Original Article

Expression and functional role of the prorenin receptor in the human adrenocortical zona glomerulosa and in primary aldosteronism

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Objectives: Prorenin can be detected in plasma of hypertensive patients. If detected in patients with primary aldosteronism could implicate prorenin in the development of primary aldosteronism. To address this issue, we measured the plasma prorenin levels in primary aldosteronism patients, the expression of the prorenin receptor (PRR) in the normal human adrenocortical zona glomerulosa and aldosterone-producing adenoma (APA), and we investigated the functional effects of PRR activation in human adrenocortical cells.

Method: Plasma renin activity, aldosterone, and active and total trypsin-activated renin were measured in primary aldosteronism patients, essential hypertensive patients, and healthy individuals, and then prorenin levels were calculated. Localization and functional role of PRR were investigated in human and rat tissues, and aldosterone-producing cells.

Results: Primary aldosteronism patients had detectable plasma levels of prorenin. Using digital-droplet real-time PCR, we found a high PRR-to-porphobilinogen deaminase ratio in both the normal adrenal cortex and APAs. Marked expression of the PRR gene and protein was also found in HAC15 cells. Immunoblotting, confocal, and immunogold electron microscopy demonstrated PRR at the cell membrane and intracellularly. Renin and prorenin significantly triggered both CYP11B2 expression (aldosterone synthase) and ERK1/2 phosphorylation, but only CYP11B2 transcription was prevented by aliskiren.

Conclusion: The presence of detectable plasma prorenin in primary aldosteronism patients, and the high expression of PRR in the normal human adrenal cortex, APA tissue, CD56+ aldosterone-producing cells, along with activation of CYP11B2 synthesis and ERK1/2 phosphorylation, suggest that the circulating and locally produced prorenin may contribute to the development or maintenance of human primary aldosteronism.

Keywords: adrenal, primary aldosteronism, prorenin receptor, renin

Abbreviations: Ang II, angiotensin II; APA, aldosterone-producing adenoma; BP, blood pressure; Elk, Ets-like gene; MAPK, mitogen-activated protein kinase; PAC, plasma aldosterone concentration; PBGD, porphobilinogen deaminase; PRA, plasma renin activity; PRR, prorenin receptor

INTRODUCTION

Primary aldosteronism is a common cause of high blood pressure (BP), particularly among patients who are resistant to drug treatment [1]. It is characterized by apparently autonomous overproduction of aldosterone [1]. As the levels of angiotensin (Ang) II – a major secretagogue of aldosterone – are low to undetectable, and the high BP and hypokalaemia are expected to blunt the renin–angiotensin system and the aldosterone secretion in primary aldosteronism [2,3], the mechanism(s) of the persistent hyperaldosteronism yet remain(s) to be elucidated.

The discovery of the (pro)renin receptor (PRR) by Nguyen and Muller [4] in 2002 was followed by the demonstration that binding of renin and/or prorenin to this receptor induces an increase in catalytic activity of renin by a non-proteolytic activation of prorenin [5]. The discovery that this binding also leads to activation of signalling pathways, as extracellular signal-regulated kinases (ERK1/2) and p38, that are involved in cell proliferation and apoptosis, led to the contention that PRR plays a role in hypertrophy and hyperplasia, and ultimately in organ damage in high BP [6].

The detection of prorenin in the plasma of patients with inappropriately raised plasma levels of aldosterone, as

Journal of Hypertension 2015, 33:1014–1022

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Received 27 July 2014 **Revised** 28 November 2014 **Accepted** 28 November 2014

DOI:10.1097/HJH.0000000000000504

heart failure and diabetes mellitus [7–11], suggested a role of prorenin not only in organ damage but also in the development of hyperaldosteronism [12], and the recent finding of prorenin in patients with primary aldosteronism well agreed with such suggestion [13].

As a streamlined approach to testing the hypothesis that prorenin may play a role in the development of hyperaldosteronism, we performed a study aimed at measuring the plasma levels of prorenin in patients with primary aldosteronism, searching for the expression of PRR gene in the normal human adrenocortical zona glomerulosa and in aldosterone-producing adenoma (APA), and investigating the functional responses induced by renin or prorenin binding to the PRR in human adrenocortical cell lines.

MATERIALS AND METHODS

Plasma levels of prorenin in patients with primary aldosteronism

For this study, the patients with primary aldosteronism were recruited at the Division of Angiology and Hypertension – Department of Internal Medicine – of the University of Lausanne, Switzerland. They were compared with matched patients with primary (essential) hypertension and to healthy normotensive individuals. We measured plasma renin activity (PRA) [14], plasma aldosterone concentration (PAC) [15], and active and total trypsin-activated renin [16,17], and calculated the plasma levels of prorenin as difference between the latter and the former.

A long-acting calcium channel blocker and/or doxazosin were prescribed to control the high BP. Treatment with mineralocorticoid receptor antagonists was withdrawn at least 6 weeks before the study. Treatment with agents that affect the renin–angiotensin–aldosterone system, as diuretics, β-blockers, angiotensin-converting enzyme inhibitors and Ang II type 1 receptor (AT1) antagonists, was withdrawn for at least 2 weeks.

