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**The prorenin receptor in the adrenal gland:
expression, localization, signalling pathway and potential role in
primary aldosteronism**

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A te che hai creduto con tanto coraggio nella ricerca

A te che mi hai insegnato a non mollare mai

A te che mi hai dedicato con immenso amore tutta la tua vita

A te, Mamma, io dedico questo traguardo

Ti amerò per sempre

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RIASSUNTO

L'iperaldosteronismo primario (PA) colpisce l'11.2% dei pazienti ipertesi inviati ai centri dell'Ipertensione. Una quota considerevole di essi presenta un'iperplasia bilaterale del surrene, nota anche come iperaldosteronismo idiopatico, che richiede una terapia medica per tutta la vita. Nonostante l'alta prevalenza di questa patologia i meccanismi che ne stanno alla base sono tuttora sconosciuti. Tuttavia dati aneddotici suggeriscono che nel PA, a differenza dei livelli plasmatici di renina che sono ridotti o indosabili, i livelli di prorenina potrebbero essere aumentati. Ciò suggerisce la possibilità che la prorenina giochi un ruolo nella fisiopatologia del PA.

Nel 2002 la scoperta del recettore della prorenina (PRR) ha aperto una nuova finestra sulla fisiopatologia del sistema renina-angiotensina: il PRR può legare sia la renina che la prorenina inducendo, rispettivamente, un aumento nell'attività catalitica della renina e un'attivazione non proteolitica della prorenina. Fino ad allora la prorenina era considerata solo il precursore inattivo della renina. La preprorenina viene convertita a prorenina nelle cellule juxtaglomerulari renali. Il clivaggio del prosegmento amino-terminale della prorenina permette l'esposizione del sito attivo nei granuli secretori generando renina. Tuttavia, la maggior parte (75%) della prorenina viene secreta costitutivamente; pertanto i livelli plasmatici di prorenina nell'uomo sono 10 volte maggiori di quelli della renina. Essi sono inoltre aumentati in diverse condizioni, tra cui il diabete mellito, dove potrebbero svolgere un ruolo fisiopatologico.

Il legame di renina e prorenina al PRR attiva vie di signalling quali MAP (mitogen activated protein) chinasi-ERK 1/2 (extracellular signal regulated kinase) e p38 indipendentemente dalla generazione di angiotensina II (Ang II). Queste vie sono implicate nella proliferazione e apoptosi, il che ha fatto ipotizzare che renina e prorenina possano indurre ipertrofia e iperplasia e quindi danno d'organo ed eventi cardiovascolari *via* PRR.

In questa ricerca abbiamo valutato l'espressione genica e proteica del PRR nel surrene e scoperto elevati livelli di espressione di mRNA del PRR negli APA, nella corteccia surrenalica umana normale e in due linee cellulari di carcinoma corticosurrenalico umano: H295R e HAC15. Abbiamo quindi investigato la

presenza e localizzazione del PRR a livello proteico mediante immunoistochimica su surrene di ratto e umano e immunocitochimica. Ciò ha evidenziato un marcato immunostaining del PRR nel surrene, a livello midollare e sub capsulare, e in cellule di ZG umana secernenti aldosterone ottenute mediante immunoseparazione.

Per localizzare il PRR, sono stati condotti esperimenti di immunoblot su frazioni di cytosol e membrana di cellule HAC15 e H295R ed esperimenti di microscopia confocale. Questi risultati hanno mostrato che il PRR è principalmente, ma non esclusivamente, localizzato a livello di membrana ove colocalizza parzialmente con la molecola di adesione CD56.

Attraverso esperimenti di microscopia confocale immunogold abbiamo poi indagato la localizzazione subcellulare del PRR e scoperto che il PRR è localizzato anche nel nucleo, nei mitocondri e nelle vescicole del Golgi.

Abbiamo quindi utilizzato H295R e HAC15 per studiare la rilevanza funzionale di questo recettore. La stimolazione con angiotensina II [100 nM], renina [50 nM] e prorenina [50 nM] ha indotto fosforilazione di ERK 1/2. In presenza dell'antagonista del recettore dell'angiotensina II (AT1) irbesartan [5 μM], la fosforilazione indotta da renina e angiotensina II era abolita in entrambe le linee cellulari mentre quella indotta dalla prorenina era abolita solo nelle H295R.

Questi risultati suggeriscono un ruolo del PRR nella proliferazione, differenziamento e apoptosi del corticosurrene e quindi un possibile ruolo funzionale di questo recettore nella fisiopatologia dell'iperaldosteronismo primario.

ABSTRACT

Primary aldosteronism (PA) comprises about 11% patients referred to specialized centers for Arterial Hypertension. A considerable part of them carry a bilateral adrenocortical hyperplasia, also known as, idiopathic hypertension, which require life-long medical treatment. Notwithstanding this high prevalence rate the mechanisms leading to PA are unknown. However, anecdotal reports suggest that prorenin levels could be increased in PA, although the low or undetectable levels of active plasma renin, plasma prorenin levels could be increased. This suggests a possible role of prorenin in the pathophysiology of PA.

The discovery of the prorenin receptor (PRR) in 2002, show a new way forward to the pathophysiology of the renin-angiotensin system: PRR can bind renin and prorenin inducing respectively an increase in renin catalytic activity and a non-proteolytically activation of prorenin. Till that discovery, prorenin was only seen as the inactive precursor of renin. Preprorenin is converted to prorenin in the juxtaglomerular cells of the kidney. The cleavage of the amino terminal 43-amino acid prosegment allows exposure of the active site in the secretory granules where generate renin. However the majority (75%) of prorenin is secreted constitutively; therefore The prorenin levels of human blood plasma are approximately 10-fold higher than those of renin. Further, they are increased in several conditions, including diabetes mellitus, where they could play a pathophysiologic role.

PRR binding triggers activation of the mitogen activated protein (MAP) kinase–extracellular signal regulated kinase (ERK)1/2 and p38 signalling pathway independent from a possible generation of angiotensin II (Ang II).

Activation of these signalling pathways, that are associated with cell proliferation and cell death has led to hypothesize that renin and prorenin could lead, *via* PRR, to hypertrophy and hyperplasia and ultimately to organ damage and cardiovascular events.

In this study we first evaluated the gene and protein expression of PRR in adrenal gland and founded high expression of the PRR mRNA in APA, in human normal adrenal cortex and two human adrenocortical carcinoma cell lines: H295R and HAC15.

Then, we sought for the expression of the PRR at the protein level with immunohystochemistry experiments on rat and human normal adrenal gland and with immunocytochemistry experiments. These experiments revealed a strong immunostaining for PRR in the adrenal gland at medulla and sub capsular level, and in aldosterone-secreting cells from the normal human ZG obtained by immunoseparation.

In order to localize PRR we carried out immunoblot experiments on HAC15 and H295R cytosolic and membrane fractions and confocal microscopy experiments. These results showed that, PRR is mainly, but not exclusively, localized at the membrane level where partially colocalizes with the adhesion molecule CD56.

We then investigated the subcellular localization of the PRR by immuno-gold electron microscopy experiments, and found that PRR is localized also in the nucleus, mitochondria and Golgi's vesicles.

We used H295R and HAC15 cell lines to investigate the functional relevance of this receptor. Stimulation with angiotensin II [100 nM], renin [50 nM] and prorenin [50 nM] induced ERK 1/2 phosphorylation.

The renin and angiotensin II induced phosphorylation was abolished in presence of the angiotensin II (AT1) receptor antagonist irbesartan [5 μM] in both cell lines, whereas the phosphorylation induced by prorenin was abolished only in the H295R cells.

These results suggest a role of PRR in proliferation, differentiation, and apoptosis of adrenal cortex and, therefore, a functional role of PRR in the pathophysiology of PA.

INTRODUCTION

Primary aldosteronism and Adenoma producing aldosterone

Primary aldosteronism (PA) is the most common endocrine form of secondary arterial hypertension among the patients referred to specialized centers for Arterial Hypertension (Rossi et al., 2006) and among patients with resistant hypertension (Douma et al., 2008). In PA he increase of blood pressure, the consistent suppression of the renin-angiotensin system and the common hypokalaemia would be expected to blunt aldosterone secretion, which instead remains paradoxically increased (Rossi and Medscape, 2011). Hence, the mechanisms responsible of the persistent aldosteronism have puzzled investigators for decades and yet remain largely unknown.

The first cases of PA was reported by Litynski (LITYNSKI, 1953), but Conn was the first to well characterize the disorder (CONN, 1955); in 1956 he reported a 34-year-old woman case: the patient presented hypertension, intermittent paralysis, hypokalaemia and metabolic alkalosis. Further biochemical analyses detected increased activity of urinary salt retaining corticoid hormone. The patient was cured by removal of a benign adrenal adenoma(CONN and LOUIS, 1956).

The main causes of PA entail aldosterone-producing adenoma (APA) and idiopathic hyperaldosteronism (IHA). Other causes of PA include aldosterone producing ovarian tumours, aldosterone-producing carcinomas (APCs) (Dluhy and Lifton, 1999; Seccia et al., 2005) and familial hyperaldosteronism forms. The differential diagnosis between APA and IHA is crucially important for the choice of treatment: in APA patients, adrenalectomy can cure (55%), or markedly ameliorate hypertension (Stowasser et al., 1994), whereas patients with IHA can be pharmacologically treated with mineralocorticoid receptor antagonists (Rossi, 2006).

In 2006 the Primary Aldosteronism Prevalence in Italy (PAPY) Study showed that PA involves 11.2% of 1125 consecutive patients with newly diagnosed hypertensive patients referred to hypertension (HT) centers, which was leaded by a prevalence respectively of 4.8% and 6.4% of all patients for aldosterone-

producing adenoma (APA) and idiopathic hyperaldosteronism (IHA) (Rossi et al., 2006).

