Angiotensin II signaling via type 2 receptors in a human model of vascular hyporeactivity: implications for hypertension

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Objective **Angiotensin II (Ang II) signaling via type 1 receptor (AT1R) has been extensively characterized, whereas Ang II signaling via type 2 receptors (AT2R), although counteracts actions mediated by AT1R, is still not completely understood. Bartter's/Gitelman's patients (BS/GS) have intrinsically blunted AT1R signaling, making them a good model to examine Ang II signaling via AT2R with particular emphasis on mitogen-activated protein kinase phosphatase 1 (MKP-1) that interacts with the Ang II-stimulated ERK pathway of cell signaling.**

Methods **BS/GS and healthy controls fibroblasts AT1R and AT2R level and the time course of Ang II's effect on MKP-1 levels and ERK1/2 phosphorylation over 1-h time course were assessed by western blot. The time course of Ang II's effect on MKP-1 levels and ERK1/2 phosphorylation alone or in the presence of either PD123319, an AT2R blocker, or Losartan, an AT1R blocker, or in combination was characterized.**

Results **AT1R and AT2R levels did not differ between BS/GS and healthy controls. Ang II induced ERK1/2 phosphorylation in BS/GS fibroblasts, but peak ERK1/2 phosphorylation declined more rapidly than that in control and BS/GS fibroblasts also exhibited increased MKP-1 levels at 30-min incubation. PD123319, an AT2R blocker in BS/GS fibroblasts, abolished the increased MKP-1 and ERK1/2 phosphorylation time course became same as that for control. Losartan, an AT1R blocker, alone altered the time course of control fibroblast MKP-1 to mimic the increase seen in BS/GS fibroblasts, whereas ERK1/2 declined concomitantly. Treatment with Losartan and PD123319 in controls reduced MKP-1 and elevated ERK1/2 phosphorylation to the level observed in BS/GS patients treated with PD123319.**

Conclusion **ERK1/2 phosphorylation time course found in BS/GS fibroblasts tracked changes in MKP-1 levels and incubation with an AT2R blocker, PD123319, abrogated those responses. Losartan, an AT1R blocker, reproduced these changes in healthy controls, whereas the presence of both AT1R and AT2R inhibitors in controls abolished these changes. These data strongly suggest that MKP-1 is a major effector in altering ERK1/2 phosphorylation status. Moreover, the results provide insight into the blunted responses in BS/GS reported for Ang II short-term and long-term effects, the mechanisms responsible, and thereby yield additional support for the role of AT2R signaling in the proposed effects of Ang II AT1R blockers beyond AT1R blockade.** *J Hypertens* **28:111–118 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Abbreviations: ACEI, angiotensin converting-enzyme inhibitor; ARB, angiotensin II type I receptor blocker; AT1R, angiotensin II type 1 receptor; ERK, extracellular signal-regulated kinase; IMT, intima–media thickness; MAPK, mitogen-activated protein kinase; RAAS, renin–angiotensin–aldosterone system

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Introduction

The involvement of angiotensin II (Ang II), the major effector peptide of the renin–angiotensin–aldosterone system (RAAS), in the induction and progression of cardiovascular diseases, is drawing increasing scrutiny [1–4]. Many of the Ang II-related signaling events are mediated via activation of its AT1 receptor (AT1R) followed by signaling via members of the mitogen-activated protein kinase (MAPK) family, including the extracellular signal-regulated kinase (ERK), the p38 and the c-Jun NH2-terminal kinase (JNK) [1]. Among the MAPKs, ERK has been strongly linked to hypertrophic response in addition to JNK and p38, which were reported to regulate cardiac hypertrophy [5]. ERK1/2, a member of the MAPK family, elicits a hypertrophic response via phosphorylation of nuclear targets (e.g., c-myc, c-jun, and ATF-2), leading to transcriptional reprogramming and the altered gene expression associated with hypertrophy [5]. Clinically, RAAS suppression with either Ang II AT1R blockers (ARBs) or angiotensin-converting enzyme inhibitors (ACEIs) has a favorable impact on left ventricular hypertrophy [6,7], reduces common carotid artery intima–media

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thickness (IMT) [8,9], and is thought to occur in addition to or independent of lowering blood pressure.

Although Ang II signaling via the AT1R has been the subject of intensive study with many of its effects and effectors characterized, Ang II signaling via AT2R stimulation has been suggested to counteract many actions mediated by AT1R. The role of AT2R in inducing vasodilation, antiproliferation, and apoptosis [10] remains to be completely elucidated. For example, AT2R-related events may improve the remodeling of resistance arteries [8,11] as well as cardiac hypertrophy [6,7] beyond blood pressure control as demonstrated in hypertensive as well as type 2 hypertensive diabetic patients upon selective AT1R antagonism. Stimulation of these receptors evokes pathways that involve tyrosine or serine/threonine phosphatases. Three such AT2R-associated phosphatases that interact with the ERK pathway have been identified: mitogen-activated protein kinase phosphatase 1 (MKP-1), protein phosphatase 2A (PP2A), and SH2 domain-containing phosphatase (SHP-1) [12].