Adrenal samples

Consecutive patients with a conclusive diagnosis of APA by the 'four corner approach' were recruited for this study [1]. Patients underwent laparoscopic adrenalectomy, and the excised adrenal gland was cut into halves; one was used for the pathologic examination and the other one was divided into two pieces – one rapidly frozen in liquid nitrogen and stored for the molecular studies, and the other one immediately processed for the isolation of CD56+ cells. Normal human adrenocortical tissue was obtained under sterile conditions during surgery from patients with clear cells cancer of the lower renal pole undergoing unilateral nephrectomy and ipsilateral adrenalectomy.

All patients gave an informed consent and the Ethics Committee approved use of the tissues. For space limitations, details of the methods used are given in an expanded section as online supplement (please see Supplemental Materials and methods, http://links.lww.com/HJH/A459).

Immuno-magnetic isolation of CD56+ cells

Dispersed zona glomerulosa or APA cells were obtained by sequential enzymatic digestion and mechanical disaggregation. A pure population of aldosterone-secreting cells from the normal human zona glomerulosa was obtained by immuno-separation of CD56+ cells on magnetic beads precoated with an antibody specific for neural cell adhesion molecule (NCAM) (CD56) as reported in detail [18].

HAC15 cell culture

HAC15 adrenocortical carcinoma cell line was provided by Dr W.E. Rainey. HAC15 cells were cultured in DMEM/F12 medium supplemented with 10% cosmic calf serum (HyClone, Logan, Utah, USA), antibiotics and 1% insulin/transferrin/selenium Premix (BD Biosciences, Milan, Italy).

Adrenal rat tissues

Adult male Sprague–Dawley rats (140–160 g body weight, Charles-River, Como, Italy) were housed in temperature and humidity-controlled conditions, exposed to a 12-h light/dark cycle, and given tap water *ad libitum* and a standard diet. They were sacrificed and the adrenals were immediately removed and fixed in paraformaldehyde solution 4% overnight and embedded in paraffin. The local Ethics Committee for Human and Animal Studies approved the study protocol.

Prorenin receptor gene expression

The PRR mRNA was measured with Droplet Digital PCR (QX100 Droplet Reader; Bio-Rad Laboratories, Segrate, Italy). Absolute levels of the target DNA were expressed as number of copies per microgram RNA. As a confirmatory approach, the PRR mRNA was also measured using real-time RT-PCR and Universal Probe Library in the LightCycler 480 Instrument (Roche, Monza, Italy). In these experiments standard curves were preliminarily performed to assess the reaction efficiency. The PRR expression was calculated relative to porphobilinogen deaminase (PBGD) used as an internal control.

Prorenin receptor localization

Immunohistochemistry was used to localize the PRR in 4-μm thick serial sections from paraffin blocks of normal human and rat adrenal gland tissues. Moreover, immune-separated zona glomerulosa CD56+ cells were seeded on cover slips, fixed, and processed for immunocytochemistry.

To further localize the PRR at the cellular and sub-cellular level, we used confocal and immunogold electron microscopy. Isolation of cell membranes and cytosol protein by centrifugation followed by immunoblotting experiments was also undertaken (please see Supplemental Materials and methods, http://links.lww.com/HJH/A459).

CYP11B2 gene expression

CYP11B2 expression was measured using RT-PCR and Universal Probe Library in the LightCycler 480 Instrument (Roche). Quantification was performed by the Delta Delta Ct method using the housekeeping gene PBGD as an internal control.

ERK1/2 phosphorylation

To investigate the functional role of the PRR activation, we measured ERK1/2 phosphorylation by immunoblotting in HAC15 cells exposed to 100 nmol/l Ang II (Sigma, Milan,

Italy), 50 nmol/l prorenin (Cayman, Milan, Italy), 50 nmol/l renin (Cayman) with or without 30-min pre-incubation with the angiotensin AT1 receptor irbesartan (5 μmol/l) or the direct renin inhibitor Aliskiren (5–50 μmol/l) (Novartis, Basel, CH) (please see Supplemental Materials and methods, http://links.lww.com/HJH/A459).

Statistical analysis

Results were expressed as mean ± standard error (SE) of at least three separate experiments in which each sample was assayed in triplicate. Group differences were analysed by *t* test or one-way analysis of variance (ANOVA), followed by Bonferroni's correction for multiple group comparison, or Mann–Whitney or Kruskall–Wallis tests, when distribution was not normal. Statistical significance was set at *P* value less than 0.05. Analyses were performed with SPSS for Mac (version 20.0; SPSS Italy Inc., Bologna, Italy).

RESULTS

Plasma levels of prorenin in patients with primary aldosteronism

The plasma levels of active renin, total renin, prorenin and plasma aldosterone were measured in randomly selected patients with primary hypertension ($n = 13$), patients with surgically confirmed primary aldosteronism ($n = 12$) and healthy normotensive individuals ($n = 16$). The primary aldosteronism patients had low levels of both PRA (not shown) and active renin, and raised levels of PAC, which translated into a high aldosterone-to-renin ratio (ARR) (not shown), as compared to both primary hypertension patients and healthy normotensive individuals (Fig. 1). Active and total renin showed differences among groups that parallel those of PRA. Of note, the plasma levels of prorenin were clearly detectable in all groups, including the primary aldosteronism patients.

