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From Angiotensin to Renin to Prorenin and from the Adrenal to the Kidney to the Placenta and the Lungs: An Historical Journey

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Abstract

In 1966 I carried out a study on the role of calcium on angiotensin's stimulant effects on the adrenal medulla. Since then I have been studying the renin-angiotensin system (RAS) for over a half-century in a wide variety of biological preparations, while awareness of its complexity has exploded. My journey has involved studies on genes, proteins, organelles, cells, tissues, glands, organs and whole animals. This chapter reviews what my colleagues and I have learned from these different levels of organization and is not meant to be an update on all features of the RAS. My studies have included experiments on: perfused cat adrenal glands; genetic and second messenger control of catecholamine synthesis and secretion from cultured bovine chromaffin cells and from rats *in vivo*; renin storage and release in the rat kidney and secretory granules; properties of isolated renin, prorenin and renin-like proteins; hormonal and second messenger control of prorenin secretion from human utero-placental tissues; renin/prorenin in a variety of tumors; and the effect of RAS drugs in a rodent model of pulmonary fat embolism. This most recent study has direct clinical application. I conclude with what I have learned about biomedical research and lessons for the future.

Keywords: angiotensin, renin, prorenin, prorenin receptor, renin-angiotensin system, adrenal medulla, lungs, kidney, placenta, chromaffin granules, calcium, fat embolism

1. Introduction

Awareness of the complexity of the renin-angiotensin system (RAS) has increased exponentially since it was initially considered relevant only to hypertension and has led to an explosion of understanding in biochemistry, molecular biology, cell physiology, anatomy, pharmacology, and pathophysiology. I have been involved in studies at all these levels in a wide variety of experimental models in animals and humans for over 50 years. This chapter is a review of what my colleagues and I have learned over the course of this half-century. This is not meant to be an update on all features of the RAS but rather the advances over the years in my personal research journey. It represents almost half of my total research publications.

This has included studies on the effects of angiotensin on the adrenal medulla in intact cat adrenal glands and cultured bovine chromaffin cells, renin storage and release in the rat kidney and secretory granules, properties of isolated proteins, hormonal and second messenger control of prorenin presence and secretion from human utero-placental tissues and renin/prorenin in a variety of tumors. These studies have implicated the RAS in a rodent model of pulmonary fat embolism syndrome (PFE) and showed that drugs acting at different steps in the RAS provided protection, suggesting that this approach could be useful in treating/preventing this potentially fatal condition. Investigating the RAS in many models in animals and humans should increase our understanding of normal and pathological processes and thus improve therapy/prevention of a variety of diseases. For the sake simplicity the term renin will be understood to mean total renin (renin + prorenin) unless specified otherwise. In extrarenal sites, prorenin may be the only one present and it can have some catalytic activity even without processing to the smaller protein renin, especially when bound to its membrane receptor.

2. Angiotensin and the adrenal medulla

2.1 Mechanism of short-term effects on the intact adrenal gland

In the early 1960s, evidence was presented that angiotensin II (Ang) could evoke the release of aldosterone from the adrenal cortex and catecholamines from the adrenal medulla, but the cellular mechanisms had not been completely identified. Since my colleagues and I had been mining the role of calcium in adrenal medullary secretion in response to acetylcholine [1], we decided to examine peptides in our studies and found that extracellular calcium was required for the stimulant effect of Ang [2]. Interestingly, one of the earliest demonstrations of the direct effect of Ang on adrenomedullary hormone secretion was carried out in 1963 in the laboratory of Wilhelm Feldberg at the National Institute for Medical Research in London [3] at which time I was working in the same lab on a different project during my postdoctoral training. Their study and our later one were carried out on the isolated perfused adrenal glands of cats. This is when I gained experience and insight into the value of using intact tissues in experimental studies without disruption of their cellular connections and revealed the immediate effect of treatment with peptides and amines. We could also stimulate the splanchnic nerve in these preparations to more closely simulate the natural signaling condition. Further studies on the role of calcium in stimulus-secretion coupling revealed its fundamental importance in exocytosis in exocrine secretion and neurosecretion [4–6]. This was summarized in several review papers [7–9].