Renin and its precursor Prorenin

Renin is an aspartyl protease essential for the control of blood pressure: its only known substrate is angiotensinogen that is cleaved in at the N terminus site for the transformation to angiotensin I (Ang I), which is then converted, by soluble or endothelian cell-associated angiotensin converting enzyme (ACE), in angiotensin II (Ang II). In the heart, Ang I is mainly converted by chymase (Wolny et al., 1997). Ang II acts on vascular smooth muscle cells as a potent vasoconstrictor via Ang II receptors that are widely distributed (Nguyen et al., 2002). Renin is synthesized as prorenin, an inactive proenzyme that contains a prosegment that consists in an additional 43-ammino acid N-terminal fragment. Maturation of prorenin into active renin takes place exclusively in the juxtaglomerular cells of the kidney (Sealey et al., 1977). The juxtaglomerular epithelioid cells, located in the walls of renal afferent arterioles, at the entrance of the glomerular capillay network, are the main source of renin in the body. Renin is synthesized as preprorenin, which is converted to prorenin upon insertion into the endoplasmatic reticulum. The majority (75%) of prorenin is secreted constitutively, while the remainder is targeted to dense core secretory granules. In these granules, an acidic pH is created to optimize the activity of the proteases (cathepsin B, prohormone convertases) that processes prorenin to renin by cleavage of the amino terminal 43-amino acid prosegment, which allows exposure of the active site of renin (Hsueh and Baxter, 1991; Krop and Danser, 2008). Prorenin can be activated in two ways: proteolytic or non-proteolytic (Danser and Deinum, 2005). Proteolytic activation is irreversible because it involves removal of the propeptide. Non-proteolytic activation of prorenin is reversible, presumably due to the unfolding of the propeptide from the enzymatic cleft. Non-proteolytic activation can be induced by exposure to low pH (pH 3.3) or cold (4 ºC) (Danser and Deinum, 2005). Non-proteolytically activated prorenin is enzymatically active and can be recognized by monoclonal antibodies that are specific for the active site. Kinetic

studies of the non proteolytic activation process have indicated that an equilibrium exists between the closed (inactive) and open (active) forms of prorenin. The inactivation step is highly temperature dependent and occurs very rapidly at neutral pH and 37 ºC. Consequently, under physiological conditions, <2% of prorenin is in the open, active form, i.e. displays enzymatic activity, and >98% is closed and inactive. Prorenin and renin levels are highly correlated but do not always change in the same way. Acute stimuli of renin will not affect prorenin levels, whereas chronic stimuli (like a decrease in Ang II) increase both renin and prorenin. This suggests that renin is stored as active enzyme and is released immediately upon stimulation of the juxtaglomerular apparatus. In contrast, prorenin release occurs constitutively and not stored. Chronic stimulation causes more prorenin to be converted to renin, leading to an increased renin/prorenin ratio in plasma (Schalekamp et al., 2008). However, some exceptions to this rule exist. A well-known example is diabetes mellitus complicated by retinopathy and nephropathy (Luetscher et al., 1985). In microalbuminuric diabetic subjects, prorenin is increased out of proportion to renin. This increase starts before the occurrence of microalbuminuria, and the prorenin level, in conjunction with the glycated haemoglobin level, might even be used to predict the occurrence of later microalbuminuria (Deinum et al., 1999). The reason for the elevated prorenin levels in diabetic subjects is unknown. One possibility is that prorenin originates outside the kidney. Indeed, it is prorenin, and not renin, that remains detectable in blood following a bilateral nephrectomy, although its levels are lower than in normal subjects (Danser et al., 1998; Krop et al., 2008). This suggests that, although the kidney is the main, if not the only, source of renin in the body, there are other tissues releasing prorenin into the circulation. For instance, pregnant women have high plasma prorenin levels, derived from the ovaries (Derkx et al., 1987). The function of this prorenin is unknown, as is the function of prorenin in amniotic fluid, in which prorenin was discovered. The reproductive organs, together with the adrenal, eye, and submandibular gland, are sites of extrarenal renin gene expression (Krop and Danser, 2008). For reasons that are not understood, these tissues exclusively release prorenin.

The renal vasodilator response to captopril in diabetic subjects correlated better with plasma prorenin than with plasma renin (Stankovic et al., 2006). Possibly,

therefore, prorenin (and not renin) is responsible for tissue angiotensin generation. Obviously, this would require local conversion prorenin to renin, for which no evidence exists (Lenz et al., 1991). In support of this concept, however, transgenic rodents with inducible prorenin expression in the liver display increased cardiac ang I levels, cardiac hypertrophy, hypertension and/or vascular damage without evidence for increased renin or angiotensin levels in blood (Peters et al., 2008; Prescott et al., 2002; Veniant et al., 1996). Interestingly, increased tissue ang I formation occurred even when expressing a non-cleavable prorenin variant, i.e. a prorenin variant that cannot be enzymatically cleaved to renin (Methot et al., 1999). Based on these data, it seems that tissues are capable of sequestering prorenin, e.g. via a receptor-dependent mechanism, and that this binding results in prorenin non-proteolytic activation. The PRR is the most promising candidate for the tissue uptake of circulating renin/prorenin.

The prorenin levels of human blood plasma are approximately 10-fold higher than those of renin. Renin and prorenin have been detected in plasma in several conditions, including diabetes mellitus, where they could play a pathophysiologic role (Sealey et al., 2005a; Sealey et al., 2005b; Wilkinson-Berka and Campbell, 2009). However, whether they are involved in the pathophysiology of PA, which is characterized by suppression of the renin angiotensin system remained uncertain.

The discovery of the PRR and its biochemistry

The discovery of the prorenin receptor (PRR) by Nguyen's group in 2002 has added a further level of complexity to our knowledge of renin-angiotensin system. The messenger RNA (mRNA) of the receptor is 2034 bp long (GenBank accession number AF291814), has a long 3' untranslated region and no alternative splicing product. The mRNA encodes a 350-amino acid transmembrane protein with no homology with any known protein based on the nucleotide and the amino-acid sequence and with a single transmembrane domain at the C-terminus (Nguyen et al., 2002). The degree of homology between human, rat, and mice *PRR* is about 95% for the nucleotide sequence and over 80% at the amino-acid

level, indicating an extremely conserved protein. A multispecies protein sequence comparison reveals homologues to the human receptor in many species, including rat, mouse, chicken, frog, zebrafish, mosquito, and drosophila, and in species as remote from humans as *C. elegans* and the bacteria, *Ehrlichia chaffeensis*. The highest homology is in the transmembrane and cytoplasmic regions, pointing to an important function for this fragment of PRR (Bader, 2007; Burckle and Bader, 2006; Wakeel et al., 2009). Indeed, inactivation of the prorenin receptor gene before the end of embryogenesis is lethal in zebrafish (Amsterdam et al., 2004). The extracellular, prorenin-binding and renin-binding part of the receptor retains high similarity between mammals only (Burckle and Bader, 2006). This part of the prorenin receptor might therefore have developed later in evolution, for instance, because there are advantages upon its binding with prorenin or renin (Bader, 2007).

Homologies in the tertiary structure have not yet been determined owing to lack of knowledge of the crystal structure of PRR due to the difficult to generate recombinant PRR in native form (Nguyen and Danser, 2008; Nguyen and Muller, 2010). In 2002 Nguyen et al. found that between the analized tissue samples, the highest levels of the receptor mRNA are detected in heart, brain, placenta, and lower levels in kidney, liver, and pancreas. The receptor mRNA is barely detectable in lung and skeletal muscle. Immunofluorescence studies on normal human kidney and heart frozen sections showed that the receptor is localized in the mesangium of glomeruli and in the subendothelium of coronary and kidney artery. Furthermore, double staining with anti–smooth muscle cell alpha actin or with anti-renin antibodies and analysis by confocal microscopy showed that the receptor is associated with smooth muscle cells and is co-localized with renin. In human glomeruli, because there is no specific marker for normal mesangial cell, the mesangial localization of the receptor was confirmed by the absence of co-localization of receptor antibody with anti-CD31 or with anti-neutral endopeptidase, which are specific markers of endothelial cells and of epithelial cells, respectively (Nguyen et al., 2002). The receptor binds both renin and prorenin, with affinities in the nanomolar range, and the encoding gene, called *ATP6ap2,* is located on the X chromosome in locus p11.4.

Interestingly, prorenin receptor expression appears to be upregulated under pathological conditions; for example, at cardiac tissue sites of stroke-prone spontaneously hypertensive rats on a high salt diet (Ichihara et al., 2006b) and in the clipped kidneys of Goldblatt rats (Krebs et al., 2007).

Binding PRR: renin and prorenin activation

The PRR specifically binds renin and prorenin. In 2002 Nguyen and colleagues have observed that binding of renin to its receptor increases renin catalytic activity for its substrate angiotensinogen (Aogen). The comparison of the kinetics of aogen cleavage by receptor-bound renin and renin in solution showed that Aogen cleavage is four- to fivefold more efficient when renin is associated to its receptor ($kcat/Km$ 9.3 vs 2.2 µM-1s-1 for receptor-bound and soluble renin, respectively). They hypothesized that this could be due to the complex renin-receptor formation of a new structure that is more favourable to renin-angiotensinogen interaction, and that this complex formation would induce modification of renin conformation (Nguyen et al., 2002). They also observed that receptor bound prorenin was able to cleave Aogen in a manner comparable with that of fully active renin in solution in the absence of cleavage of the prosegment. These results underline that receptor binding activates prorenin non-proteolytically, most likely via a conformational change induced by binding *per se* that does not require cleavage of the prosegment (Nguyen et al., 2002).

The sequence of the ectodomain responsible for the interaction with prorenin have not yet been determined by structure-function studies (Nguyen and Muller, 2010).

The human PRR, when expressed in rat vascular smooth muscle cells, bound prorenin with a Kd of 6 nmol l^{-1}. Renin binding occurred with much lower affinity (Kd $\geq$ 20 nmol l^{-1}) (Batenburg et al., 2007; Batenburg et al., 2008; Nabi et al., 2006). Binding was not apparent at 4 °C, most likely because the majority (90%) of the PRR is located intracellularly, (Saris et al., 2006) whereas cycling between the intracellular compartment and the cell surface occurs at 37 °C only. The binding affinities of prorenin and renin for their receptor are 3–4 orders of

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magnitude above the normal levels of prorenin and renin in blood plasma (≈ 5 and 0.5 pmol l^{-1}) (Danser et al., 1998). Thus, significant prorenin binding to the PRR *in vivo* will most likely only occur at tissue sites where prorenin is produced locally, that is, in the kidney, ovaries, testis, adrenal and eye (Krop and Danser, 2008).

PRR binding and signaling pathways activation

Nguyen and colleagues observed in 2002 that binding of the PRR triggers activation of the mitogen activated protein (MAP) kinase–extracellular signal regulated kinase (ERK)1/2 signalling pathway in transfected human fetal mesangial cells (HMC) stably expressing the receptor. The activation of ERK1/2 was observed by stimulating cells with prorenin, in the presence of losartan (1 μM), indicating that this activation was independent from a possible generation of Ang II (Nguyen et al., 2002).

Further studies on the signalling pathways involved in PRR activation confirmed ERK1/2 phosphorylation and showed that it was due to ERK kinase and provoked Ets-like gene (Elk) phosphorylation (Feldt et al., 2008; Huang et al., 2006; Huang et al., 2007b; Sakoda et al., 2007). Moreover, ERK 1/2 activation resulted in the upregulation of transforming growth factor β1 gene expression, the subsequent upregulation of genes coding for profibrotic molecules, such as plasminogen-activator inhibitor-1, fibronectin and collagens, and the induction of mesangial cell proliferation (Huang et al., 2006; Huang et al., 2007a; Huang et al., 2007b).

The ERK1/2 pathway is not the only signalling pathway linked to the PRR, since the receptor also appears to activate the MAP kinase p38–heat shock protein 27 cascade (Ichihara et al., 2006c; Saris et al., 2006) and the phosphatidylinositol-3 kinase-p85 (PI3K-p85) pathway (Schefe et al., 2006). Interestingly, the latter pathway results in the nuclear translocation of the promyelocytic zinc finger transcription factor, which downregulates the expression of the PRR itself (Schefe et al., 2006). This observation suggests that high prorenin levels might suppress PRR expression, thereby preventing excessive receptor activation.

Activation of signalling pathways, as ERK 1/2 and p38, that are associated with cell proliferation and cell death has led to hypothesize that renin and prorenin can lead to hypertrophy and hyperplasia and ultimately to organ damage and cardiovascular events in hypertension (Feldman et al., 2008).