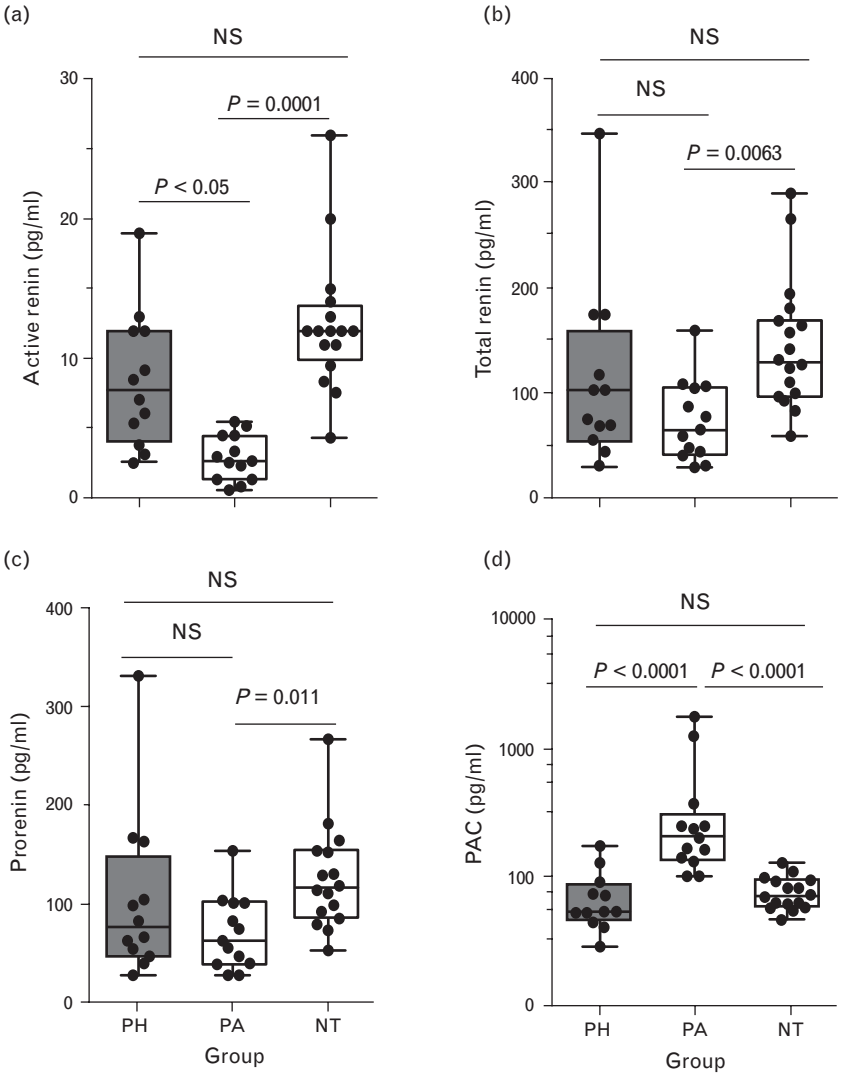

FIGURE 1 Plasma levels of active renin (panel a), total renin (panel b), prorenin (panel c), and plasma aldosterone (panel d) in patients with primary hypertension (PH, $n = 13$), primary aldosteronism (PA, $n = 12$), and in normotensive (NT, $n = 16$) individuals.

Prorenin receptor gene and protein expression in the adrenal gland

Absolute quantification of PRR gene showed similar expression (P = NS for comparison) in APAs {n = 11, 29 550 [95% confidence interval (CI) 19 185–39 914] copies per μg RNA} and in the normal human adrenal cortex [n = 8, 24 650 (95% CI 24 275–27 585)].

Prorenin receptor mRNA levels were also high in HAC15 cells (54 500, 95% CI 44 250–56 500). RT-PCR (please see Supplemental Materials and methods, http://links.lww.com/HJH/A459) confirmed the high expression of PRR in the normal adrenocortical tissue, in APA and the cell line, the PRR-to-PBGD ratio being 15, 12.3 and 5.0, respectively.

Immunohistochemistry confirmed the PRR expression in the normal adrenal gland of both humans and rats: staining was marked in the sub-capsular zone adrenal cortex and the medulla (Fig. 2, panels a and c). In the human adrenal gland the expression of PRR was scarce in the zona fasciculata, even more so than in the rat (Fig. 2, panels a–d).

Immunocytochemistry on CD56+ cells isolated from normal zona glomerulosa (Fig. 2, panel e) or from APA (Fig. 2, panel e) confirmed an intense PRR staining in the aldosterone-secreting cells. The specificity of the reaction was confirmed in each experiment by the lack of staining upon omission of the primary antibody and also after its precipitation with excess antigen.