Bartter's and Gitelman's syndromes (BS/GS), rare diseases caused by gene defects in specific kidney transporters and ion channels, present a puzzling clinical picture characterized by hypokalemia, sodium depletion, activation of the RAAS, with increased plasma levels of Ang II and aldosterone and yet normo/hypotension, reduced peripheral resistance, and hyporesponsiveness to pressor agents [13]. Therefore, we have proffered these patients as a good system to explore the signaling pathways of Ang II. The results of an extensive series of studies have provided mechanistic explanations for these patients' vascular hyporeactivity [14–16]. Along with these findings has come the recognition that BS/GS offer a means to explore the mechanisms responsible for maintaining/controlling vascular tone and cardiovascular remodeling involved in the Ang II signaling. Understanding why they do not develop hypertension and its complications, such as cardiovascular remodeling and atherogenesis, in spite of high Ang II and activation of the RAAS, should shed considerable light on the cellular basis of hypertension and its complications [14–16].

We have recently reported on cardiac and vascular remodeling in BS/GS patients and found that both carotid IMT growth and cardiac hypertrophy are absent in these patients compared with patients with hypertension [17,18]. This was expected, given our findings that demonstrate that BS/GS represents the opposite with respect to the status of the signals in vascular regulation typical of hypertension [14–16]. In fact, the large body of data reported by our laboratory points to BS/GS as being an example of endogenous Ang II AT1R antagonism. Thus, these findings provided some of the first human clinical data supporting a direct remodeling role for Ang II and the potential involvement of AT2R in mediating the absence of cardiac and vascular remodeling in these patients [17,18].

The current study directly addresses the issue of AT2R-mediated effects by making use of model system that the BS/GS patients offer. The study explored the mechanisms regulating the Ang II-induced ERK1/2 stimulation by examining the response of MKP-1, whose protein expression was shown to correlate with ERK1/2 activation [19–21] in fibroblasts from BS/GS patients in comparison with fibroblasts from healthy individuals.

Methods

Patients

We recruited five patients (two men and three women, age range 26–54) with either BS ($n = 1$) or GS ($n = 4$) from our cohort of BS/GS patients, the same patients evaluated in previous studies [14–16]. For the BS patient, the genetic characterization is still pending, whereas a full biochemical characterization is available. All GS patients have a full biochemical and genetic characterization.

Five normotensive healthy individuals were used as controls (two men and three women, age 43.2 ± 10.6 years), who were recruited from the staff of the Department of Clinical and Experimental Medicine, University of Padova.

None of the patients or controls had been taking drugs, with the only exception of potassium supplements for BS/GS patients, for at least 2 weeks prior to the study.

Clinical and laboratory data of our BS/GS patients and controls and genetic characterization for the patients are shown in Tables 1 and 2, respectively.

The study protocol was approved by our institutional authorities and informed consent was obtained from all study participants.

Cell culture

Fibroblasts from BS/GS patients and healthy controls were derived from a skin biopsy taken from the anterior surface of the left forearm by excision, under topical anesthesia with ethyl chloride. The biopsy was then cultured in nutrient mixture F-10 HAM medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, and 4 mmol/l glutamine, as previously described [22,23]. Fibroblasts obtained from each participant and grown separately were used for the experiments after the third passage.

Analysis of AT1R, AT2R, ERK1/2, and MPK expression and ERK1/2 phosphorylation

The response to Ang II of ERK1/2 phosphorylation and ERK1/2 and MKP-1 expression was evaluated by western blot analysis using 250 nmol/l Ang II and cell samples were taken at time zero, 2, 30, and 60 min. The effect of

Table 1 Clinical and laboratory data of the patients included in the study

	Sex	Age (years)	BP (mmHg)	Plasma electrolytes (mmol/l)				Urinary electrolytes (mmol/day)				PRA (ng Ang I/ml/h)	Aldosterone (mmol/l)
				Na^+	K^+	Cl^-	Mg^{2+}	Na^+	K^+	Cl^-	Ca^{2+}		
Bartter's patient													
1	F	26	105/70	138	2.3	96	0.93	190	38.6	190	3.8	12	0.94
Gitelman's patients													
1	M	44	110/70	140	2.8	98	0.60	198	30.8	220	2.0	7	0.88
2	F	31	115/75	138	2.7	98	0.60	297	42.8	289	2.1	10	0.78
3	F	52	115/70	138	3.0	99	0.56	200	81.5	239	2.0	6	0.67
4	M	54	120/70	140	3.0	100	0.55	190	42.5	220	2.0	6	0.70
Controls ($n=5$)	2 M/3 F	43.2 ± 10.6	129.5/82 ± 5.2/2.8	140 ± 1.1	4.2 ± 0.2	98 ± 0.96	0.98 ± 0.2	180 ± 15.5	53.8 ± 4.7	180.8 ± 18.9	4.6 ± 0.6	0.77 ± 0.15	0.20 ± 0.03

The table reports single data of the patients. Normal values for PRA and plasma aldosterone in our laboratory are 0.2–2.8 ng Ang I/ml per h and 0.08–0.29 nmol/l, respectively. Normal values for plasma Na^+, K^+, Cl^-, and Mg^{2+} are 136–145, 3.5–5, 96–108, and 0.65–1.05 mmol/l, 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/day respectively. BP, blood pressure; F, female; M, male; PRA, plasma and rennin activity.