PRR Blocking

Unfortunately no selective PRR blocker has been synthesized so far. Hence, the renin inhibitor aliskiren that blocks the active site of renin was used to investigate PRR system: *in vitro* studies do not reveal any blocking effects of aliskiren toward the direct effects of renin/prorenin through their receptor. Aliskiren affected neither the binding of renin/prorenin to the receptor, nor their signalling cascade following receptor activation (Batenburg et al., 2008; Feldman et al., 2008; Feldt et al., 2008). Aliskiren, however, did block angiotensin generation by receptor-bound prorenin (Batenburg et al., 2008).

Since studies with aliskiren showed that blocking of the active site of renin and prorenin do not alter their binding to PRR or subsequent ERK1/2 activation, the discover of the "handle" region by Suzuki et al. (Suzuki et al., 2003) arouse interest. Suzuki et al. they observed that an antibody against a sequence of the prosegment of human prorenin (I11PFLKR15P) was able to open the profragment to yield a 'non-proteolytically' activated prorenin, in a manner similar to the putative mechanism of PRR binding-induced prorenin activation. They named this region of the prosegment the 'handle' region. Based on this observation, Ichihara et al. (Ichihara et al., 2004) tested a 10-amino-acid peptide which encompassed the handle region (HRP) as a blocker of prorenin–PRR binding. In a clever set-up of studies in diabetic rodents, they reasoned that diabetes would increase prorenin synthesis, thus creating optimal conditions to test the efficacy of HRP *in vivo*. Indeed, HRP prevented or even reversed diabetic nephropathy (Ichihara et al., 2004; Ichihara et al., 2006c; Takahashi et al., 2007), and blocked ischaemia-induced retinal neovascularization and ocular inflammation in endotoxin-induced uveitis (Satofuka et al., 2006; Satofuka et al., 2007). Moreover, it diminished cardiac fibrosis in stroke-prone spontaneously hypertensive rats (Ichihara et al.,

2006b). Taken together, these data strongly suggest that the prorenin–PRR axis plays an essential role in end organ damage in diabetic and inflammatory pathologies. However, enthusiasm was rapidly replaced by scepticism when the *in vivo* data could not be reproduced It has subsequently been argued that the apparent lack of effect of HRP could be explained by the notion that HRP exerts its effect only in diseases associated with high prorenin and low renin levels because HRP blocks prorenin and not renin binding to PRR. *In vitro* results have also been discrepant. Some groups using straightforward methods such as inhibition of binding of radiolabeled prorenin and ERK activation in the presence of HRP found no inhibitory effects, even at HRP concentrations as high as 10 µmol/ L (Batenburg et al., 2007; Feldt et al., 2008; Krebs et al., 2007; Muller et al., 2008). Others report that HRP inhibits not only prorenin binding (Susic et al., 2008) but also renin binding to recombinant PRR (Kato et al., 2008; Nabi et al., 2009), or that HRP stimulates ERK1/2 by itself (Ichihara et al., 2006a).With these latter findings, it is difficult to understand how HRP could totally inhibit ERK1/2 phosphorylation in the kidney of HRP-treated animals when it could itself stimulate ERK, and why HRP would not be effective in high renin models when it could inhibit renin binding *in vitro*. The possible mode of action of HRP is far from here and the discrepancy between *in vitro* and *in vivo* data cannot be explained at this time. Therefore, it seems reasonable not to call HRP a "PRR blocker" until more clarity emerges.

Transgenic animals

All arguments supporting a role of PRR in hypertension, cardiovascular and renal diseases come from animals overexpressing PRR or from studies that use the still disputed putative PRR blocker HRP. Ubiquitous overexpression of human PRR transgenic rats shows that the rats remain normotensive but develop proteinuria and a slowly progressive nephropathy, suggesting a direct pathologic role of PRR in renal damage. The glomeruli of these transgenic rats show a measurable degree of ERK1/2, p38, and c-Jun N-terminal kinase activity, but not EGF receptor phosphorylation compared with controls, and renal levels of Ang II are normal

(Kaneshiro et al., 2007). A second transgenic model overexpressing the human *PRR* gene exclusively in smooth muscle cells, including vascular smooth muscle cells, provides further insight into the genesis of hypertension. After 6 month of age, transgenic rats develop a cardiovascular phenotype with elevated systolic blood pressure (BP) and augmentation in heart rate. Kidney function is normal with increased levels of plasma aldosterone and a rise in the aldosterone/renin ratio. These alterations also progressively increase with age (Burckle et al., 2006). To study whether increased prorenin alone could induce fibrosis, two groups generated transgenic animals with inducible or constitutive overexpression of prorenin, and their results clearly showed that increased prorenin (up to 200 times the normal concentrations) over a period of 18 months was not associated with any cardiac or kidney fibrosis as assessed by histologic examination and by PCR analysis for TGF-β and collagens. However, the animals had severe hypertension correlating with the level of expression of prorenin; the hypertension was attributed to increased generation of angiotensin II, as it was controlled by an angiotensin-converting enzyme inhibitor (Mercure et al., 2009; Peters et al., 2008).

There should be two ways to establish the role of a receptor in pathology: the use of an antagonist specific for the receptor; and studies in mice knocked-out for the receptor gene. As discussed before, a specific blocker of the PRR does not exist, for this reason several groups have tried to generate a *PRR*-null mouse despite numerous attempts, possibly because *PRR* $^{-/-}$ embryonic stem cells do not form chimeras after blastocyst injection (Nguyen and Muller, 2010). *PRR* deletion in *C. elegans* and zebrafish (Amsterdam et al., 2004) yields embryos that die before the end of embryogenesis, thus supporting an essential, but still unknown, cellular function for PRR.

PRR and the V-ATPase

Native PRR undergoes intracellular processing to generate three different molecular forms of PRR, a full-length integral transmembrane protein (PRR), a soluble PRR (sPRR), and a truncated form composed of the transmembrane and

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cytoplasmic domains associated with the V-ATPase. The truncated transmembrane/cytoplasmic (TM–IC) form of PRR was identified when Ludwig et al. (Ludwig et al., 1998) were looking for proteins associated with the V-ATPase. V-ATPases are ATP-dependent proton pumps that acidify intracellular compartments and transport protons across plasma membranes in intercalated cells, osteoclasts, macrophages and tumour cells. The structure of the V-ATPase is well established: they are multi-subunit proteins composed of a V1 domain containing eight different subunits (A–H), which is responsible for ATP hydrolysis, and a V0 domain composed of six different subunits (a, c, c", d, e and the accessory protein subnit Ac45 in mammals), which is responsible for proton translocation. Some subunits in the V1 and V0 domains may be present in multiple copies and most subunits have tissue-specific isoforms (Cipriano et al., 2008; Nishi and Forgac, 2002). In mammals, there are two accessory subunits binding to the V0 sector that have been described to regulate V-ATPase activity: Ac45 encoded by the gene *ATP6ap1* and the truncated form of PRR encoded by the gene *APT6ap2*. If Ac45 is a genuine accessory protein, because it is found as an integral component of the V0 sector, and therefore deserves to be named a V-ATPase subunit (Toei et al., 2010), PRR is mistakenly considered and named a V-ATPase subunit because it has never been found as an integral component of the V0 sector. However, Ac45 and PRR share some similarities. First, they are processed by furin. This furin processing appears to be a prerequisite forAc45 function, as reduced proteolytic processing of Ac45 impairs acidic vesicle formation and is responsible for defective insulin secretion in pancreatic *β*-cells (Louagie et al., 2008). This could explain why the majority of PRR is also cleaved by furin (Cousin et al., 2009) to generate the truncated TM–IC necessary for V-ATPase assembly. Secondly, *ATP6ap1/Ac45* knockout in mice is embryonic-lethal (Schoonderwoert and Martens, 2002) and *PRR*-knockout mice could not be generated. Thirdly, mutation of *ATP6ap1/Ac45* and *ATP6ap2/PRR* in zebrafish gives a similar phenotype: oculocutaneous albinism, small head and eyes, CNS (central nervous system) necrosis and embryonic lethality (Amsterdam et al., 2004; Nuckels et al., 2009). A functional link between PRR and V-ATPase was established for the first time in renal intercalated cells by Advani et al.; they have shown that blocking V-ATPase function with bafilomycin in MDCK (Madin–

Darby canine kidney) cells of a collecting duct/distal lineage also inhibited PRR activation and ERK phosphorylation induced by prorenin (Advani et al., 2009).

PRR intracellular processing

PRR is a multifunctional protein existing in different molecular forms with an interesting cellular localization. Two different immunofluorescence studies of Advani et al. and Cousin et al. on endogenous PRR (Advani et al., 2009; Cousin et al., 2009) and one of Schefe et al. on transfected PRR show that most of the protein is intracellular (Schefe et al., 2008). Nguyen et al. recently proposed a model of intracellular processing of the PRR (Nguyen and Muller, 2010): they suggest that the PRR accumulates in the trans-Golgi, where a part of it is cleaved by furin to generate a 28-kD soluble form of the PRR, which is secreted, and a 10-kD transmembrane/cytoplasmic fragment that likely represents the truncated, which remains associated with V-ATPase. The other part of PRR remains intact, can complex with the V-ATPase like the truncated form, and is addressed to the plasma membrane.

AIMS

General aim

The present study was designed to investigate the presence, expression level localization and functional role of the PRR in the adrenal cortex and to get information on its potential involvement in the pathophysiology of human hyperldosteronism.

Specific aims

The specific aims of this study are:

a) Evaluate the gene expression of the PRR in human adrenal gland and APA.

b) Verify the expression of the PRR protein in adrenal gland specimen and in a pure population of aldosterone-secreting cells from the normal human ZG.

c) Determinate the subcellular localization of the PRR protein.

d) Investigate if the two adrenocortical carcinoma cell lines HAC15 and H295R are appropriate tools to investigate the role of PRR in the adrenal gland

e) Evaluate if prorenin and renin induce ERK 1/2 activation in H295R and HAC15 cells., and if ERK 1/2 activation may be prevented by antagonizing angiotensin II AT1 subtype receptor

MATERIALS AND METHODS

a) Adrenal tissues and cells

Human Adrenal samples

The study was approved by Ethics Committee and each patient consented to the study. Aldosterone Producing Adenomas (APA) were diagnosed by the "four corner approach" (Rossi, 2011), which include 1) biochemical diagnosis of PA, 2) lateralization of aldosterone secretion at minuscolo bilaterally selective Adrenal Vein Sampling, 3) evidence of adrenocortical nodule at histopathology, and more importantly, 4) cure or improvement of hypertension, and correction of the biochemical picture of PA at follow-up after adrenalectomy (Rossi et al., 2006). Cure was defined as a systolic blood pressure <140 mm Hg and diastolic blood pressure <90 mm Hg without medications, and improvement as a systolic and diastolic blood pressure <140/90 mm Hg, respectively, on the same or a reduced number of medications and/or a reduced number of defined daily doses (Anonymous, 1999).

Patients underwent laparoscopic adrenalectomy. Tissues were obtained under sterile conditions at surgery, in the operating room and the excised adrenal gland was cut into halves according to the lane of the APA. Half of the adrenal was sent to the Pathology department for histology; the remaining tissue was divided into 3 pieces: the first was rapidly frozen in liquid nitrogen and stored for molecular studies, another part was processed for the isolation of CD56 positive cells and the third was fixed in neutral formalin and included in paraffin.