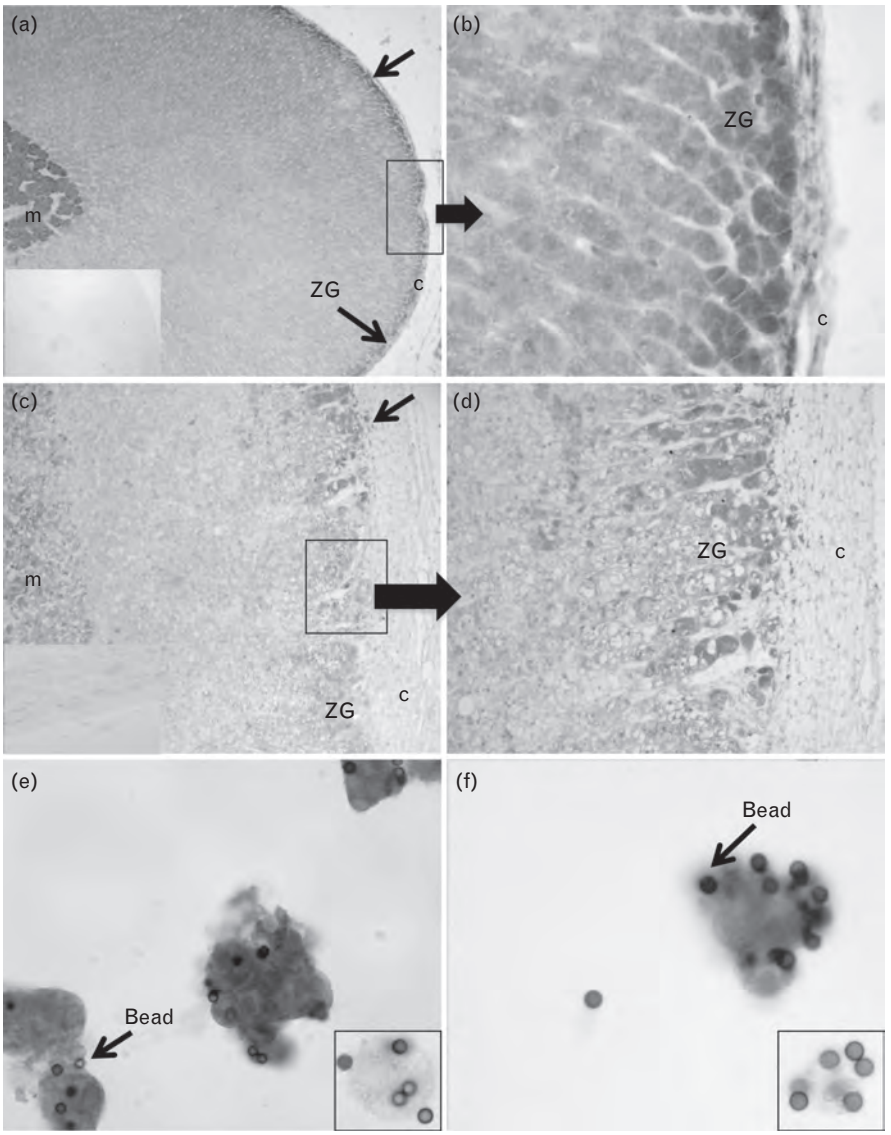

FIGURE 2 Prorenin receptor (PRR) expression in the adrenal rat (panels a and b) and normal human (panels c and d) gland. Sub-capsular zone (arrow); medulla (m); capsule (c); zona glomerulosa (ZG). Bottom insets show lack of immunostaining when the primary antibodies were omitted. The magnification of the middle insets of panels a and c clearly shows the marked immunostaining in the sub-capsular zone and ZG. The immunostaining is markedly evident in the sub-capsular zone and progressively weakens throughout the zona fasciculata. A marked staining is evident also in the medulla (panels a and c). Magnification: panel A, 5×; panels B–D, 40×. Immunostaining for the PRR in CD56+ cells immune-separated from normal human ZG (panel e) and APA (panel f). The arrows indicate the magnetic beads used for the immuno separation of the CD56+ cells. Omission of the primary antibody provided no specific immunostaining (insets). 40× magnification. APA, aldosterone-producing adenoma.

Intracellular localization of prorenin receptor

Immunoblotting experiments evidenced a band of the molecular weight expected for the PRR in HAC15; furthermore, it suggested localization of the PRR in the plasma membrane (Fig. 3, panel a).

Confocal microscopy studies confirmed that PRR was mainly, albeit not exclusively, located in the membrane in HAC15 cell line. Moreover, it showed that PRR co-localized in part with the neural cell adhesion molecule (NCAM) CD56 (Fig. 3, panel b). Immuno-gold electron microscopy experiments allowed a more precise sub-cellular localization of the PRR in zona glomerulosa CD56+ cells (Fig. 3, panels c–f), with detection of the PRR staining in the nuclei, mitochondria and Golgi's vesicles.

Prorenin receptor-mediated CYP11B2 transcription

Both 50 nmol/l prorenin and renin markedly enhanced CYP11B2 gene expression (Fig. 4) in HAC15 cells, and the increase was prevented by both irbesartan and 5–50 μmol/l aliskiren.

Prorenin receptor-mediated ERK1/2 phosphorylation

Exposure of HAC15 cells to 100 nmol/l Ang II, 50 nmol/l renin or 50 nmol/l prorenin increased ERK1/2 phosphorylation, compared to the vehicle-treated cells (control). ERK1/2 phosphorylation induced by renin or Ang II, but not by prorenin, was abrogated by pre-incubation with

