: $P<0.0001$): 10.52 ± 2.22% in BS/GS patients *vs.* 5.99 ± 1.68% in HPs ($P=0.0001$) *vs.* 7.99 ± 1.13% in C ($P=0.006$). FMD in hypertensive patients was also significantly reduced compared with healthy subjects ($P=0.003$) (Figure 3).

In BS/GS patients, SIRT1 protein levels and FMD showed a strong direct correlation ($r^2=0.63$, $P=0.0026$); no such correlation was observed in healthy subjects ($r^2=0.07$, $P=0.46$) or in the essential hypertensive patients ($r^2=0.16$, $P=0.24$) (Figure 4).

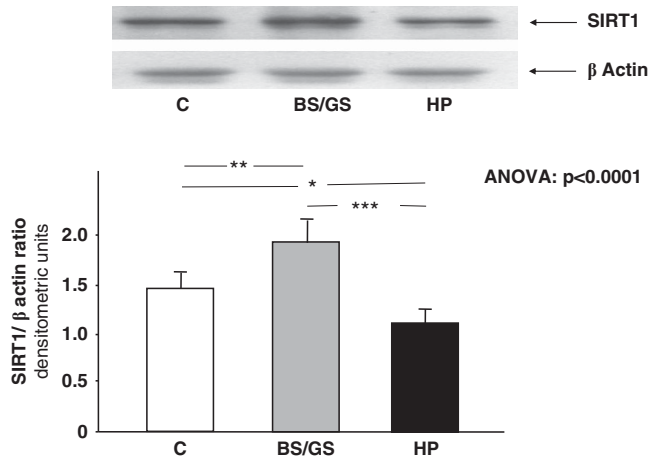

Figure 1 Densitometric analysis of SIRT1 protein expression in mononuclear cells of Bartter's/Gitelman's patients (BS/GS), healthy subjects (C) and essential hypertensive patients (HPs).The top part of the figure shows representative SIRT1 western blot products from 1 patient with BS/GS, 1 C and 1 patient with HP. *0.025; **0.013; ***0.001.

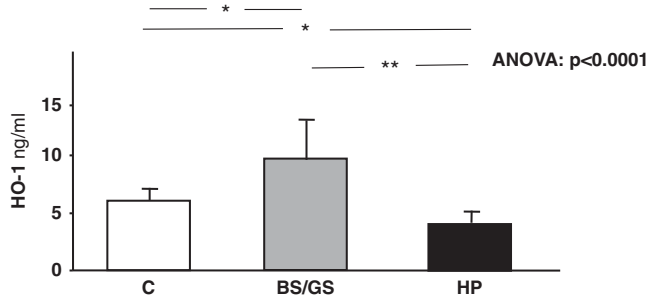

Figure 2 HO-1 protein levels in Bartter's/Gitelman's patients (BS/GS), healthy subjects (C) and essential hypertensive patients (HPs). *0.003; **0.001.

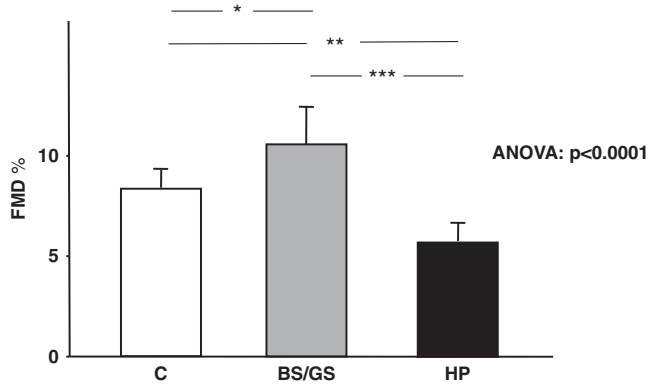

Figure 3 FMD in Bartter's/Gitelman's patients (BS/GS), healthy subjects (C) and essential hypertensive patients (HPs). *0.006; **0.003; ***0.001.