Human normal adrenal cortex tissue was obtained under sterile conditions at surgery from patients with renal cancer carcinoma undergoing unilateral nephrectomy and ipsilateral adrenalectomy.

All patients gave an informed consent and the use of tissues followed our institutional guidelines.

Immuno-magnetic isolation of CD56 positive cells from the adrenal gland

Human normal adrenal tissue and APA tissue were immediately processed under sterile condition after surgery. The samples were cleaned from the fat and connective tissue, were minced into small pieaces. Dispersed Zona Glomerulosa (ZG) or APA cells were obtained by sequential enzymatic digestion and mechanical disaggregation. Digestion was performed at 37 °C with gentle shaking for 40 min in a digestion solution composed of 5 mL of Krebs Ringer solution containing 2 mg/mL collagenase-I, 0.1 mg/mL deoxyribonuclease-I and 4% bovine serum albumin (BSA). Disaggregation was favoured by gentle pipetting, aspirating with syringe and filtering with a 40 μm cell strainer. Cells were centrifuged for 10 min at 100 x g at 4 °C, resuspended and washed with 5 ml 0,5 % BSA, Krebs Ringer solution by three centrifugation for 10 min at 100 x g.

Dispersed cells were counted and their viability was assessed by trypan blue coloration.

CD56 positive cells were isolated with a method developed in our lab which provides a pure population of aldosterone-secreting cells from the normal human ZG (Caroccia et al., 2010). This method is based on precoating magnetic beads with an antibody specific for NCAM (CD56).

In our studies we used Dynabeads Goat anti-Mouse IgG that are uniform, superparamagnetic polystyrene beads (4.5 μm diameter) coated with polyclonal goat anti-mouse IgG antibodies. The binding to the surface of the beads, of target-specific antibodies allows specific cell isolation directly from a complex suspension.

Before using for cell separation, Dynabeads were washed three times with wash solution PBS, 0.1% BSA, 2mM EDTA and then coated with purified mouse antihuman CD56 IgG (Biolegend, San Diego, CA) by overnight (o.n) incubation at 4 °C with 4 µg of antibody per $2x10^7$ beads under gentle tilting and rotation. Beads were recovered with a magnet, washed for 3 times with PBS, 0.1% BSA, 2mM EDTA and then used for cell separation.

CD56 Precoated beads were mixed with ZG and APA cells for 40 min at 4°C by gentle shaking and rotation. Dynabeads were used at a ratio of five beads per cell.

Beads bound cells, CD56 positive cells, were separated with a magnet. Immunoseparated CD56+ cells were seeded on coverslips and fixed after adhesion

for immunocitochemistry, confocal microscopy and electron microscopy immuno gold experiments.

b) PRR gene expression

In order to evaluate PRR gene expression, Real time RT-PCR experiments were performed. Total RNA was extracted from Aldosterone Producing Adenomas (APAs), human normal adrenal cortex and two human adrenocortical carcinoma cell lines: HAC15 and H295R, using RNeasy Mini Kit (Qiagen) following the manufacturer's protocol.

Cells were collected by trypsinization and lysed in 350 µl buffer RLT with 10 µl/ml β-mercaptoethanol. Cell lysate was mixed with 70% ethanol and than transferred into column filter that selectively bound RNA. Purified RNA was eluted in 30 µl RNase-free water.

On the other hand, cryoconserved adrenal tissue was mixed in 600 µl lysis buffer (594 µl buffer RPE, 6 µl β-mercaptoethanol). Tissue was omogenized with Roche Tissue Lyser, the disgregated sample was centrifuged and supernatant was mixed with 70% ethanol. Sample was transferred into column filter that selectively bound RNA. Purified RNA was eluted in 40 µl RNase-free water.

The quality of the RNA was assessed with a laboratory-on-chip technology in an Agilent Bioanalyzer 2100 with the RNA6000 Nano assay (Agilent Technologies, Santa Clara, CA). The instrument uses capillary electrophoresis to analyze nucleic acid and results are visualized as electropherograms. The RNA amount and quality was evaluated with spectrophotometric readings at 260/280/230 nm.

One µg of total RNA was reverse transcribed with iScript ™ cDNA Synthesis Kit (Bio-Rad, Milan, Italy) in a final volume of 20 µl following the manufacturer's recommendations. Samples were incubated for 5 minutes at 25 °C, for 30 minutes at 42 °C and for 5 minutes at 85 °C.

PRR and porphobilinogen deaminase (PBGD) primers for RT-PCR were designed using Roche-applied-science Universal Probe Library site, Operon Biotechnologies and BLAST site. PRR mRNAs was measured with a real time RT-PCR with Universal ProbeLibrary Probes in the LightCycler 480 Instrument

(Roche, Monza, Italy). The reaction was performed following manufacturer's protocol in 20 µl composed of 10 µl Probes Master (Roche), primers forward and reverse 200 nM, probe 200 nM and 3 µl of cDNA sample.

The PRR expression was calculated relative to porphobilinogen deaminase, used as an internal control. Quantification of gene expression was carried out by comparative Ct ($2-\Delta\Delta Ct$) method (Livak and Schmittgen, 2001).

c) PRR protein expression

To verify PRR expression at protein level 4 µm thick serial sections from paraffin blocks of rat and human normal adrenal gland were processed for immunohistochemistry and immunoseparated ZG CD56+ cells were seeded on coverlips, fixed and processed for immunocitochemistry.

Immunohistochemistry

Four µm thick serial sections from paraffin blocks of rat and human normal adrenal gland were processed for immunohistochemistry. The sections were dewaxed with 5 minutes incubations with decreasing concentration of ethanol and rehydrated with distilled water. Antigen was retrieved by incubation with MS-UNMASKER (DIAPATH) 1:10 in distilled water at 96 °C for 30 minutes. Sections were washed twice in distilled water and once in PBS for 5 min at RT. Endogenous peroxidase was inhibited by 5 minutes incubation with 0.5% hydrogen peroxide. Sections were washed three times with PBS for 5 min. Blocking of non-specific sites was obtained by 30 min incubation with PBS, 0.2% BSA, 0.2% Triton, 1:50 Rabbit serum. Sections were incubated overnight at 4 °C with a Goat polyclonal antibody specific for ATP6IP2 (PRR) (ab5959 Abcam) diluted 1:100 in PBS, 0.2% BSA, 0.2% Triton. Sections were washed three times with PBS for 5 min and incubated for 1h with a secondary antibody labelled with horseradish peroxidase diluted 1:200 in PBS, 0.2% BSA, 0.2% Triton. Antigen was detected by diaminobenzidine (DAKO) incubation and reaction was blocked with distilled water. The sections were dehydrated with 5 minutes washing with

increasing concentration of ethanol and mounted. Negative controls were processed in the same way but with omission of the primary antibody.

Immunocitochemistry

Immunoseparated ZG CD56+ cells were seeded on glass coverlips and fixed. Cells were washed, after adhesion, with PBS and fixed with 4% PFA pH 7 for 30 min at 4 °C. Coverlips were washed three times with PBS for 5 min and endogenous peroxidase was inhibited by 5 minutes incubation with 3% hydrogen peroxide. Blocking of non-specific sites was obtained by 30 min incubation with PBS, 5% Milk, 0.5% Triton. Glass coverlips were incubated 1h room temperature with a Goat polyclonal antibody specific for ATP6IP2 (ab5959 Abcam) and than washed three times with PBS for 5 min. After washing, cells were incubated 1h at RT with a secondary antibody labelled with horseradish peroxidise and than washed three times with PBS for 5 min. Antigen was detected by and diaminobenzidine (DAKO) incubation, reaction was blocked with distilled water and glass coverlips were mounted. Negative controls were processed in the same way but with omission of the primary antibody.

d) PRR localization

To localize the PRR at the cellular and subcellular level we performed confocal microscopy, immunogold electron microscopy and cell membrane and cytosol separation followed by immunoblotting experiments.

Confocal microscopy

H295R, HAC15 cells and immunoseparated ZG CD56 positive cells were fixed with 4% PFA for 30 min at 4 °C. Cells were incubated with primary antibodies anti-CD56 and anti-PRR (ab5959 Abcam) and secondary antibodies anti-mouse IgG Alexa Fluor 488 (Invitrogen) and anti-goat Alexa Fluor 594 (Invitrogen). The

fluorescence was detected using the confocal system Leica TCS SP5 with a 488 nm filter and the images were acquired using the software LAS AF.

Cells membrane and cytosol separation and Immunoblotting for PRR

H295R and HAC15 were seeded and cultivated in 6 well plates, respectively with RPMI medium supplemented with 10% fetal bovine serum (Sigma), antibiotics and 1% insulin/transferrin/selenium Premix (BD Biosciences) and of DME/F12 medium supplemented with 10% cosmic calf serum (HyClone, Logan, UT), antibiotics and 1% insulin/transferrin/selenium Premix (BD Biosciences). Medium was eliminated and cells were washed with PBS, proteins were extracted by 5 min incubation on ice with 200 μl Lysis buffer (Euroclone) and scraped. Samples were sonicated and supernatants were collected after centrifugation. Sample supernatants were ultracentrifuged for 1h at 100000 g at 4 °C. Supernatants were collected (cytosol fraction) and pellets (membrane fraction) were resuspended with Lysis buffer. Protein quantification was performed with BCA kit (Thermo Pierce), 50 μg of proteins were loaded in a 10% acrylamide gel and SDS-PAGE was carried out. Non-specific sites were blocked by overnight membrane incubation at 4 °C with T-PBS, 5% Milk. Membranes were incubated for 1h room temperature with a Goat polyclonal antibody specific for ATP6IP2 (ab5959 Abcam). Antigen was detected by 1h room temperature incubation with the secondary antibody Donkey anti-Goat (sc2020 Santa Cruz) and ECL kit (Thermo Scientific). Images were acquired and analyzed with Versadoc (Biorad).

Electron Microscopy Immuno Gold

Monolayer cells (CD56 positive cells, H295R and HAC15) were fixed in 3% paraformaldehyde–1% glutaraldehyde in 0.1 M sodium cacodylate buffer with $CaCl_2$, Tannic acid (Fluka) 0.5% in Maleate buffer and P-phenylenediamine (Fluka) for 2 h (Berryman et al., 1992), dehydrated and then embedded in an epoxy resin.

Sixty nm-ultrathin sections were cut with a Reichert-Jung Super Nova ultramicrotome and collected on 400-mesh nickel grids. The epoxy resin was removed by exposing the sections to 0.2% (w/v) NaOH in 35% (v/v) aqueous ethanol, rinsing in 35% ethanol and in bi-distillate water. Nickel grids, were incubated for 15 min at 90 °C in order to unmask antigen and washed in PBS. Ultrathin sections were pre-incubated in Blocking buffer (0.2% normal goat serum, 2% BSA, 0.2% Tween-20 in PBS) for 60 min at room temperature, and then incubated with the primary Goat Polyclonal antibody anti-ProRenin Receptor (ab5959 Abcam) (1:50 dilution) overnight at 4 °C. After repeated PBS washing, ultrathins sections were incubated for 60 min at room temperature with 18 nm colloidal gold-labelled antiGoat (Jackson Immunoresearch) (1:15 dilution) in blocking buffer. After washing in PBS and bi-distilled water, grids were counterstained with lead hydroxide.