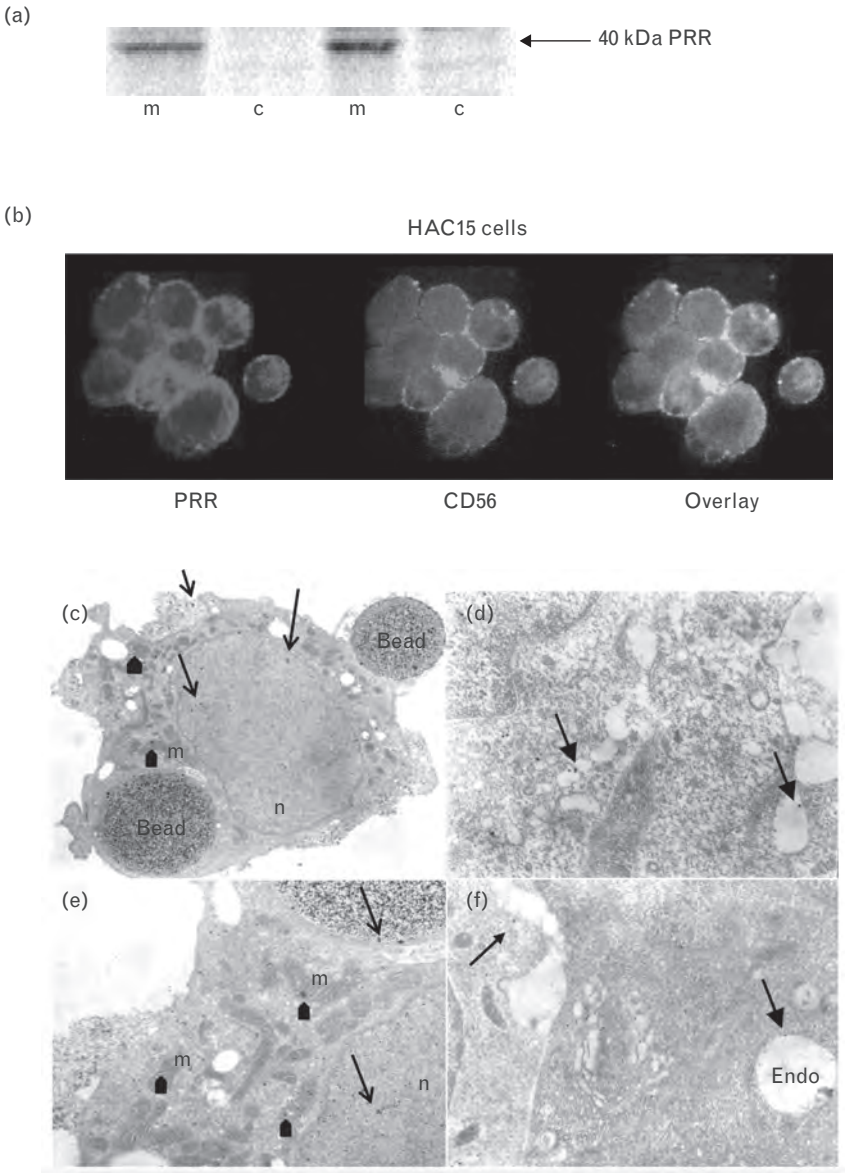

FIGURE 3 Immunoblotting of membrane (m) and cytosol (c) fractions from HAC15 cells (panel a). The band of 40 kDa corresponds to the expected PRR molecular weight. Confocal microscopy (panel b). Localization of PRR (originally labelled in red) at the membrane level in HAC15 cell line, similarly for the ZG cell marker CD56 (originally labelled in green). The overlay of PRR and CD56 (originally visualized as yellow) shows colocalization of PRR and CD56 proteins. No immunofluorescence was seen by omitting the primary antibodies or by prior immunoprecipitation of them (not shown). Immunogold electron microscopy of CD56+ cells immunoseparated from normal ZG. PRR expression at the cell membrane (short arrow), nucleus (n, long arrows), mitochondria (m, arrowheads), and endoplasmic vesicles or endosomes (Endo, arrows with fill heads, panel c). Magnification: panel c, 7000×; panel d, 30 000×; panel e, 15 000×; panel e, 20 000×. PRR, prorenin receptor; ZG, zona glomerulosa.

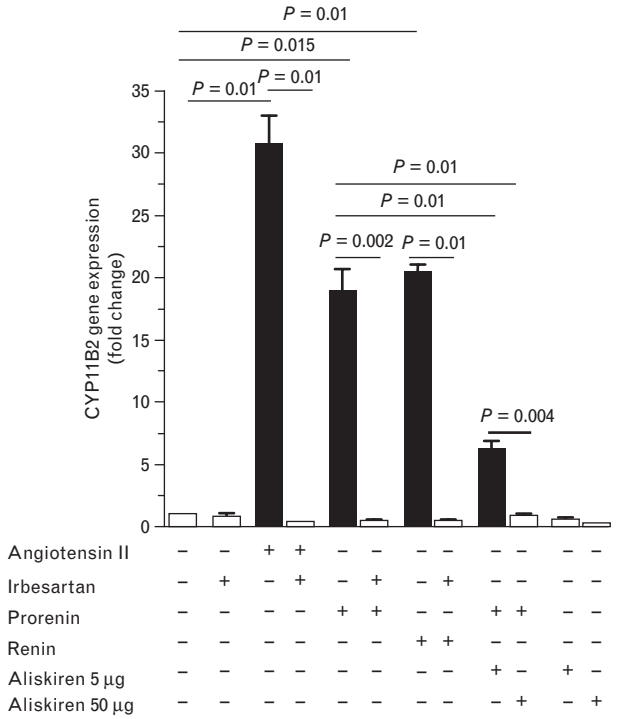

FIGURE 4 CYP11B2 expression in HAC15 cells after stimulation with 100 nmol/l angiotensin II, 50 nmol/l prorenin, or 50 nmol/l renin, in presence or absence of 5 μmol/l irbesartan, 5 μmol/l, or 50 μmol/l aliskiren. Whenever not indicated, difference between treatments was not significant. Results were obtained from two separate experiments in which each sample was assayed in duplicate.