2.2 Mechanisms of long-term effects on adrenal chromaffin cells

The next time that I had occasion to study the effects of Ang was over 20 years later when I was working on sabbatical in the laboratory of my former student, Dr. J.S. Hong, at the National Institute of Environmental and Health Sciences. His lab was interested in the long-term effects of agents on the adrenal medulla (as a surrogate for postganglionic sympathetic nerves) and the potential feedback on enzymes and peptides. In a series of experiments on isolated chromaffin cells, our group found that a stable Ang peptide (S-Ang) increased the secretion and expression of catecholamines and met-enkephalin as well as the mRNA expression of several catecholamine synthetic genes (tyrosine hydroxylase and phenylethanolamine

N-methyltransferase) [10, 11]. The time course of the response to S-Ang showed both short-term and long-term effects and revealed the increased expression of the oncogene c-fos [12] and its role in nuclear stimulation. These changes were mimicked by in vivo stimulation in rats by insulin [12]. These experiments directly implicated intracellular calcium as a second messenger leading to nuclear mRNA synthesis that required a lag time that followed catecholamine release [11].

2.3 Evidence for endogenous generation of Ang in chromaffin cells

We found evidence that endogenously released Ang from chromaffin cells could initiate the secretion of catecholamines and met-enkephalin from bovine chromaffin cells [13, 14]. It is likely that the enzyme responsible for this is renin since renin has been found in the adrenal medulla and in chromaffin cells [15]. Prorenin was not found in these cells in control animals. These results suggest that there may be some autocrine regulation of adrenomedullary secretion mediated by the RAS.

3. Renin and the kidney

3.1 Properties of renin substrate

In the 1970s, we turned our attention to studies on renin and the analytical method of the day was to measure the generation of angiotensin I using a protein or polypeptide substrate. The literature indicated that renin activity from various sources was not inhibited by the usual SH-targeting agents but was potentiated in some cases by the SH-protecting agent dithiothreitol (DTT). Since we intended to find a useful substrate, we first studied the interaction of DTT with renin and/or several renin substrates (angiotensinogen). The commercially substrate available at the time was hog renin substrate but we also prepared a semi-purified bovine substrate. We found that the potentiating effect of DTT was exerted on the substrate (bovine or porcine) and not on renin [16].

3.2 Subcellular storage of renin

Using our sensitive enzymatic assay and radioimmunoassay (RIA) for angiotensin I, we were able to initiate a number of studies on the storage of renin in the kidney using knowledge gained from our previous studies on secretory granules from the adrenal medulla [17] and the posterior pituitary [18]. The goal was to understand secretory mechanism for renin utilizing the secretory granules from the juxtaglomerular cells of the renal cortex. There had been a few studies on storage of renin but no systematic studies to understand their physical properties and the effect of isolation techniques. In our initial study we took into consideration what we had learned about the influence of temperature and pH on other secretory granules and found that crude rat kidney renin secretory granules were more stable when isolated at room temperature (22–25°) than at 0° and were most stable at pH 6.0 [19]. They were also somewhat stabilized by MgATP unlike adrenal chromaffin granules [20]. Later studies with more purified granules confirmed that the granules were more stable at room temperature but were labile if transferred from hyperosmotic density gradient media back to physiological tonicity [21]. When these purified granules were incubated at 37° instead of room temperature they again showed lability when they were subsequently incubated at 0° [22]. In order to avoid the problems with isolation in hypertonic media, we employed density

gradients with lower osmotic properties and at room temperature. These granules were stable as long as there were not transferred to 0° media and kept not far from pH 6.0 [23]. Granules prepared in isotonic density gradient media showed two peaks with short term centrifugation that was resolved with longer term preparation, suggesting that renin granules are of two sizes with the same density [23].