Negative controls were obtained performing the same protocol but omitting the primary antibody.

Samples were examined by a Hitachi H-300 electron microscope.

e) Functional Studies

To investigate the functional role of the PRR activation H295R and HAC15 cells were exposed to 100 nM Angiotensin II (Sigma), 50 nM Prorenin (Cayman), 50 nM Renin (Cayman) with or without 30 min preincubation and co-incubation with 5 µM Irbesartan. The effect of those stimuli on ERK 1/2 phosphorylation was analysed by immunoblotting.

Cell culture and stimulation

H295R and HAC15 cells were seeded in 6 wells plates, growth media consisted respectively of RPMI medium supplemented with 10% fetal bovine serum (Sigma), antibiotics and 1% insulin/transferrin/selenium Premix (BD Biosciences) and of DME/F12 medium supplemented with 10% cosmic calf serum (HyClone, Logan, UT), antibiotics and 1% insulin/transferrin/selenium Premix (BD

Biosciences). Cells were stimulated 30 min at 37 °C with 100 nM angiotensin II (Sigma), 50 nM prorenin (Cayman), 50 nM renin (Cayman) with or without 30 min preincubation and co-incubation with 5 µM irbesartan.

Immunoblotting and analysis of ERK 1/2 phosphorylation

Proteins of H295R and HAC15 previously stimulated 100 nM angiotensin II (Sigma), 50 nM prorenin (Cayman), 50 nM renin (Cayman) with or without 5 µM irbesartan, were extracted by 5 min incubation on ice with 200 µl Lysis buffer (Euroclone) and scraped. Samples were sonicated and supernatants were collected after centrifugation. Protein quantification was performed with BCA kit (Thermo Pierce), 50 µg of proteins were loaded in a 10% acrylamide gel and SDS-PAGE was carried out. Non-specific sites were blocked by overnight membrane incubation at 4 °C with T-PBS, 5% BSA. Membranes were incubated over night room temperature with an antibody specific for ERK phosphorylated form and total ERK (Cell Signalling). Antigen was detected by 1h room temperature incubation with the anti rabbit secondary antibody (Amersham) and ECL kit (Thermo Scientific). Images were acquired and analyzed with Versadoc (Biorad).

RESULTS

b) PRR gene is highly expressed in human adrenal gland

To clarify a possible role of the PRR in PA pathophysiology we beforehand investigated its expression in adrenal samples: APA, normal adrenal cortex and in two human adrenocortical carcinoma cell lines.

Real time PCR evidenced that PRR gene is highly expressed, when compared to the housekeeping gene PBGD, in all investigated adrenal samples: APA (n=11), normal human adrenal cortex and the two human adrenocortical carcinoma cell lines, H295R and HAC15 cells.

We evaluated the ratio between PRR and PBGD expression, and we observed that the average PRR expression in APAs and normal human adrenal cortex was similar, being 12 and 15 fold higher than PBGD, respectively (Fig. 1).
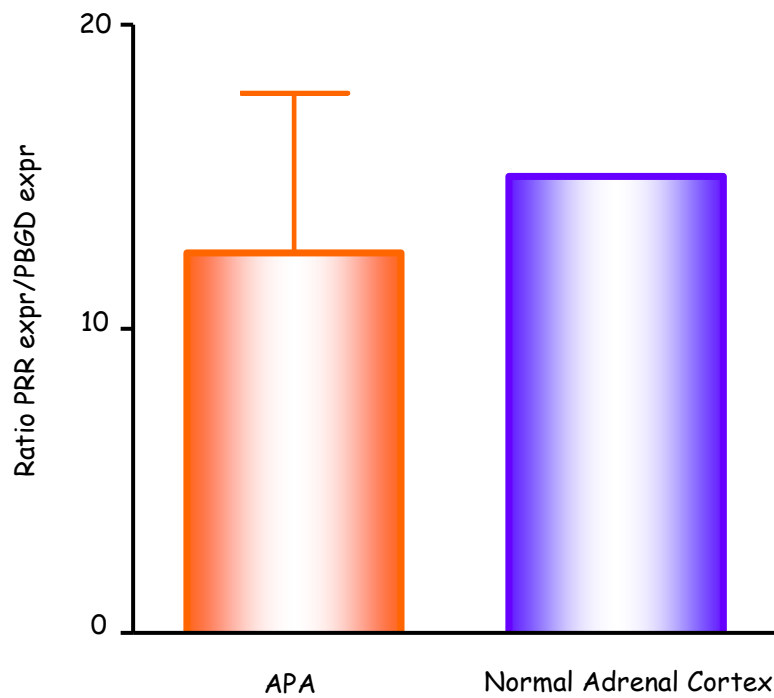
Fig. 1 PRR gene expression in APA (n=11) and in human normal adrenal cortex. The PRR gene expression is shown as the ratio of the expression of the PRR and the expression of the housekeeping gene PBGD.

Real Time RT-PCR Amplification Curves (Fig. 2) show in fact that the Ct (cycle threshold) for PRR, which is inversely proportional to the amount of target nucleic acid in the sample, is markedly lower than that of PBGD (PRR Ct = 22,69 ± 1,11; PBGD Ct = 27,49 ± 1,69 p<0.0001).
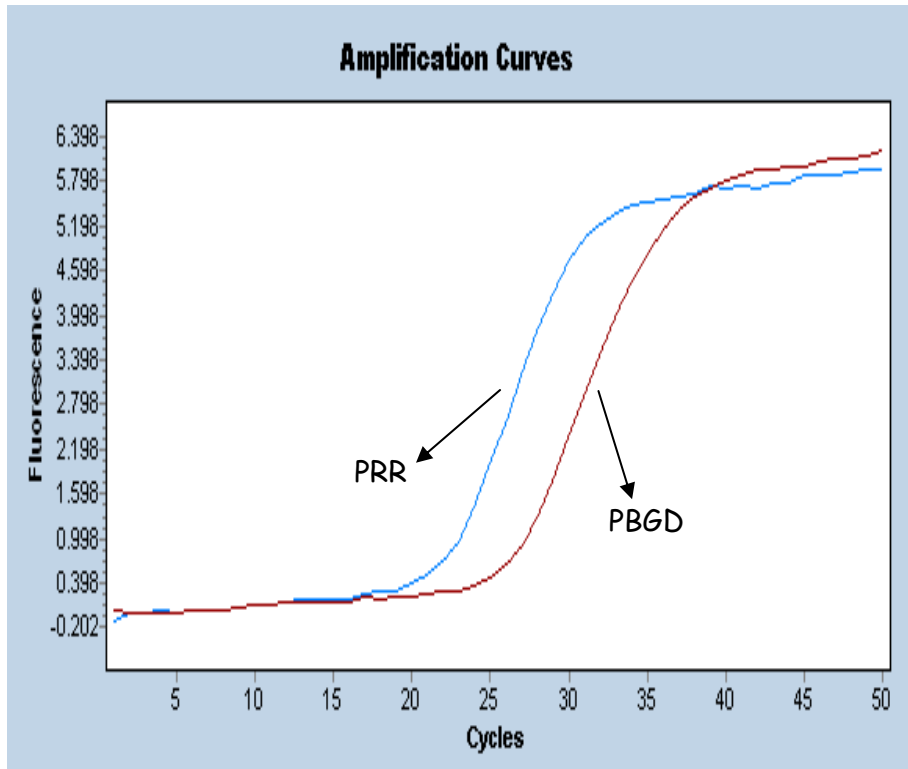
Fig. 2 Real Time RT-PCR Amplification Curves for PRR transcript and the PBGD transcript in APA tissue.

We also performed Real time RT-PCR in HAC15 and H295R cell lines, and we found that PRR gene was highly expressed in both. These human adrenocortical cercinoma cell lines contain all the enzymes required for aldosterone biosynthesis and therefore are commonly used as model system for studying the molecular and biochemical mechanism controlling adrenal steroidogenesis (Parmar et al., 2008; Rainey et al., 2004).

In particular, the average PRR expression in H295R cells was about 7 fold higher than PBGD and in HAC15 was about 16 fold higher (Fig. 3).
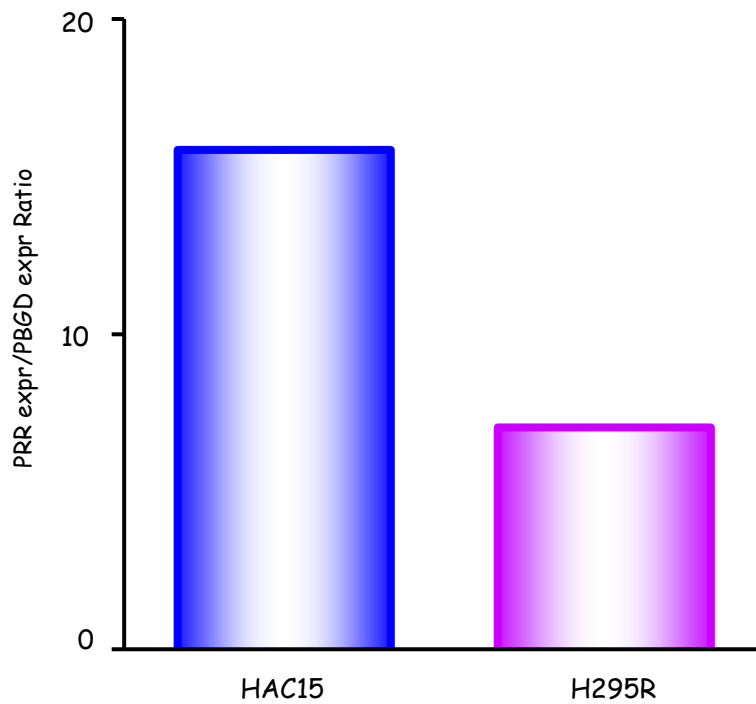
Fig. 3 PRR gene expression in two human adrenocortical carcinoma cell lines: HAC15 and H295R. The PRR gene expression is shown as the ratio of the expression of the PRR and the expression of the housekeeping gene PBGD.

c) PRR protein is expressed in human adrenal gland

We evaluated PRR protein expression in adrenal gland by performing immunohytochemistry and immunocytochemistry experiments.