PD123319, an AT2R blocker, in BS/GS patients and healthy controls, and Losartan, an AT1R blocker alone and plus PD123319 in healthy controls, on ERK-1/2 and MKP-1 expression and ERK1/2 phosphorylation was tested by preincubating the cells with 1 μmol/l PD123319 or 100 μmol/l Losartan or Losartan plus PD123319 for 30 min and then samples were treated as detailed below.

Total protein extract was obtained, electrophorized, and blotted as previously described [23,24]. The membranes were incubated overnight with antiphospho-ERK-1/2 (Cell Signaling, Danvers, Massachusetts, USA), anti MKP-1 (Santa Cruz Biotechnologies, Santa Cruz, California, USA), and anti-GAPDH (Chemicon International, Temecula, California, USA). Specific secondary antibodies were horseradish peroxidase (HRP)-conjugated (Amersham Pharmacia, Uppsala, Sweden) and immunoreactive proteins were visualized with chemiluminescence using SuperSignal WestPico Chemiluminescent Substrate (Pierce, Rockford, Illinois, USA).

Primary polyclonal antibody anti-AT1 receptor, anti AT2R (Santa Cruz Biotechnologies), and specific HRP-conjugated secondary antibodies (Amersham Pharmacia) were used for the determination of AT1R and AT2R levels.

ERK1/2 phosphorylation and protein expression were quantified using a densitometric semiquantitative analysis using NIH image software. The ratio between phospho-ERK1/2 and ERK1/2 were used as indexes of ERK1/2 activation. MKP-1 levels were normalized using the housekeeping protein GAPDH levels.

Statistical analysis

Data were evaluated statistically using a repeated measures ANOVA (Statistica; Statsoft Inc., Oklahoma City, Oklahoma, USA). Posthoc comparisons were made using Tukey's Honestly Significant Difference (HSD). Results with P less than 0.05 were considered significant and data values are presented as mean ± SD.

Results

Figure 1 demonstrates that the levels of AT1R and AT2R do not differ between healthy individuals and BS/GS patients.

Figure 2, panel a, shows the time course of Ang II-induced ERK1/2 phosphorylation, expressed as the ratio of phosphorylated ERK1/2 and total ERK1/2 for control and BS/GS fibroblasts in culture with or without preincubation with PD123319, an ATR2 blocker. Upon statistical analysis by repeated measures ANOVA, ERK1/2 phosphorylation showed a statistically significant three-way interaction ($P < 0.00003$) between time × drug (i.e., ±PD123319, an AT2R blocker) × disease (control versus BS/GS). Further, time, drug, disease × drug, and the time - × drug interactions effects were statistically significant

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

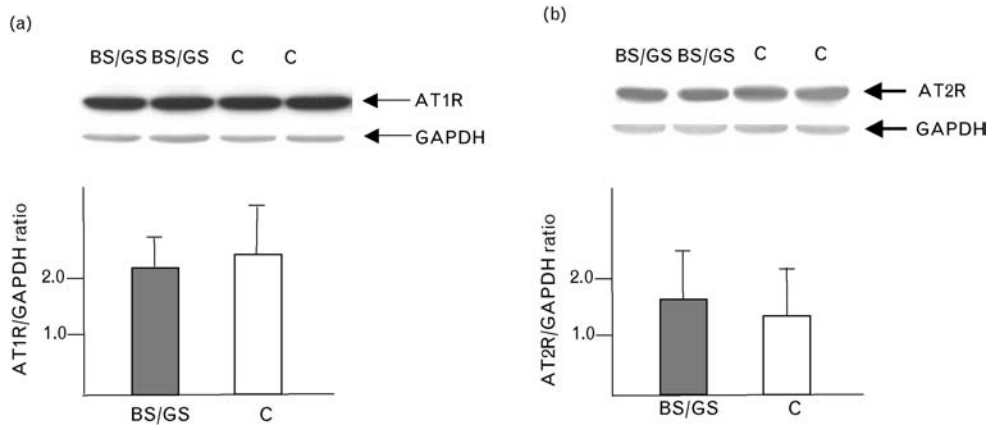
Patient	Exon	Mutation at nucleotide	Homo/heterozygous	Predicted effect on protein
1	23	2736G→A	Homozygous	Arg904Gln
2	22	2579C→T	Heterozygous	Arg852Cys
	23	2736G→A	Heterozygous	Arg904Gln
3	15	1950G→A	Heterozygous	Arg642His or splice donor site truncated SLC12A3 protein
	18	2246G→A	Heterozygous	Gly741Arg
4	22	2579C→T	Heterozygous	Arg852Cys
	23	2736G→A	Homozygous	Arg904Gln

($P < 0.00001$, $P < 0.00004$, $P < 0.00002$, and $P < 0.00001$, respectively). Upon posthoc testing within time-$\times$ drug $\times$ disease using Tukey's HSD, ERK1/2 phosphorylation in BS/GS patients differed from time zero at 2 min (0.62 ± 0.02 vs. 1.12 ± 0.04, $P < 0.002$) but did not differ statistically from time zero at either 30 or 60 min. In controls, ERK1/2 phosphorylation also differed from time zero at 2 min but, unlike the results in BS/GS patients, remained statistically different and elevated at 30 min before becoming not significantly different from time zero at 60 min.