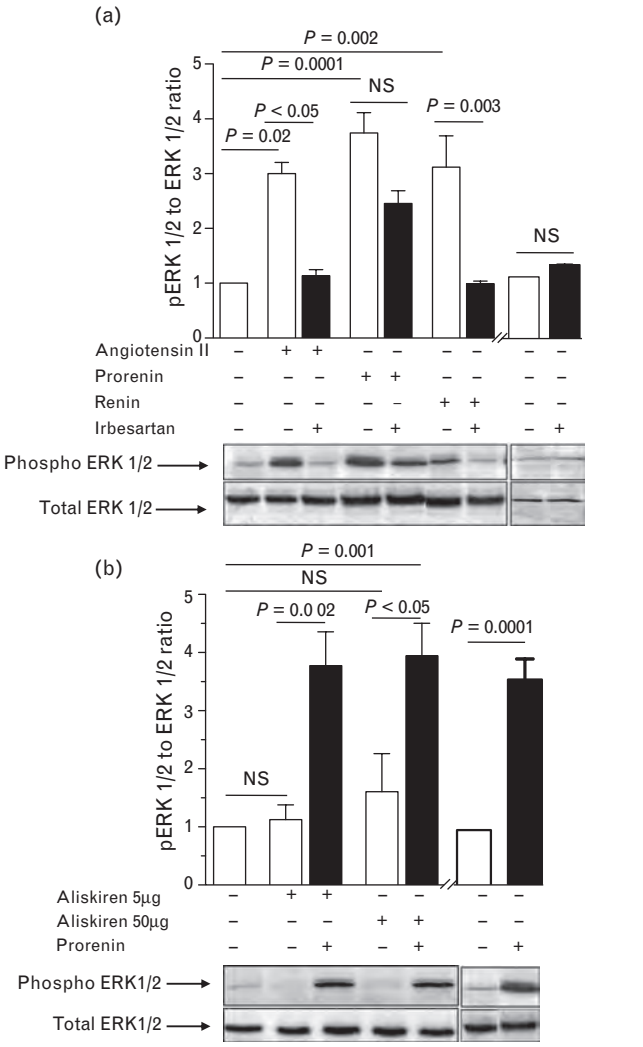

FIGURE 5 ERK1/2 phosphorylation in HAC15 cells after stimulation with 100 nmol/l Ang II, 50 nmol/l prorenin, or 50 nmol/l renin, in presence or absence of 5 μmol/l irbesartan (panel a), and 5 μmol/l or 50 μmol/l aliskiren (panel b). ERK1/2 phosphorylation is expressed as the ratio of phosphorylated to total ERK1/2 protein in both panels. Whenever not indicated, difference between treatments was not significant. Results were obtained from three separate experiments in which each sample was assayed in triplicate, and ANOVA followed by Bonferroni's correction tests for multiple group comparison was used for the analysis. Ang II, angiotensin II; ANOVA, analysis of variance.

5 μmol/l irbesartan (Fig. 5, panel a). Pre-treatment with 5–50 μmol/l aliskiren, which *per se* did not affect phosphorylation, left unaffected ERK1/2 phosphorylation induced by prorenin (Fig. 4, panel b).

DISCUSSION

We herein show that prorenin is detectable in plasma of patients with surgically confirmed primary aldosteronism, even though its levels paralleled the low levels of active renin (Fig. 1). This observation agrees with findings in a smaller cohort [13]. Along with the detection of the PRR in human adrenocortical tissues (see later), it suggests that prorenin plays a role in the adrenocortical cells, as shown by the marked activation of CYP11B2 transcription.

A vast array of tissues, including heart, brain, placenta, liver, and pancreas, were found to express the PRR [19], but information on the human adrenal gland is limited [20]. Yamamoto *et al.* [20] recently described the PRR in the human adrenals and adrenocortical tumours including APAs, but functional data lack, which is somewhat surprising given the key role of this gland in BP regulation. We herein provide evidence of a high level of the PRR mRNA in the adrenal cortex of the normal human adrenal gland and APA. Moreover, using immunoblotting, immunohistochemistry, and confocal microscopy, we show that the PRR mRNA was translated into a functional protein particularly in the sub-capsular zona glomerulosa, the main site of aldosterone production (Fig. 2 panels a–d), suggesting that this regulates aldosterone production or favours the transformation of the normal adrenocortical cells into APA cells. The finding of such a high PRR expression also in aldosterone-producing adenoma, a common cause of human hypertension, supports this contention. Further support to this contention was provided by the detection of the PRR also in CD56+ cells, entailing aldosterone-producing cells isolated from both the normal zona glomerulosa and from APA (Fig. 2 panels e and f).

Sub-cellular localization of prorenin receptor

Confocal microscopy along with immunoblotting of the cytosol and membrane fractions of HAC15 cells showed that the PRR is mainly expressed in the cell membrane where it co-localized with the adhesion molecule CD56.

Apart from confirming these findings, the ultrastructural analysis of a pure population of human zona glomerulosa cells evidenced a sub-cellular localization of the PRR in the nuclei, mitochondria, and Golgi's vesicles, similar to that reported also in the kidney [21–23], and consistently with the aforementioned association of a PRR fragment with V-ATPase [4].