3.3 Renin-like activity in the rat *in vivo*

When we began to study renal granules, we became aware of a study of another angiotensin I-generating enzyme that had a lower pH optimum and preferred the tetradecapeptide substrate rather than the protein substrate. They called it pseudorenin [24]. It was found in rat plasma and a wide variety of tissues and in much higher concentrations in the salivary gland and the spleen than in the kidney. Since we could not find any physiological studies of pseudorenin in intact animals, we examined the changes in plasma pseudorenin and renin in rats after nephrectomy and in response to converting enzyme inhibition and beta-adrenergic receptor blockade. We found that, unlike renin, plasma pseudorenin increased after nephrectomy and treatment with propranolol but did change after angiotensin converting enzyme treatment [25]. Later we examined bovine spleen and provided evidence that pseudorenin is cathepsin D [26].

4. Renin in human tumors

4.1 Enzymatic analysis

Although there had been several reports of increased renin activity in serum or tissues of patients with renal tumors using bioassays for analysis [27, 28], none had followed the clinical course and biochemical evaluation of the patients and utilized agents interfering with angiotensin to modulate the course of the disease. We reported on a patient with bilateral Wilms tumor (nephroblastoma) who exhibited congestive heart failure, hypertension and elevated serum renin using a more contemporary radioimmunoassay and an international standard [29]. We found the patient's clinical course and tumor size in response to surgery and chemotherapy were paralleled by serum renin concentrations (PRC) and his hypertension was ameliorated by saralasin, a peptide angiotensin receptor antagonist (this was before non-peptide ARBs were available). We were able to assay samples with exceedingly high renin concentrations that would not be possible with simple plasma renin activity (PRA) assays since these high dilutions would reduce the available endogenous substrate to suboptimal levels. For our assays, we used substrate from nephrectomized sheep that was known to be a better substrate for human renin. Plasma renin concentration was over 4600 $\mu\text{U}/\text{ml}$ before therapy (normal 30–90 in our lab) and fell to 69 after chemotherapy and surgery. A few months later the tumor size increased and so did the renin concentration [29]. A partially resected tumor mass was found to contain renin by immunohistochemical and biochemical analysis (1245 $\mu\text{U}/\text{g}$).

4.2 Immunohistochemical analysis

To utilize a non-enzymatic method to localize renin, we utilized a specific antirenin antiserum and examined several non-renal tumors. In preliminary studies we found renin and prorenin in complete and incomplete hydatidiform moles [30, 31]. By 1990, prorenin was a widely accepted name for what had one

time been called “inactive renin” and we reported on its presence in cyst fluid and ascites in patients with ovarian tumors [32].

5. Renin/prorenin in human amniotic fluid and amnion

5.1 Purification and properties

When considering the possibility of alternative forms of renin, we noted the report of high concentrations of renin in human amniotic fluid [33] and sought to purify the enzyme from this source. We noted that the original description of renin in amniotic fluid included a step of acidification and that it was subsequently found that this caused an activation of “inactive renin”. We compared chromatographic and kinetic properties of endogenous renin, acid- and pepsin-activated renin using bovine and hog substrate and found differences between acid- and pepsin-activated renin [34]. Further purification of the inactive renin allowed separation from the pseudorenin mentioned above that was similarly inhibited by pepstatin [35, 36]. We developed an assay utilizing a single tube for renin-generated angiotensin I and the subsequent radioimmunoassay which greatly facilitated these studies [37]. We also showed that both prorenin and active renin were inactivated by ethyl diazoacetyl glycinate, a compound known to inactivate aspartyl proteases but not pepsinogen [38].

5.2 Localization, synthesis and release from amnion

We initially demonstrated the presence of the renin and prorenin with both acid and trypsin activation using nephrectomized sheep plasma substrate. This showed, like amniotic fluid, that the bulk of the potential angiotensin-generating activity was in the inactive (IR)-prorenin form (about 70% in these samples) [39]. Our immunofluorescence study, using antiserum to human kidney renin, showed that the positive cells were the amniotic epithelial cells and not contaminating chorionic cells [39]. We noted at the time that early initial attempts to show synthesis by cultured amniotic cells were negative [40]. Those studies included bioassays of samples that had initially been treated at pH 3.0 so some prorenin would have been activated and recorded as renin [40]. In our study where we did not expose samples to low pH and used trypsin activation to assay IR, we found no IR or R in medium from cultured amniotic cells although similarly cultured chorionic cells produced enormous quantities of IR/prorenin that sometimes required a dilution of 1000× to bring the samples into the assay range [41].