Preincubation with 1 μmol/l PD123319, an AT2R blocker, was found to have a marked statistically significant interaction effect with disease and time as noted above and posthoc analysis of this, using Tukey's HSD, found that the AT2R blocker effects are restricted to BS/GS patients at later time points, as only the posthoc comparisons of ERK1/2 phosphorylation at 30 and 60 min with or without PD123319 in the BS/GS patients showed statistically significant differences ($P < 0.00015$, $P < 0.00014$, respectively), whereas control ± 1 μmol/l PD123319 did not differ significantly at any time points. In addition, PD123319 abolished the statistically significant difference in ERK1/2 phosphorylation between BS/GS patients and control at 30 min, whereas a statistically significant difference ($P < 0.00014$ versus controls and $P < 0.025$ versus controls +PD123319) in ERK1/2 phosphorylation between BS/GS patients and controls at 60 min remained after the ERK1/2 phosphorylation increase found at the 60 min BS/GS time point upon PD123319 treatment.

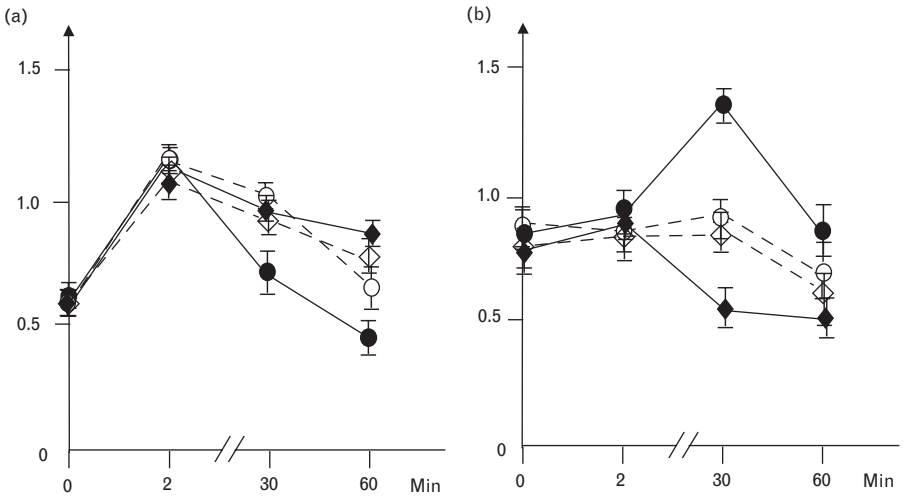
Figure 2, panel b, shows the time course of Ang II-induced MKP-1 protein levels for controls and BS/GS fibroblasts with or without preincubation with PD123319, an ATR2 blocker. Upon statistical analysis by repeated measures ANOVA, MKP-1 protein showed a statistically significant three-way interaction ($P < 0.000001$) between time $\times$ drug $\times$ disease. Further, time and time $\times$ drug interaction effects were statistically significant ($P < 0.00001$, $P < 0.00001$, respectively). Upon posthoc comparison within time $\times$ drug $\times$ disease using Tukey's HSD, BS/GS MKP-1 protein at 30 min differed from time zero ($P < 0.0002$) and then declined at the 60 min time point compared with the 30 min time point ($P < 0.0002$), whereas control MKP-1 protein showed no differences between time zero and the rest of the time points but levels did differ statistically between the 30 and 60 min time points ($P < 0.04$).

Fig. 1



Densitometric analysis of the ratio of angiotensin II type 1 receptor (AT1R) (panel a) and of angiotensin II type 2 receptor (AT2R) (panel b) to GAPDH in fibroblasts of patients with Bartter's/Gitelman's syndromes (BS/GS) and healthy controls (C) ($N = 5$ in each case, done in triplicate). The top of each panel shows representative AT1R and AT2R immunoblots products from two BS/GS patients and two C.