Immunohystochemistry on rat normal adrenal gland revealed a strong and clearly evident immunostaining for PRR in the medulla and the subcapsular zone (Fig. 4).
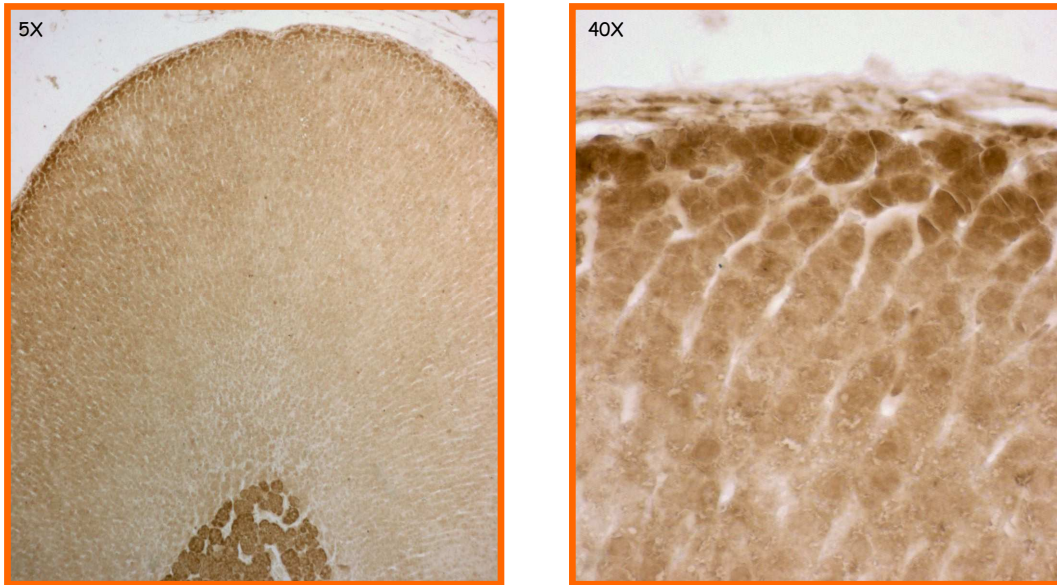
Fig. 4 Immunohystochemistry for PRR in rat adrenal gland. Left panel 5X magnification; right panel 40X.

The same experiments were performed on human adrenal gland samples and, as shown in Fig. 5, we confirmed the PRR expression at protein level with the highest positive labelling at the medulla and sub capsular levels.

The specificity of the reactions was confirmed by lack of staining when the primary antibody was omitted.
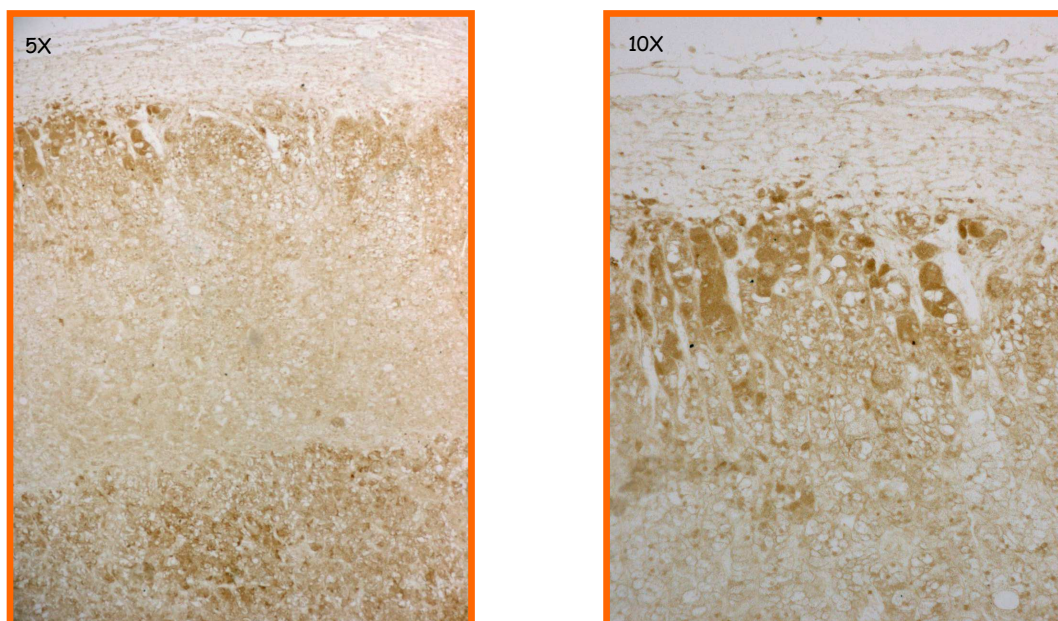
Fig. 5 Immunohystochemistry for PRR in human adrenal gland; Left panel 5X magnification and right panel 10X.

To better determinate PRR expression at protein level, we performed immunocytochemistry experiments on ZG immunoseparated CD56+ cells. Our results clearly confirm that the PRR is expressed in this pure population of aldosterone-secreting cells from the normal human ZG (Fig. 6 right panel).

The specificity of the reaction was tested by omission of the primary antibody (Fig. 6 left panel).
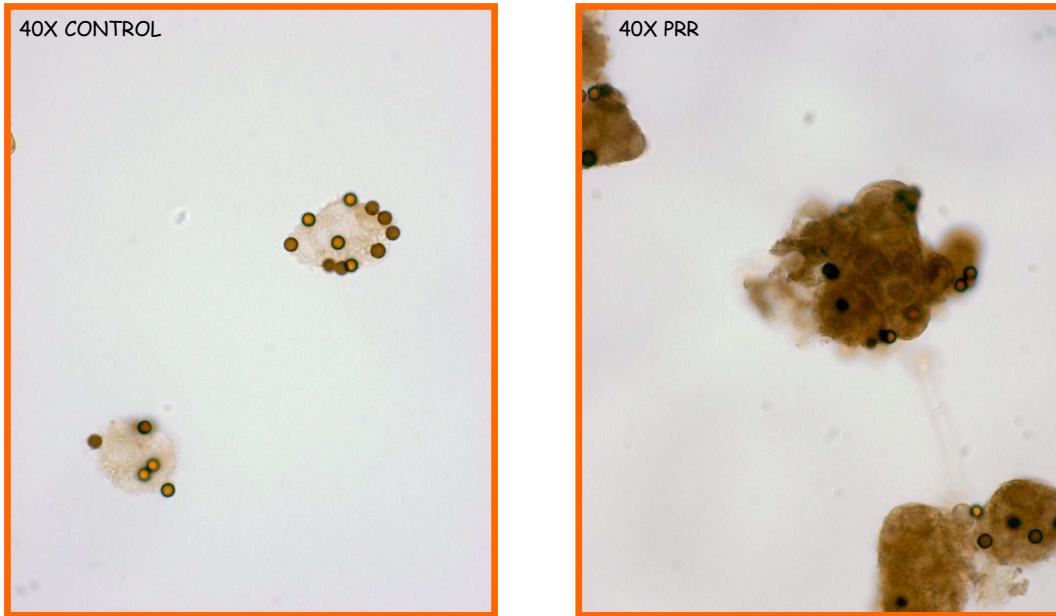
Fig. 6 Immunocytochemistry for PRR in ZG CD56+ cells. In the left panel representing the control reaction (omission of the primary antibody) please note the magnetic beads and no immunostaining. In the right panel the positive reaction for the PRR is demonstrated. The images are taken at 40X magnification.

d) PRR localization

A further demonstration of the presence of the PRR is given by immunoblot experiments performed on HAC15 and H295R cytosolic and membrane fractions. After obtaining the two different fractions by ultracentrifugation, western blot results identified a band at the expected molecular weight (40 kDa) only in the membrane fraction of each cell line (Fig. 7).
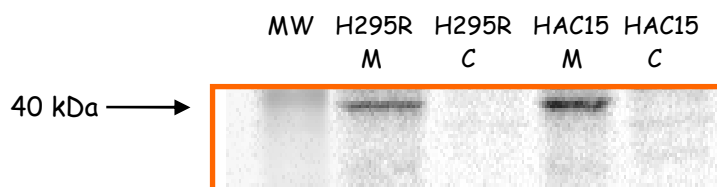

Fig. 7 Western Blot for PRR performed on membrane (M) and cytosol (C) fractions of H295R and HAC15 cell lines.

Experiments of confocal microscopy confirm the presence of the PRR in the adrenocortical carcinoma cell lines. In particular, the images show that PRR is mainly localized at membrane level. In these experiments we have also found that PRR partially colocalizes with the adhesion molecule CD56 in HAC15 (Fig. 8 upper panel) and H295 cell lines (Fig. 8 lower panel).
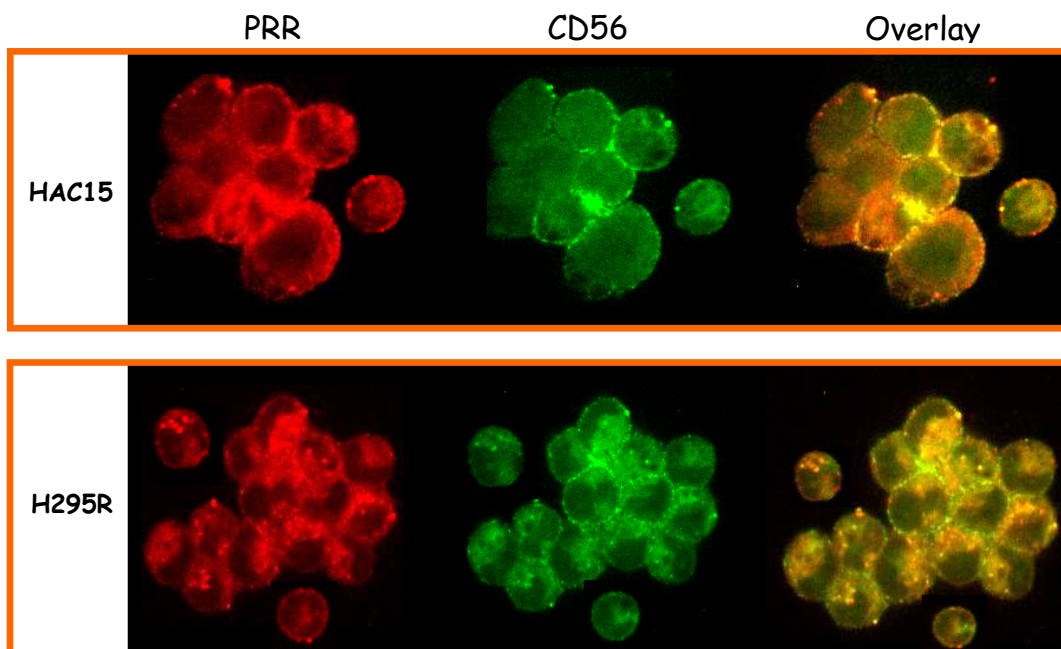

Fig. 8 Confocal microscoy images: Localization of PRR *(red)*, CD56 *(green)* and overlay of PRR and CD56 *(yellow)* in HAC15 (upper panel) and H295R (lower panel).

A more precise subcellular localization was obtained by Immuno-gold electron microscopy performed on ZG CD56+ cells, on HAC15 and H295R.