Both prorenin and renin trigger CYP11B2 expression via angiotensin II activation of the AT1 receptor

HAC15 cells exposed to either prorenin or renin exhibited a marked increase of CYP11B2 (aldosterone synthase) expression. This huge increase was abolished by pre-

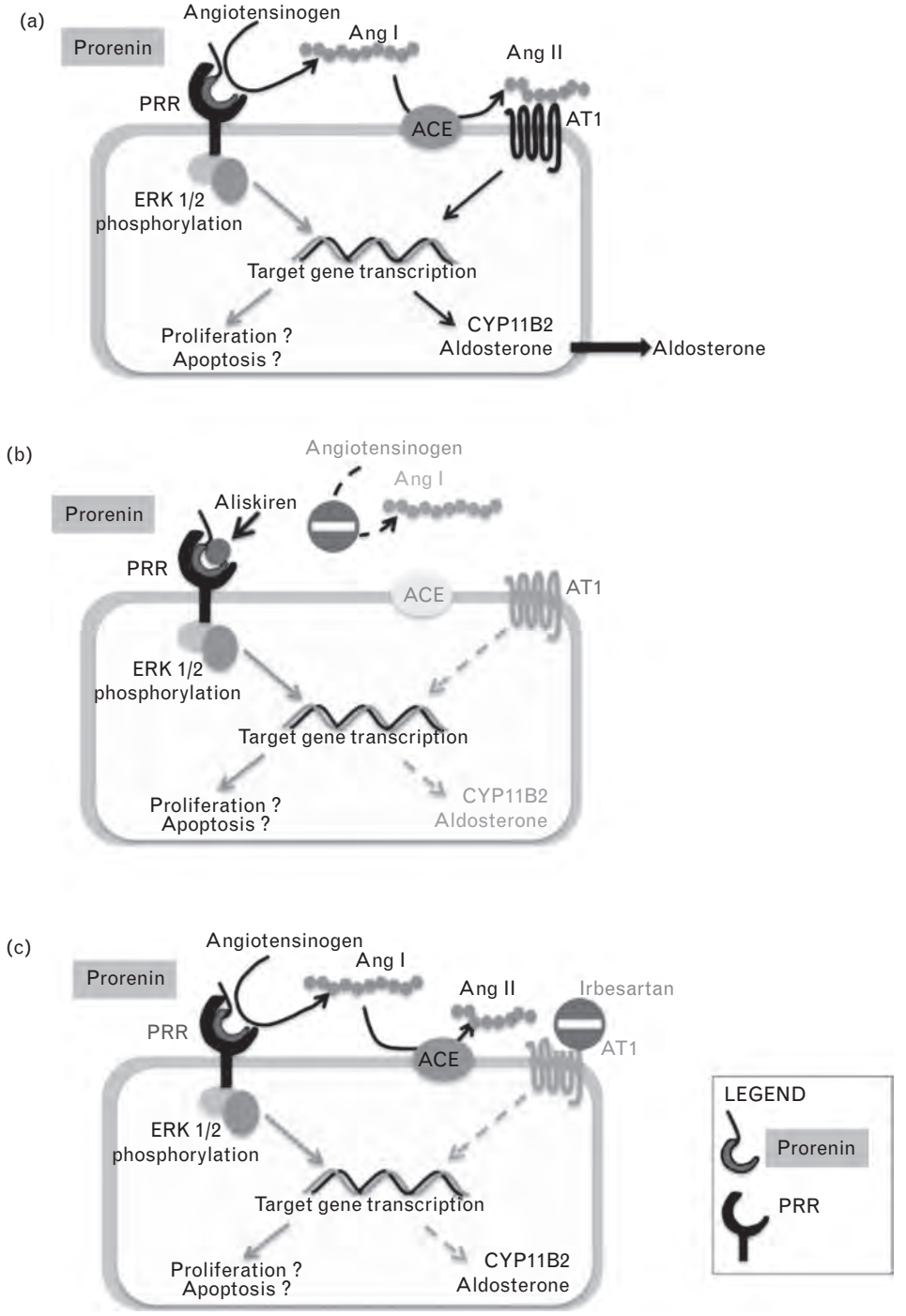

FIGURE 6 Proposed scheme of the action of prorenin in the human adrenocortical cells HAC15. The binding of prorenin to the PRR triggers the generation of angiotensin (Ang) I, with ensuing formation of Ang II and activation of the angiotensin AT1 receptor leading to transcription of CYP11B2 and aldosterone production (panel a). In the presence of aliskiren (panel b), Ang I generation is prevented, thus precluding activation of AT1 and therefore transcription of CYP11B2. The same result is induced by irbesartan (panel c). At variance, when bound to the PRR, prorenin activates ERK1/2 phosphorylation and downstream target genes transcription in a way that was not inhibited by aliskiren (panel b). Ang, angiotensin; AT1, angiotensin II type 1 receptor; PRR, prorenin receptor.

treatment with either irbesartan or aliskiren, thus indicating that aldosterone synthesis was strictly dependent on Ang II generation and its activation of the AT1 receptor (Fig. 6).