Fig. 2

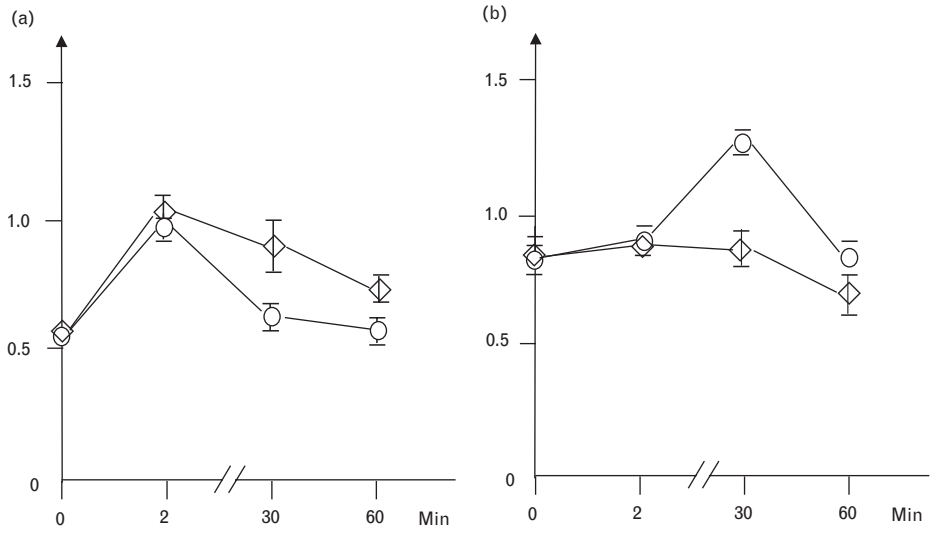


Time course of Ang II induced ERK1/2 phosphorylation (panel a) and MKP-1 levels (panel b), in fibroblasts of Bartter's/Gitelman's syndromes (BS/GS) and healthy controls and effect of the AT2R blocker PD123319 (1 μmol/l). Phospho-ERK1/2 and MKP-1 levels are expressed as the ratio of phospho-ERK1/2 and ERK1/2 and MKP-1 to GAPDH in densitometric units. ● and filled lines, BS/GS without PD123319; ◆ and filled lines, BS/GS plus PD123319; ○ and dashed lines, healthy controls without PD123319; ◇ and dashed lines, healthy controls plus PD123319 ($n = 3$ replicates for sample). For statistic significances see text.

Preincubation with 1 μmol/l PD123319, an AT2R blocker, was found to have a marked statistically significant interaction effect with disease and time as noted above and posthoc analysis of this using Tukey's HSD found that the differences were restricted to BS/GS and AT2R blocker as controls MKP-1 protein did not differ significantly ±PD123319 at any time point, whereas posthoc comparison using Tukey's HSD of MKP-1 protein at 30 min plus PD123319 in BS/GS showed statistically significant ($P < 0.0003$) decreased MKP-1 protein relative to BS/GS untreated at 30 min.

Figure 3, panel a, shows that upon statistical analysis by repeated measures ANOVA, ERK1/2 showed a statisti-

Fig. 3



Time course of Ang II induced ERK1/2 phosphorylation (panel a) and MKP-1 levels (panel b) in fibroblasts of healthy controls and effect of preincubation of Losartan (100 μmol/l) (○), an AT1R blocker and Losartan plus PD123319 (1 μmol/l) (◇), an AT2R blocker ($n = 3$ replicates for sample). For statistic significances see text.

cally significant two-way interaction between time and drug ($P < 0.000002$). Further, time and drug effects were statistically significant ($P < 0.00001$, $P < 0.00001$, respectively). Upon posthoc comparison within time × drug using Tukey's HSD, Losartan addition only resulted in the ERK level at 2 min as only time point that differed from time zero ($P < 0.0002$) in the Losartan-treated cells. In contrast, addition of Losartan and PD123319 resulted in ERK levels at 2, 30, and 60 min as different from time zero ($P < 0.0002$, $P < 0.0002$, and $P < 0.0002$, respectively). Time zero and time 2 min ERK levels did not differ between Losartan and Losartan + PD123319-treated cells, whereas the ERK levels upon Losartan + PD123319 treatment differed from the Losartan-only levels at both 30 and 60 min ($P < 0.00019$, $P < 0.00018$, respectively).

Figure 3, panel b, shows that upon statistical analysis by repeated measures ANOVA, MKP-1 protein levels showed a statistically significant two-way interaction between time and drug ($P < 0.019$). Further, time was statistically significant ($P < 0.004$). Upon posthoc comparison within time × drug using Tukey's HSD, Losartan addition only resulted in a peak of MKP-1 protein at 30 min, which differed from time zero, time 2, and time 60 ($P < 0.0008$, $P < 0.02$, $P < 0.005$, respectively) in the Losartan-treated cells. In contrast, no such peak was evident in Losartan + PD123319 added to controls cells as no time point comparisons reached statistical significance. Therefore, in healthy controls, Losartan alone altered the time course of MKP-1 cells to mimic the increase seen in BS/GS fibroblasts, whereas ERK1/2 declined concomitantly. In addition, in healthy controls, the treatment with Losartan + PD123319 reduced MKP-1 and elevated ERK1/2 phosphorylation to the levels observed in BS/GS cells treated with PD123319.