This fine technique evidence that in immunoseparated ZG CD56+ cells, PRR is localized in the nucleus, mitochondria and cytoplasm (Fig. 9).
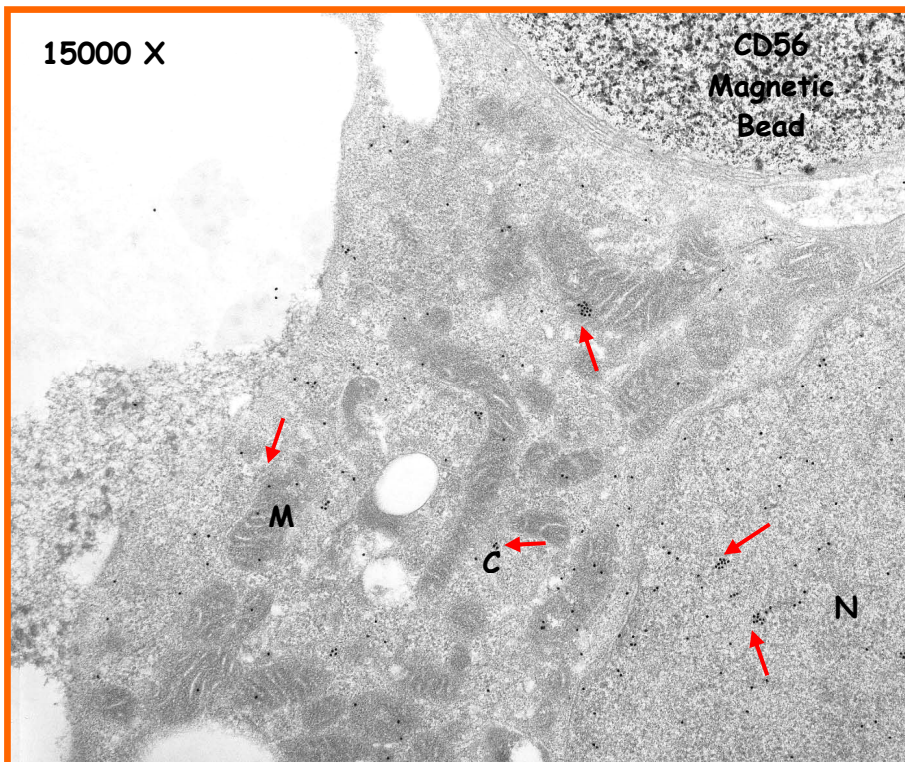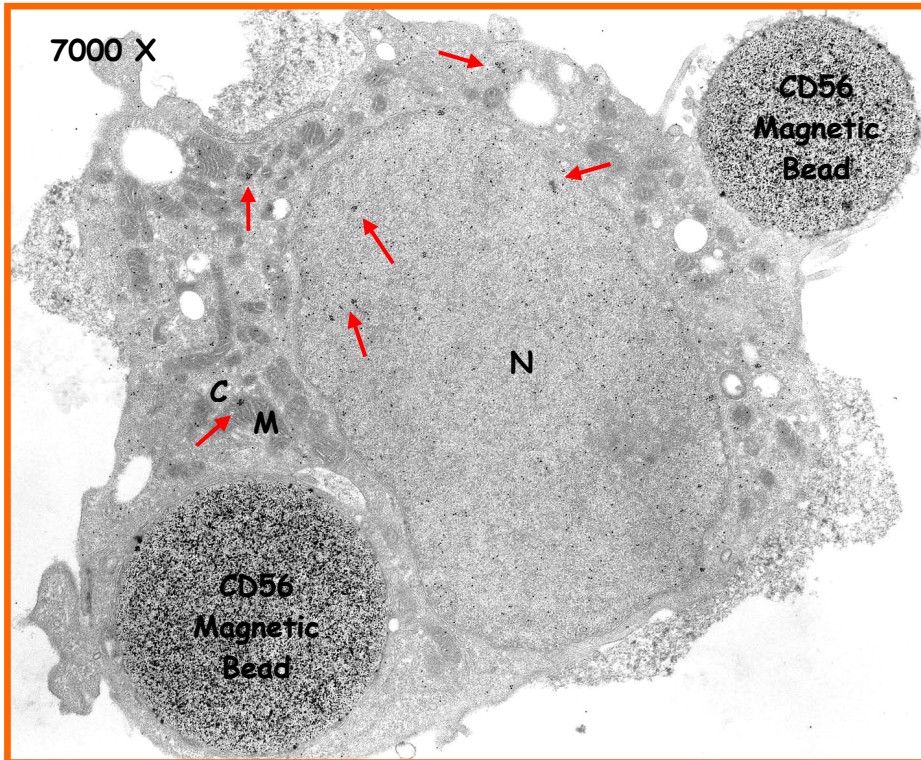
Fig. 9 PRR subcellular localization in immunoseparated ZG CD56+ cells obtained by immunogold electron microscopy. Upper panel is 7000X magnification and lower panel is 15000X magnification.

The same experiments were carried out in HAC15 and H295R cell lines. The results reveal that PRR is localized in the nucleus, mitochondria, cytoplasm and in Golgi's vesicle (Fig. 10).
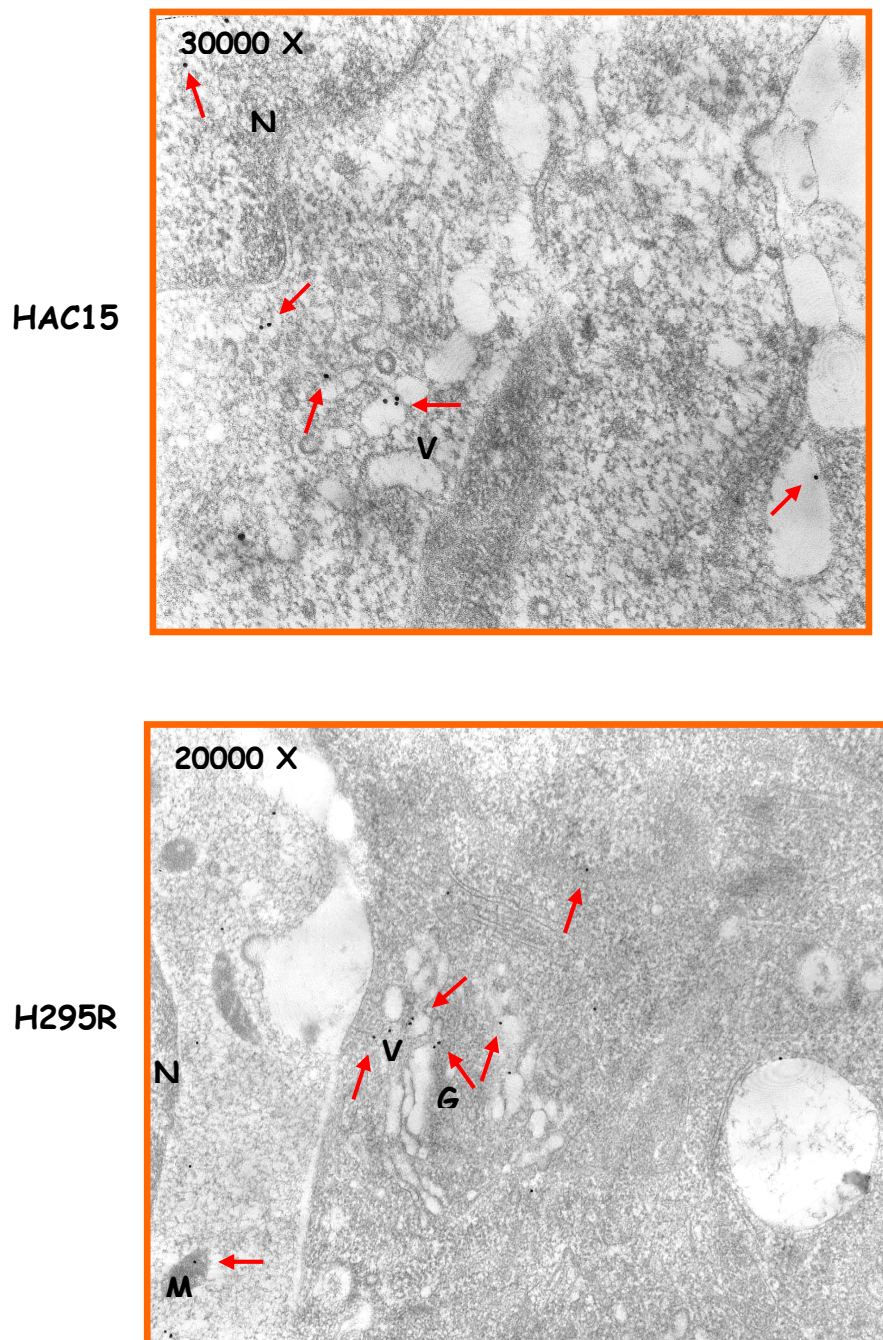

Fig. 10 PRR subcellular localization in HAC15 (upper panel, 30000X magnification) and in H295R (lower panel, 20000X magnification).

e) Functional Studies

Functional studies performed on HAC15 and H295R cells evidenced that 30 min stimulation with Ang II 100 nM, renin 50 nM or prorenin 50 nM induce marked ERK 1/2 phosphorylation. Interestingly activation of ERK 1/2 after Ang II and renin can be totally prevented by 30 min preincubation with irbesartan 5 µM in both cell lines (Fig. 11-14). ERK 1/2 phosphorylation induced by prorenin incubation can be completely abolished by irbesartan preincubation in H295R cells (Fig. 13, 14), but only partially inhibited by irbesartan preincubation in HAC15 cells (Fig 11, 12).
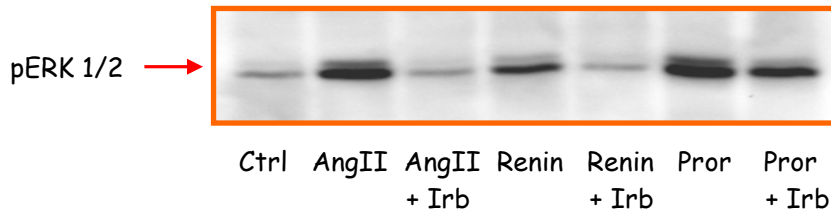

Fig. 11 Western Blot representing ERK 1/2 phosphorylation in HAC15 cells stimulated with Ang II 100 nM, renin 50 nM, prorenin 50 nM in presence or absence of irbesartan 5 μM.