DISCUSSION

Recent evidence suggests that SIRT1 expression is reduced in atherosclerotic vessels and in the coronary vessels of aged rats.[32] In addition, inhibition of SIRT1 expression has been linked to decreased NO bioavailability, inhibited endothelium-dependent vasorelaxation and premature endothelial cell senescence.[9] Conversely, overexpression of SIRT1 in endothelial cells attenuated

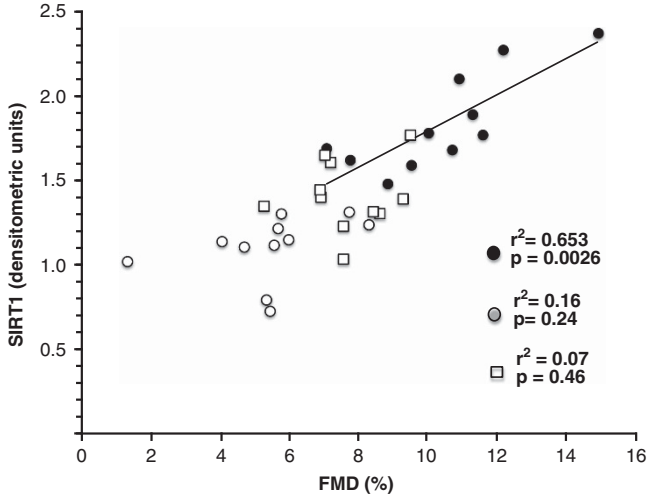

Figure 4 Correlation between SIRT1 and NO-mediated vasodilation in subjects from the three groups considered in the study. ● = Bartter's/Gitelman's patients; ○ = healthy subjects; □ = essential hypertensive patients.

oxidative stress[33] and prevented the loss of vasorelaxation, as well as decreased atherosclerotic plaques, in an apoE −/− model of atherogenesis,[34] and it inhibited endothelial activation.[35]

Most recently, Donato *et al.*[36] reported that impaired endothelium-dependent dilation with aging was associated with reduced SIRT1 expression, along with increased acetylated eNOS, this latter outcome resulting in decreased eNOS activity. These results demonstrate that SIRT1 in humans is lower in the vascular endothelial cells of older adults and that this decrease is positively correlated with *in vivo* decreases in endothelium-dependent dilation. Combined with previous results in animal experiments,[37] these findings lead these authors to suggest a possible role for reduced SIRT1 in mediating vascular endothelial dysfunction in aging.

Our main finding is that in BS/GS, a human model opposite to that of hypertension, SIRT1 is increased and is directly associated with increased endothelium-dependent vasodilation. The association with BS/GS patients, who have increased NO levels, increased gene expression of the endothelial subunit of NOS and activation of the NO system,[19–21] and who are normo- or even hypotensive despite increased vasoconstrictor levels,[19,28–30] provides further evidence linking SIRT1, NO and vascular function in humans. Moreover, as we previously reported, increased endothelium-independent dilation in BS/GS patients[24] provides further evidence that SIRT1 activity is an important mechanism of enhanced NO-dependent vasodilation. This finding is strengthened by the strong and significant correlation between SIRT1 levels and endothelium-dependent dilation found in BS/GS patients. We propose that the elevation of SIRT1 in BS/GS is related to changes in cAMP levels because Park *et al.*[38] reported that SIRT1 activation by resveratrol proceeded via increased cAMP as a result of resveratrol-related inhibition of phosphodiesterase (PDE)1, PDE3 and PDE4. Our contention that BS/GS patients have increased plasma levels of cAMP[39] as a result of increased SIRT1 activity is supported by a recent report regarding cAMP's effect on the regulator of G-protein signaling (RGS) 2,[40] increased levels of which occur in normo- and hypotensive BS/GS patients,[28,29] in line with the relationship between reduced RGS2 levels and hypertension.[41,42] In addition to the finding that cAMP induces RGS2,[43] Xie *et al.*[40] recently identified a cAMP-responsive element in the regulator of

RGS2 promoter as a key *cis*-regulatory element for RGS2 transcriptional regulation by ang II. That RGS2 is elevated along with SIRT1 activity suggests that the normo/hypotension of BS/GS patients, despite increased levels of ang II and other vasopressors, likely results from the ang II-related negative feedback loop that serves to modulate its pressor effects.[19,28,29] Hence, the current study and previous findings[38,40] suggest that a key factor in ang II signaling is the balance between the activities of adenyl cyclases synthesizing cAMP and those of cyclic nucleotide PDEs, which hydrolyze cAMP or cGMP to AMP or GMP, respectively. Finally, as reduced SIRT1 expression, reduced NO bioavailability and impaired NO-mediated vasodilation have been shown to be associated with premature endothelial cell senescence, the increased number of circulating endothelial progenitor cells recently reported in BS/GS patients[26] further strengthens the link among SIRT1, NO and vascular function in humans.[9,44] This link among SIRT1, NO and vascular function might also contribute to highlighting further the pathophysiology of endothelial dysfunction in hypertension: in our hypertensive patients, the reduced SIRT1 protein levels paralleled reduced endothelium-dependent vasodilation, as well as the reduced circulating endothelial progenitor cells' numbers and functions described in these patients.[18]