Discussion

The role of AT2R signaling in the control of cardiovascular remodeling and vascular tone remains to be fully defined. In fact, AT2R stimulation has been suggested to oppose AT1R effects by inducing vasodilation, antiproliferation, and apoptosis [25] through a completely different signaling pathway [26]. The results of the current study sought to provide insight into this system by making use of the unique characteristics of the BS/GS patients [13–16]. AT2R-mediated signaling is active in BS/GS patients based on the increased activation of NO system [17,27,28] as well as on the reduced activity of RhoA/Rho kinase system [16,29,30], which are both AT2R signaling-related [26,31]. In addition, BS/GS patients have a markedly higher insulin sensitivity along with the absence of microalbuminuria and endothelial dysfunction [32,33], the opposite of the Ang II signaling/insulin–glucose metabolism relationships found in diabetes and hypertension, all this in the presence of high level of Ang II. Furthermore, AT2R stimulation has been reported to downregulate ERK1/2 phosphorylation, which, in turn, results in reduced proliferation and apoptosis [34]. Thus, it would appear that BS/GS represent a unique human model of endogenous Ang II AT1R antagonism. This is further suggested by the fact that the regulator of G protein signaling (RGS)-2, which regulates Ang II AT1R function via downregulation of Gαq signaling [35,36], is upregulated in BS/GS [24], whereas altering RGS-2 via its silencing [22] produces in these patients on Ang II signaling effects that mirror those seen in fibroblasts from hypertensive patients [23]. The existence of endogenous AT1R signaling blockade in BS/GS patients then suggests that they may provide clearer insight into Ang II signaling mediated by Ang II AT2R.

In the present study, we found that the time course of ERK1/2 phosphorylation in response to Ang II in fibroblasts in cell culture differed between BS/GS patients and healthy individuals. Control fibroblasts upon incubation with 250 nmol/l Ang II exhibited a prolonged increase in ERK1/2 phosphorylation compared with the short duration of the increase in ERK1/2 phosphorylation seen in BS/GS patients. Differences in ATR2 receptor levels cannot account for this, as these are similar to the levels in controls. The presence of the ATR2 inhibitor, PD123319, extended the ERK1/2 phosphorylation response in BS/GS fibroblasts to the same level as the prolonged ERK1/2 phosphorylation increase found in the control fibroblasts. However, PD123319 did not alter the ERK1/2 phosphorylation responses to Ang II in the fibroblasts from healthy individuals. These findings confirm the existence of AT2R signaling in BS/GS. The existence of AT2R signaling in BS/GS may contribute to explain the lack of the long-term complications associated with elevated Ang II levels, for example, IMT and left ventricular hypertrophy, but not found in BS/GS despite their elevated Ang II levels [17,18].

Given the alterations noted in ERK1/2 phosphorylation, the fact that MKP-1 protein expression has been shown to negatively correlate with ERK1/2 activation [19–21] and is associated with AT2R signaling [13], this study also characterized the response of MKP-1 to Ang II. MKP-1 showed no difference at the basal and 2 min time points in both controls and BS/GS patients. However, a significant increase only appears at 30 min, matched by a corresponding decrease in ERK1/2 phosphorylation, and then returns to basal levels at 60 min in the BS/GS patients, unlike the MKP-1 protein levels in the control fibroblasts, which did not change. PD123319, an AT2R blocker, abolished the 30 min increase in MKP-1 in BS/GS patients, whereas it had no effect on MKP-1 protein levels in the controls. Others have previously reported that MKP-1 activity and gene expression are linked to AT2R signaling [37]. Our results, in our knowledge, are the first to report that AT2R signaling controls MKP-1 protein levels. The decline in ERK1/2 phosphorylation in

BS/GS occurs at the same time as the increase in MKP-1 levels and PD123319 abolishes both effects. The effect of inhibition of AT1R by Losartan in healthy controls shifted their MKP-1 response to resemble that of BS/GS. When Losartan and PD123319 were used in combination, the healthy controls response became similar to that of BS/GS fibroblasts preincubated with PD123319. These similarities between BS/GS and healthy controls in response to the various Ang II receptor blockers further bolster the contention that BS/GS have endogenous AT1R inhibition, as well as indicate that AT2R signaling is active in BS/GS or that in these patients AT2R signaling is not opposed or masked by AT1R signaling, as is the case in normal control. These data also suggest that the increase in MKP-1 observed in BS/GS may help explain the short duration of the ERK1/2 phosphorylation found in these patients' fibroblasts and strongly suggests that MKP-1 is a major effector in altering ERK1/2 phosphorylation status.

In conclusion, the results of the present study demonstrate that AT2R signaling is present in BS/GS patients. This presence of AT2R signaling may provide another means whereby the absence of AT1R signaling produces the blunted responses to both short-term and long-term Ang II effects reported in these patients such as the reduced vascular tone [13] and reduced vascular tone regulation [14,24,38,39], reduced oxidative stress [40,41], and cardiovascular remodeling [17,18] despite their high levels of Ang II. The interactions between AT1R and AT2R signals noted in the current study may provide further insight into the mechanisms involved in the Ang II signaling as they suggest that MKP-1 is a significant effector in the control of ERK1/2 phosphorylation. Moreover, they offer additional evidence to support the proposed role of AT2R signaling in the effects of Ang II AT1R blockers (ARBs) beyond AT1R blockade [42].

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There are no conflicts of interest.