In another model, to assess the potential synthesis of IR from the amnion, we superfused separately the amnion and the chorion from a clamped fetal membrane in a Ussing chamber device. We found that there was a dramatic and increasing release from the chorion side but a low and decreasing amount from the amnion side [41]. Furthermore, when a high concentration of IR previously released on the chorion side, was superfused on the chorion side, there was no increase on the amnion side, thus excluding leakage or transport [41]. Since there are many sources of prorenin in the human uteroplacental complex at term pregnancy [42], there could be more than one source of its presence in amniotic fluid. That could include uptake from fetal urine since prorenin has been found in urine [43] and an uptake system in amnion has been reported [44]. The latter group used amnion explants and found very low levels of renin mRNA and extremely low levels of prorenin protein release but considered that the decidua could be the source of amniotic prorenin. There was no evidence of *de novo* synthesis in our experiments using undigested amnion [41].

6. Prorenin production and release from human chorion and chorionic trophoblasts

6.1 Purification and properties

After determining that the amnion was not a likely source of prorenin in the amniotic fluid, we turned our attention to the other major fetal membrane, the chorion, and more specifically the chorion leave (free chorion). In our initial report [45], we noted the early work that suggested that Hofbauer cells (fetal macrophages) and not trophoblasts or fibroblasts were the renin-containing cells, using the Bowie stain that showed renin in the kidney. We used an immunofluorescence technique and employed an antiserum to a highly purified renin preparation from human kidney that we subsequently realized recognized both renin and prorenin. We found that the renin immunoreactivity in the chorion was strictly localized to the cytotrophoblast layer [45]. Subsequent biochemical studies showed that the “inactive renin” from chorion and culture medium from chorionic cells is definitely prorenin [46]. In addition, we found that this same cell layer was positive for hCG [47] and there was a relatively constant ratio of renin/hCG in purified chorionic cells: 5.14 μ U renin/mIU hCG. Here and elsewhere the terms renin and prorenin and IR are used interchangeably except where noted since all or almost all the renin is present as prorenin.

6.2 Release of prorenin and hormones from intact membranes and isolated cells

We used our superfused membrane preparation to show that both prorenin and hCG were released at a constant or increasing rate even after 80 minutes [47]. An interesting finding from our studies on the superfused chorion was the short term release of prorenin by angiotensin II [48]. We also found that angiotensin induced the release of LHRH (GnRH)-like activity from this preparation [49]. In parallel experiments we demonstrated specific binding of angiotensin II to these cells [49].

Since the purified chorionic cells could be grown in tissue cultures for many days, we were able to examine factors that might modify synthesis and release in the short or long term. We could even grow these cells for periods up to 3 months without them losing their capacity to synthesize and release prorenin [41, 48]. When we examined these cells for the steroid hormone progesterone that had been reported to be present in the chorion, we found they indeed did contain progesterone and its synthesis and release could be promoted by various precursor steroids. (pregnenolone and 25HC) [50]. The amount of progesterone released greatly exceeded the amount initially found in these cells and the synthesis and secretion were both promoted by agents acting to raise cyclic AMP (cAMP) [50]. These included dibutyryl cAMP, methyl isobutyl-xanthine (MIX), forskolin and cholera toxin. Prorenin secretion by these cultured cells was also promoted by MIX and cholera toxin and especially by cholera toxin in the presence of phorbol myristate acetate (PMA), a protein kinase C agonist. In some cases, the concentration after 72 hours of incubation with these agents reached 700,000 IU/ml. This was not due to an increase in cell numbers since these were confluent monolayers [48]. The dramatic potential for term chorion to synthesize and release prorenin clearly differentiates the secretory process from the renal secretion of renin where it is stored primarily in dense secretory granules and is presumably released by conventional regulated exocytosis. We have found prorenin in the chorion to be mostly in the cytoplasmic fraction of the tissue and not in particulate fractions [49].