Prorenin activates ERK1/2 phosphorylation independently of angiotensin II generation in HAC15

The PRR is currently viewed as a multifunctional receptor capable of binding both renin and prorenin: the binding of renin increases (by 4–5-fold) its catalytic activity and that of prorenin confers an enzymatic activity comparable to that of renin [5]. Activation by either agonist triggers intracellular signalling and enhances cell proliferation via activation of the mitogen-activated protein (MAP) kinases (MAPKs) ERK1/2 and phosphorylation of Ets-like gene (Elk) [24–27]. In the heart and kidney these effects were found to occur independently of Ang II generation [23,27–30].

We found that both renin and prorenin induce phosphorylation of ERK1/2 in HAC15 cells likewise Ang II (Figs 4 and 5). This is at variance with the negative findings obtained using agonist concentration (10 nmol/l renin and 2.5 nmol/l prorenin) [31] 5 and 20-fold lower than those used in our experiments (50 nmol/l renin and 50 nmol/l prorenin). Since (pro)renin is synthesized in the adrenal gland, the concentrations in the adrenal tissue are presumably much greater than those in the plasma; therefore ERK1/2 phosphorylation is likely to occur *in vivo*.

We found that prorenin-mediated ERK 1/2 phosphorylation was not abolished by irbesartan in the HAC15 cells (Fig. 3a), indicating that it occurs, at least in part, via Ang II-independent mechanisms. By contrast, renin-mediated ERK1/2 activation was abrogated by irbesartan; hence, it depends on Ang II binding to the AT1 receptor.

The direct (pro)renin inhibitor aliskiren was unable to prevent prorenin-induced ERK1/2 phosphorylation in HAC15 cells, thus further supporting the notion that prorenin-induced ERK1/2 phosphorylation is not dependent on Ang II generation in HAC15 cells. Similar findings were reported in other cell types, as the human embryonic kidney cells HEK293 [23] and human podocytes [32], indicating that the downstream activation of PRR is due to the direct action of prorenin on the PRR. Given that under physiologic conditions the prorenin-to-renin ratio is high [33], the direct activation of the ERK-induced by binding of prorenin to the PRR could dominate over the renin–Ang II-induced AT1 receptor-mediated ERK phosphorylation in the adrenal cortex. Conditions known to be associated with very high prorenin levels, as diabetes mellitus, could be expected to lead to enhanced activation of activation of prorenin via binding to PRR and/or to direct PRR signalling activation. Hence, in contrast to aldosterone synthesis, which is strictly dependent on Ang II generation and AT1 receptor activation, in HAC15 cells, ERK phosphorylation is driven by prorenin binding to the PRR, and cannot be inhibited by aliskiren (Fig. 6).

Limitations and strengths of the study

Use of an adrenocortical cell line might be seen as a limitation of our study, but immortalized cells deriving from normal adrenocortical zona glomerulosa are currently unavailable. However, the finding that prorenin-induced ERK1/2 phosphorylation, either directly via PRR activation or indirectly via Ang II generation, was consistent and reproducible.

We also exploited the use of an immuno-separation technique that allowed isolation of CD56+ cells, a pure population of aldosterone-secreting cells, from both the normal human adrenal gland and from APA [18]. These cells, like HAC15 cells, were found to markedly express PRR at both the mRNA and the protein level.

In summary, we found that: prorenin is measurable in plasma of primary aldosteronism patients, and the PRR is markedly expressed in the human and rat adrenocortical zona glomerulosa, as well as in APA, and in the HAC15 cell line. The expression of a functional PRR protein occurred at both intracellular sites, and in the cell membrane level, where the PRR partially co-localizes with the adhesion molecule CD56.

Both prorenin and renin activate aldosterone synthesis through an effect involving Ang II generation and AT1 receptor activation in the HAC15 cells. Prorenin also induced phosphorylation of ERK1/2, but this effect was independent of the AT1 receptor blockade and was not prevented by aliskiren. Direct renin/prorenin receptor blockers remain therefore to be tested, but unfortunately this is not possible yet in the clinical conditions.

ACKNOWLEDGEMENTS

Funding: FORICA (The FOundation for advanced Research In Hypertension and CArdiovascular diseases www.forica.it), the Società Italiana dell'Ipertensione Arteriosa and the University of Padua to GPR and TMS.

Conflicts of interest

There are no conflicts of interest.

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Reviewers' Summary Evaluations

Referee 1

Recarti *et al.* propose that prorenin, through binding to the (pro)renin receptor, induces aldosterone synthesis in adrenocortical cells, and that this contributes to the high aldosterone levels in primary aldosteronism. Here it should be noted that, as confirmed in this study, prorenin levels in primary aldosteronism are low, and easily >1 million-fold below the levels that upregulated aldosterone synthase in vitro. Moreover, recent in-vivo studies strongly support (pro)renin receptor functions that are entirely prorenin-independent. Given the observation by Recarti *et al.* that renin inhibition prevented the effects of prorenin, a direct prorenin-(pro)renin receptor-aldosterone link remains uncertain.

Referee 2

This is a very interesting basic research study evaluating the role of prorenin in patients with primary aldosteronism. The authors conducted a well designed and highly sophisticated study demonstrating the presence of circulating prorenin in patients with primary aldosteronism (PA), the expression of prorenin receptors in the adrenal gland (PA, normals, animals), and the downstream activation of intracellular pathways with prorenin, suggesting a functional role of prorenin in patients with PA. Prorenin induced activation was not inhibited by angiotensin receptor blockers or aliskiren underlining the need to evaluate direct prorenin receptor inhibitors to unveil the impact of prorenin effects in PA and other disease conditions.