References

1 Mehta PK, Griendling KK. Angiotensin II cell signaling. Physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol* 2007; **92**:C82–C97.
2 Touyz RM. Role of angiotensin II in regulating vascular structural and functional changes in hypertension. *Curr Hypertens Rep* 2003; **5**:155–164.
3 Ruiz Ortega M, Ruperez M, Esteban V, Egido J. Molecular mechanisms of angiotensin II-induced vascular injury. *Curr Hypertens Rep* 2003; **5**:73–79.
4 Dzau VJ, Lopez-Ilasaca M. Searching for transcriptional regulators of Ang II-induced vascular pathology. *J Clin Invest* 2005; **115**:2319–2322.
5 Kim S, Iwao H. Molecular and cellular mechanisms of Ang II-mediated cardiovascular and renal diseases. *Pharmacol Rev* 2000; **52**:11–34.
6 Devereux RB, Dahlöf B, Gerdts E, Boman K, Nieminen MS, Papademetriou V, *et al.* Regression of hypertensive left ventricular hypertrophy by Losartan compared with atenolol. The Losartan Intervention for Endpoint Reduction in Hypertension (LIFE) Trial. *Circulation* 2004; **110**:1456–1462.
7 Ruilope LM, Schmieder RE. Left ventricular hypertrophy and clinical outcomes in hypertensive patients. *Am J Hypertens* 2008; **21**:500–508.
8 Olsen MH, Wachtell K, Neland K, Bella JN, Rokkedal J, Dige-Petersen H, Ibsen H. Losartan but not atenolol reduce carotid artery hypertrophy in essential hypertension. A LIFE substudy. *Blood Press* 2005; **14**:177–183.
9 Lonn E, Yusuf S, Dzavik V, Doris C, Yi Q, Smith S, *et al.* SECURE Investigators. Effects of ramipril and vitamin E on atherosclerosis: the Study to Evaluate Carotid Ultrasound Changes in Patients Treated With Ramipril and Vitamin E (SECURE). *Circulation* 2001; **103**:919–925.
10 Volpe M, Musumeci B, De Paolis P, Savoia C, Morganti A. Angiotensin II AT2 receptor subtype: an uprising frontier in cardiovascular diseases? *J Hypertens* 2003; **21**:1429–1443.
11 Schiffrin EL, Park JB, Intengan HD, Touyz RM. Correction of arterial structure and endothelial dysfunction in humans essential hypertension by the angiotensin receptor antagonist losartan. *Circulation* 2000; **101**:1653–1659.
12 Nouet S, Nahmias C. Signal transduction from the angiotensin II AT2 receptor. *TEM* 2000; **11**:1–6.
13 Naesens M, Steels P, Verberckmoes R, Vanrenterghem Y, Kuypers D. Bartter's and Gitelman's syndromes: from gene to clinic. *Nephron Physiol* 2004; **96**:65–78.
14 Calò LA. Vascular tone control in humans: the utility of studies in Bartter's/Gitelman's syndromes. *Kidney Int* 2006; **69**:963–966.
15 Calò LA, Pessina AC, Semplicini A. Angiotensin II signaling in the Bartter's and Gitelman's syndromes, a negative human model of hypertension. *High Blood Press Cardiovasc Prev* 2005; **12**:17–26.
16 Calò LA, Pessina AC. RhoA/Rho-kinase pathway: much more than just a modulation of vascular tone. Evidence from studies in humans. *J Hypertens* 2007; **25**:259–264.
17 Calò LA, Puato M, Schiavo S, Zanardo M, Tirrito C, Pagnin E, Balbi G, *et al.* Absence of vascular remodelling in a high angiotensin-II state (Bartter's and Gitelman's syndromes): implications for angiotensin II signalling pathways. *Nephrol Dial Transplant* 2008; **23**:2804–2809.
18 Calò LA, Montisci R, Scognamiglio R, Davis PA, Pagnin E, Schiavo S, Mormino P *et al.* High angiotensin II state without cardiac remodeling (Bartter's and Gitelman's syndromes). are angiotensin II type 2 receptors involved? J Endocrinol Invest 2009 E-published ahead of print 14 July 2009, DOI: 10.3275/6430.
19 Sun H, Charles CH, Lau LF, Tonks NK. MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. *Cell* 1993; **75**:487–493.
20 Brondello JM, Brunet A, Pouysségur J, McKenzie FR. The Dual Specificity mitogen-activated protein kinase phosphatase 1 and 2 are induced by the p42/p44MAPK cascade. *J Biol Chem* 1997; **272**:1368–1376.
21 Brondello JM, Pouysségur J, McKenzie FR. Reduced MAP kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation. *Science* 1999; **286**:2514–2517.
22 Calò LA, Pagnin E, Ceolotto G, Davis PA, Schiavo S, Papparella I, Semplicini A, *et al.* Silencing regulator of G protein signaling-2 (RGS-2) increases angiotensin II signaling: insights into hypertension from findings in Bartter's/Gitelman's syndromes. *J Hypertens* 2008; **26**:938–945.
23 Semplicini A, Lenzini L, Sartori M, Papparella I, Calò LA, Pagnin E, Strapazzon G, *et al.* Reduced expression of regulator of G protein signaling-2 in hypertensive patients increases calcium mobilization and ERK1/2 phosphorylation induced by angiotensin II. *J Hypertens* 2006; **24**:1115–1124.
24 Calò LA, Pagnin E, Davis PA, Sartori M, Ceolotto G, Pessina AC, Semplicini A. Increased expression of regulator of G protein signaling–2 (RGS-2) in Bartter's/Gitelman's sydrome. A role in the control of vascular tone and implication for hypertension. *J Clin Endocrinol Metab* 2004; **89**:4153–4157.
25 Hansen JL, Servant G, Baranski TJ, Fujita T, Iiri T, Sheikh SP. Functional reconstitution of the angiotensin II type 2 receptor and Gi activation. *Circ Res* 2000; **87**:753–759.
26 Savoia C, Tabet F, Yao G, Schiffrin E, Touyz R. Negative regulation of Rho/Rho kinase by angiotensin II type 2 receptor in vascular smooth muscle cells: role in angiotensin II-induced vasodilation in stroke-prone spontaneously hypertensive rats. *J Hypertens* 2005; **23**:1037–1045.
27 Calò L, D'Angelo A, Cantaro S, Bordin MC, Favaro S, Antonello A, Borsatti A. Increased urinary NO_2^-/NO_3^- and cyclic GMP levels in patients with Bartter's syndrome: relationship to vascular reactivity. *Am J Kidney Dis* 1996; **27**:874–879.