7. Prorenin production and release from decidua

7.1 Primary and second messengers

After a report in 1989 that decidual cells have the capacity to synthesize and release active and total renin from decidua [51], we turned our attention to the maternal portion of the feto-placental unit. The first potential hormonal/primary messenger that we examined was relaxin [52]. This hormonal messenger was known to be present in chorion and decidua [53]. We found that renin released from cultured purified decidual cells was 95% prorenin when we did not expose the samples to acidification as was done in the earlier report [51] and that relaxin caused a dose-dependent increase in release that was paralleled by an increase in tissue prorenin and was inhibited by cycloheximide [52]. This was consistent with new protein synthesis. We cited the views at the time on relaxin's potential effects on uterine ripening and decidual prolactin release. This was one of many pieces of our studies on the utero-placental complex that pointed to paracrine or autocrine effects in supporting local autonomy.

The next potential positive regulator of prorenin release that we examined was endothelin since it was known to be present in the placenta and had been found to modify renin release from the kidney. We found that several endothelin peptides caused a dose-dependent release of prorenin that was associated with an increase in renin mRNA [54]. The release was greater than the control content and was not associated with the release of cellular prolactin. This was another clear example of prorenin secretion by the protein synthesis-dependent constitutive secretion. Further studies on the effect of endothelin (ET-1) on prorenin release showed a clear difference from the control of prolactin (PRL) release. The calcium ionophore A-23187 stimulated basal prorenin release and potentiated ET-1 stimulated release while having no effect on PRL release; and the calcium channel blocker nifedipine blocked the effect of ET-1 on prorenin but had no effect on PRL [55]. The protein kinase C agonist PMA stimulated basal and potentiated ET-1 induced prorenin release but inhibited basal PRL release and potentiated the inhibitory effect of ET-1 [55]. Finally, the PKC inhibitor staurosporine increased basal PRL release and reversed the inhibitory effect of ET-1 on PRL release. These results indicate that prorenin and PRL release from decidua are affected in different directions by protein kinase C and that prorenin release is dependent on extracellular calcium but PRL release is not [55]. In addition to protein kinase C and calcium, we also studied the influence of cyclic AMP (cAMP). We found that agents which elevated cAMP in decidual cells also stimulate Pro release. These included forskolin, cholera toxin (CT) and dibutyryl-cAMP [56]. Ninety-eight percent of the renin was in the form of Pro. PMA potentiated the effects of CT and dibutyryl cAMP. These studies had therefore implicated cAMP as well as protein kinase C as second messengers in Pro release from decidua.

7.2 Cytokines and prorenin secretion

After a report that lipopolysaccharide (LPS) and tumor necrosis factor- α (TNF) stimulated prostaglandin production by decidua [57], we examined the effects of these agents on prorenin release from our semi-purified decidual cells. We found that LPS inhibited the synthesis and release of both Pro and PRL from the decidual cells in a time and dose-dependent manner [58]. We noted at the time that the inhibitory effect of LPS might be mediated by the release of cytokines from macrophages and then a paracrine effect on stromal cells could ensue.

We also indicated that it could also be due to a direct effect on the stromal cells. We followed up this study by an examination of the effects of two other cytokines, TNF and interleukin-1 β (IL-1 β). We reported that these cytokines inhibited synthesis and release of renin from cultured decidual cells in a dose-dependent manner [59] and noted that the cells that were initially plated were composed of 22% macrophages (CD-68-staining) and 78% PRL positive (the other major cell type in decidual cells). We therefore concluded that the effects of these two cytokines could have been mediated by their known actions on macrophages. There was no inhibition of DNA synthesis or cell number. It was of interest that the effects of these cytokines was opposite to those on the rat renal tissues where there was an increase in renin release which occurred in minutes [60] unlike the decidual release that took days [59]. The third cytokine that we examined was interferon- γ (IFN γ) which was known to have receptors on placental cells. We found that IFN γ inhibited Pro release and its mRNA expression in decidual cells. When we employed an additional step of purification using immunomagnetic beads to separate the macrophages, we found that renin release from both populations of cells was inhibited by IFN γ and TNF and the combination of these two cytokines was even more effective in producing inhibition of release. Since IFN γ mRNA was found only in the macrophage population, while the IFN γ receptor was found on both, it suggested that the effect of locally produced IFN γ on renin release from macrophages could result from both autocrine and paracrine mechanisms while effects on stromal cells would be paracrine in nature [59].