Fig. 12 Graphical representation of ERK 1/2 phosphorylation in HAC15 cells stimulated with Ang II 100 nM, renin 50 nM, prorenin 50 nM in presence or absence of irbesartan 5 μM. ERK 1/2 phosphorylation is expressed as the ratio of phosphorylated and total ERK 1/2. Each value is normalized for the control. Values are mean +/- standard error. Analysis was performed with ANOVA and Bonferroni post hoc test; *: $p \leq 0{,}05$
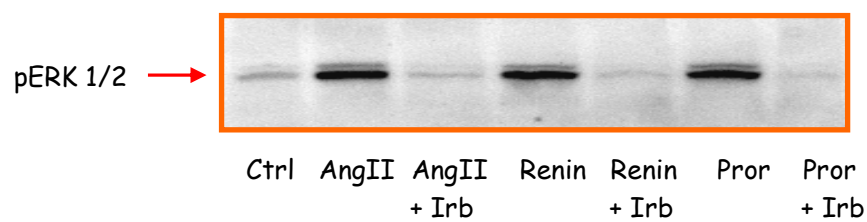
Fig. 13 Western Blot representing ERK 1/2 phosphorylation in H295R cells stimulated with Ang II 100 nM, renin 50 nM, prorenin 50 nM in presence or absence of irbesartan 5 μM.
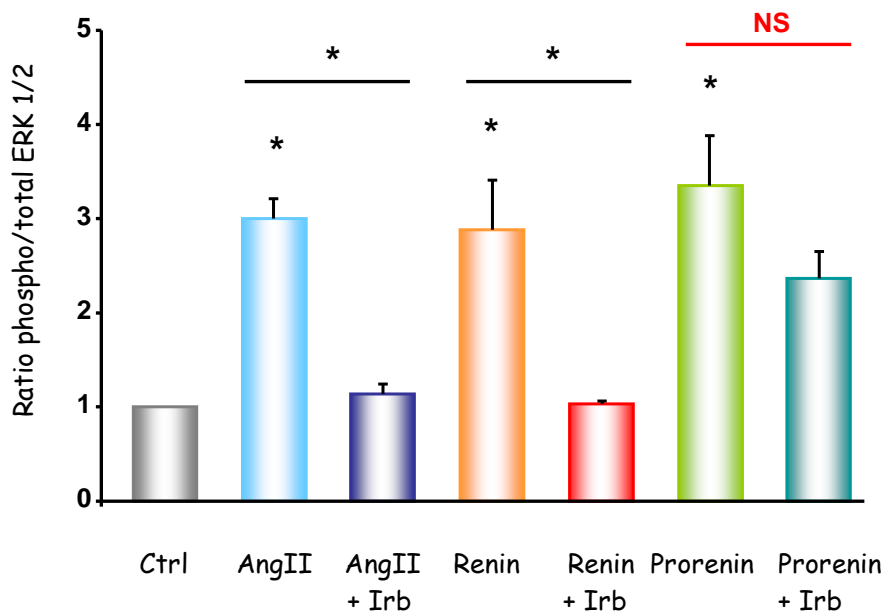
Fig. 14 Graphical representation of ERK 1/2 phosphorylation in H295R cells stimulated with Ang II 100 nM, renin 50 nM, prorenin 50 nM in presence or absence of irbesartan 5 μM. ERK 1/2 phosphorylation is expressed as the ratio of phosphorylated and total ERK 1/2. Each value is normalized for the control. Values are mean +/- standard error. Analysis was performed with ANOVA and Bonferroni post hoc test; *: $p \leq 0,05$
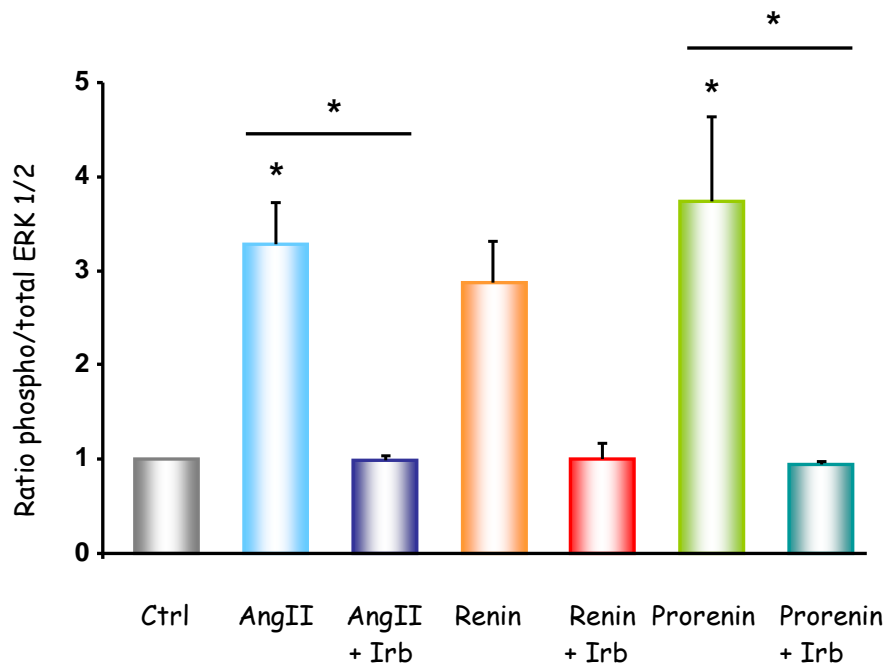
DISCUSSION

In spite of the high prevalence rate PA the underlying mechanisms leading to hyperaldosteronism are unknown. In fact, the excess aldosterone levels, with ensuing sodium and water retention and potassium waisting, and the high blood pressure that characterize PA lead to suppression of the renin angiotensin system. Therefore, the hypokalemia and the low-to-very low levels of active plasma renin, and thereby the undetectable levels of angiotensin II would be expected to blunt aldosterone secretion. As anecdotal reports suggested that prorenin levels could be increased in PA the discovery of the prorenin receptor (PRR), which is able to bind and activate both renin and prorenin and to induce ERK 1/2 and p38 phosphorylation, led us to hypothesize that PRR could play a pathophysiological role in PA. In fact the PRR was suggested to activate cellular growth and could thereby lead to adrenal hyperplasia or development of adenoma (APA).

PRR gene and protein expression in the adrenal gland

Previous studies reported PRR expression in different tissues, with the highest levels of PRR mRNA in the heart, brain, placenta, and the lowest levels in kidney, liver, and pancreas (Nguyen et al., 2002). However, no information were the expression available for the adrenal gland. Our findings of the PRR expression in normal human adrenal gland and in pathologic human adrenal samples can open new hints on the still elusive biologic role of the PRR.

Interestingly, we found high expression of PRR mRNA in the normal human adrenal cortex at a level of expression that was 15 fold higher than that of the housekeeping gene PBGD. Moreover, in APA the expression level was 12 fold higher and in two human adrenocortical carcinoma cell lines: H295R and HAC15 the level was also higher than that of PBGD (7-fold and 16-fold, respectively).

Immunohistochemistry experiments allowed us to confirm the PRR expression and also to reveal that the expression was not restricted to the human cortex. In the latter the PRR was expressed at high levels in the subcapsular zone, suggesting its implication in the cyto-physiology of the zona glomerulosa, which

is the main site of aldosterone production. However, the PRR was found to be expressed also in the medulla, thereby suggesting a role of PRR not only for ZG cell growth and aldosterone synthesis, but also a role for medullary cells and catecholamine metabolism.

Immunostaining of the entire rat adrenal cortex showed a progressive blunting of specific labelling from the sub-capsular zone to the inner cortex, presumably related to the more defined zonation in the rat as compared to the human cortex. Focusing our attention to the human adrenal cortex, we could then provide unequivocal demonstration for the presence of the PRR in the aldosterone producing ZG cells. In fact, we could demonstrate that CD56+ cells isolated from APA showed a marked PRR expression. These cells, which were obtained with an immune-separation based technique developed in our lab, entail a pure population of ZG aldosterone –producing cells (Caroccia et al., 2010).

Hence, taken together, these evidences are consistent with a role for the PRR in the normal ZG cells and in pathologic adrenal tissues from APA.

Subcellular localization of the PRR

After having successfully localizied the PRR in the adrenal tissue, we undertook immuno-blotting experiments on HAC15 and H295R after separation of the cytosolic and the membrane fractions. We also performed confocal microscopy experiments to investigate the subcellular localization of the PRR. We found that PRR is mainly expressed at the membrane level, where it partially co-localizes with the adhesion molecule CD56, which we previously showed to be expressed at the membrane level. However, since we detected PRR not exclusively at the membrane level, we analyzed two adrenocortical carcinoma cell lines and a pure population of human ZG cells with immuno-gold electron microscopy. An accurate analysis of these cells confirmed that PRR is localized not only at the cell membrane, but also in the nucleus, mitochondria and Golgi's vesicles. These findings fully agree with those obtained by other groups that investigated the PRR at the subcellular level in different tissues. In fact, both Advani et al. and Cousin et al. who focussed their attention on endogenous PRR (Advani et al., 2009; Cousin et al., 2009), as well as Schefe et al. who investigated immortalized

HEK293 cells transfected with short-interfering RNA (siRNA) against the PRR, showed that PRR is markedly expressed at the intracellular level (Schefe et al., 2008).

The recent evidence that the PRR makes complexes with the 13-subunit protein V-ATPase in the membranes of intracellular organelles suggests that also in the adrenal gland PRR may be present in this form.

Moreover, Nguyen et al. recently proposed a model of PRR processing (Nguyen and Muller, 2010), which well accounts for the intracellular PRR localization. They suggested that native PRR undergoes intracellular processing to generate three different molecular forms of PRR, a full-length integral transmembrane protein (PRR), a soluble PRR (sPRR), and a truncated form composed of the transmembrane and cytoplasmic domains associated with the V-ATPase. They proposed that a portion of the PRR is cleaved by furin in the trans-Golgi to generate a 28-kD soluble form, which is secreted, and a 10-kD transmembrane/cytoplasmic fragment that represents the truncated form, which remains associated with V-ATPase. The other part of PRR remains intact, and it may likewise complex with the V-ATPase to be addressed to the plasma membrane. These findings, independently obtained from different groups, suggest that the role of the PRR is much more complex than a simple receptor.

PRR activation induces ERK 1/2 activation

Prorenin receptor is a multifunctional receptor: PRR not only can bind renin increasing its catalytic activity by four- to fivefold, but also binds and activates prorenin by inducing enzyme activity comparable to that of renin. Moreover, PRR is able to induce a signal-transduction cascade upon ligand binding by activating the MAP (mitogen-activated protein) kinases (MAPKs) ERK1/2 and phosphorylating Ets-like gene (Elk) (Feldt et al., 2008; Funke-Kaiser et al., 2010; Huang et al., 2006; Huang et al., 2007b; Sakoda et al., 2007).

Activation of ERK1/2 was found to be independent from Ang II generation because ERK1/2 phosphorylation was observed after stimulating transformed human fetal mesangial cells (HMC) with prorenin in the presence of losartan

(Nguyen et al., 2002). These evidences collectively suggest that the PRR not only binds prorenin or renin, but is also able to convey intracellular signals.

After having showed that HAC15 and H295R cell lines are appropriate tools to functionally investigate the role of PRR in the adrenal gland, we measured ERK 1/2 activation induced by prorenin and renin in presence or absence of the angiotensin II AT1 receptor antagonist irbesartan. We found that both renin and prorenin, as well as angiotensin II, can induce marked ERK 1/2 phosphorylation. The evidence that ERK 1/2 activation caused by renin was prevented by preincubation with irbesartan in both cell lines suggests that ERK 1/2 activation is dependent on Ang I and Ang II generation and AT1 receptor binding. ERK 1/2 phosphorylation induced by prorenin was completely inhibited by irbesartan preincubation in H295R cells, but only partially suppressed (even not significantly) in HAC15 cells, thereby suggesting that the activation is independent from angiotensin I generation only in H295R cells. Different signalling pathways or different PRR expression levels might explain the discrepancies between the cell lines. However, further investigations are needed to evaluate the expression and activity of Rac, Rho and other signalling factors in the adrenal cells.

PERSPECTIVES

The results of this study suggest a role for PRR in the pathophysiology of primary aldosteronism. Further experiments are ongoing to clarify several additional questions arising from the present study. In particular we plan to better define a) the role of PRR in cell proliferation, differentiation and apoptosis; b) the PRR signalling pathways by analysing the ERK 1/2 and p38 phosphorylation induced by prorenin and renin in presence or absence of the direct renin inhibitor aliskiren; c) the levels of plasma prorenin in patients with primary aldosteronism and essential hypertension. These data will provide a better defined landscape of the role of PRR in the abnormal adrenal cell growth.

CONCLUDING REMARKS

By using a combined strategy entailing morphologic and functional studies we could demonstrate the presence of PRR at high level of expression in both the normal adrenal gland and in APA. As we could also show and ERK 1/2 intracellular activation induced by renin and prorenin, our results suggesta role for this receptor in human primary aldosteronism.

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