28 Calò L, Davis PA, Milani M, Cantaro S, Antonello A, Favaro S, D'Angelo A. Increased endothelial nitric oxide synthase mRNA level in Bartter's and Gitelman's syndrome. Relationship to vascular reactivity. *Clin Nephrol* 1999; **51**:12–17.

29 Pagnin E, Davis PA, Sartori M, Semplicini A, Pessina AC, Calò LA. Rho kinase and PAI-1 in Bartter's/Gitelman's syndromes: relationship to angiotensin II signaling. *J Hypertens* 2004; **22**:1963–1969.

30 Pagnin E, Semplicini A, Sartori M, Pessina AC, Calò LA. Reduced mRNA and protein content of Rho guanine nucleotide exchange factor (RhoGEF) in Bartter's and Gitelman's syndromes. Relevance for the pathophysiology of hypertension. *Am J Hypertens* 2005; **18**:1200–1205.

31 Kurisu S, Ozono R, Oshima T, Kambe M, Ishida T, Sugino H, Matsuura H, *et al.* Cardiac angiotensin II type 2 receptor activates the kinin/NO system and inhibits fibrosis. *Hypertension* 2003; **41**:99–107.

32 Davis PA, Pagnin E, Semplicini A, Avogaro A, Calò LA. Insulin signaling, glucose metabolism and the angiotensin II signaling system. Studies in Bartter's/Gitelman's syndromes. *Diabetes Care* 2006; **29**:469–471.

33 Calò LA, Davis PA, Palatini P, Semplicini A, Pessina AC. Urinary albumin excretion, endothelial dysfunction and cardiovascular risk: study in Bartter's/Gitelman's syndromes and relevance for hypertension. *J Hum Hypertens* 2007; **21**:904–906.

34 Abadir PM, Periasamy A, Carey RM, Siragy HM. Angiotensin II type 2 receptor bradykinin B2 receptor functional heterodimerization. *Hypertension* 2006; **48**:316–322.

35 Wieland T, Lutz S, Chidiac P. Regulators of G protein signaling: a spotlight on emerging functions in the cardiovascular system. *Curr Opin Pharmacol* 2007; **7**:1–7.

36 Le TH, Coffman TM. RGS2. a 'turn-off' in hypertension. *J Clin Invest* 2003; **111**:441–443.

37 Steckelings UM, Kaschina E, Unger TH. The AT2 receptor: a matter of love and hate. *Peptides* 2005; **26**:1401–1409.

38 Calò LA, Ceolotto G, Milani M, Pagnin E, van den Heuvel LP, Sartori M, Davis PA, *et al.* Abnormalities of Gq-mediated cell signaling in Bartter and Gitelman syndromes. *Kidney Int* 2001; **60**:882–889.

39 Calò LA, Davis PA, Semplicini A. Reduced content of alpha subunit of Gq protein in monocytes of Bartter and Gitelman syndromes: relationship with vascular hyporeactivity. *Kidney Int* 2002; **61**:353–354.

40 Calò LA, Pagnin E, Davis PA, Sartori M, Semplicini A. Oxidative stress related factors in Bartter's and Gitelman's syndromes: relevance for angiotensin II signalling. *Nephrol Dial Transplant* 2003; **18**:1518–1525.

41 Calò LA, Sartore G, Bassi A, Basso C, Bertocco S, Marin R, Zambon S, *et al.* Reduced susceptibility of low density lipoprotein to oxidation in patients with overproduction of nitric oxide (Bartter's and Gitelman's syndrome). *J Hypertens* 1998; **16**:1001–1008.

42 Siragy HM. Evidence for benefits of angiotensin receptor blockade beyond blood pressure control. *Curr Hypertens Rep* 2008; **10**:261–267.