Oxidative stress, via reduction of NO availability and consequent endothelial dysfunction,[45,46] has a major role in the pathophysiology of hypertension, target organ damage, atherogenesis and long-term complications. In addition, it is also associated with other major cardiovascular risk factors, such as hypercholesterolemia and diabetes.[47,48] As shown in this study, overexpression of SIRT1, which protects against oxidative stress,[33] in BS/GS patients is accompanied by overexpression of HO-1, a known antioxidant, anti-inflammatory and antiapoptotic enzyme,[12,13] which contributes to the regulation of vascular tone and thereby blood pressure via production of the vasodilator CO.[12,13] Reduced oxidative stress and reduced expression of oxidative stress-related proteins, such as $p22^{phox}$ and PAI-1, in BS/GS patients[22,23] suggests that the cell redox state is unaltered, and the oxidative stress-related mechanisms that mediate cardiovascular remodeling and atherosclerosis[49] are downregulated in these patients. This finding is also reflected in BS/GS by the absence of left ventricular hypertrophy[27] and carotid intima-media thickening,[24] despite high levels of Ang II and activation of the renin–angiotensin system. The increases in both SIRT1 and HO-1 found in this study point to the existence of a common mechanism involving SIRT1 and HO-1 in mediating the endothelium's anti-inflammatory, antiproliferative and cytoprotective effects resulting from the oxidative stress exerted by both SIRT1 and HO-1. The transcriptional coactivator PGC-1α might have such a role, as it has been reported to have an essential role in SIRT1's regulatory activity of lipid oxidation,[10] as well as in the upregulation of HO-1,[15] thereby having a critical role in the endothelium-cytoprotective, anti-inflammatory and antiproliferative effects of HO-1.[15]

The increased SIRT1 found in this study in BS/GS patients also contributes to underlining SIRT1's role as a regulator of the endothelial inflammatory response, as provided by the evidence in this human model. Knockdown of SIRT1 has been reported to activate the NFκB inflammatory pathway, which is important for the transcription of genes responsible for the production of factors involved in local and systemic inflammation, and in cardiovascular remodeling.[50] Activation of SIRT1 has been reported to inhibit NFκB transcription, and reduce the expression of inflammatory and acute stress molecules.[51] The increased levels of SIRT1 found in BS/GS fits, in terms of anti-inflammatory response, with the increased level of IκB, the inhibitory subunit of NFκB that we reported in these patients, compared with healthy subjects.[52]

Finally, we previously reported that BS/GS patients had increased insulin sensitivity.[25] SIRT1 gene and protein expression in humans was shown to be affected by insulin resistance and metabolic syndrome,[53] and Fröjdö's *et al.*[54] reported that SIRT1 modulated insulin responsiveness. BS/GS patients' increased insulin sensitivity and SIRT1 protein levels agree with these findings, and thereby not only provide further evidence for the elevation of SIRT1 in BS/GS but also demonstrate that the effects and/or elevation of SIRT1 in BS/GS are not limited to mononuclear cells only.

Some limitations should be acknowledged in this study, including not having performed ambulatory BP monitoring (ABPM) for the assessment of the BP phenotype, which might have exposed the study to possible observer's bias. However, BS/GS patients are known to be normo- or hypotensive; therefore, ABPM would not have increased the diagnostic accuracy in this group. In the control group of HT patients, who might have contributed some cases of white-coat hypertension, ABPM could have provided more accurate phenotyping, but unfortunately it was not performed. However, these patients' classification as hypertensives was based on repeated BP assessments on the occasions of several outpatient office evaluations, as well as on their eventual assignment to treatment with antihypertensive medications.

In conclusion, the relationship among SIRT1, NO and NO-dependent vasodilation in humans is strengthened by the data obtained in BS/GS patients. Our results suggest a role for reduced SIRT1 in the vascular endothelial dysfunction of hypertension and in its long-term complications, such as cardiovascular remodeling and atherogenesis, thus underlining the usefulness of BS/GS as a human model for exploring the mechanisms involved in cardiovascular pathophysiology.

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