7.3 Release of prorenin from macrophages and monocytes

Since we knew that macrophages represented a significant portion of decidual cells at term pregnancy and represented about 22% of our decidual cell preparation, we decided to examine directly whether these cells could also be a source of prorenin. We utilized a method employing immunomagnetic beads after coating the macrophages with HLA-DR antibody to separate the macrophages from stromal cells. This increased the portion of HLA-DR (+) cells from 22 to 93%. The purified cells no longer showed mRNA for prolactin which was abundantly expressed in the non-macrophage population [61]. These cells stained for renin with a specific antibody, expressed renin mRNA and released prorenin into culture medium during 3 days of culture. They did not release prolactin. Importantly, the non-macrophage cells also stained positively for renin and released the same amount of renin per μ g DNA per cell as the HLA-DR (+) cells. They also did not stain for a cytokine receptor that was present in the macrophage fraction [61]. These results indicated that both types of decidual cells had the capacity to synthesize and release prorenin and strengthened the case for possible autocrine/paracrine signaling. In addition, we collected some peripheral blood monocytes and demonstrated that they also showed mRNA for renin and speculated on some potential functions of the RAS within the uteroplacental complex [61].

7.4 Regulation of renin expression and secretion in differentiated monocytic cells

To study the expression and regulation of renin in a pure cell line, we employed the well-studied U-937 cells which can be differentiated into a terminal macrophage/monocyte phenotype using phorbol dibutyrate (PDBU). We found that the treatment did cause a morphological change that was identical to those reported in the literature [62, 63]. The differentiated cells expressed renin mRNA and released prorenin into culture media [64]. We first looked at the potential regulation by cAMP, which we had found to be important in prorenin release from decidual and

placental cells [56, 65] and others had found important in renal juxtaglomerular cells [66]. Renin mRNA and prorenin release were increased by dibutyryl-cAMP, and forskolin. In addition, terbutaline, a β_2 -adrenergic agonist known to increase c-AMP, also increased expression and release of prorenin [64]. The stimulation by terbutaline was potentiated by a type IV c-AMP-phosphodiesterase (PDE) inhibitor. It was known that these cells possess β_2 -adrenergic receptors and the type IV PDE. The stimulatory effect of terbutaline on renin secretion was inhibited by an angiotensin receptor agonist and also by TNF and LPS+ IFN γ [64]. Taken together with our studies on isolated decidual macrophages [61], these results reinforced the potential importance of some components of the RAS in the function of macrophages and other bone marrow-derived cells. They also highlight the possible positive and negative autocrine actions of local mediators.

8. Studies on villous placenta

8.1 Gestational differences in the RAS in placentas

The villous placenta at term has very low concentrations of renin with higher concentrations in decidua and chorion [67, 68]. We hypothesized that the renin concentration might be influenced by gestational age as influenced by alterations in hormonal milieu and found that this was indeed the case [69]. We found that there were dramatically high levels of prorenin and active renin in first-trimester pregnancies: prorenin was 1130 μ U/mg protein in the first trimester vs. 5.9 at term; the corresponding values for active renin were 330 vs. 0.15. As might be expected, the values for hCG in the first trimester were also greater than at term (2396 vs. 38.6 ng/mg protein). However the levels of hCG and prolactin in decidua did not change much during gestation and there was no detectable prolactin at any stage in placenta [69]. Placental prorenin correlated with chorionic gonadotropin but not prolactin in both groups. and could reflect similar cellular origins.

8.2 Experimental preparations to study the RAS in human placenta *in vitro*

An early preparation that we used was a superfused placental mince that allowed investigations of mostly intact cells with normal cellular contacts over a period of many hours. With this preparation we showed that there was a dramatic increase in prorenin release beginning after 12 hours, reaching levels of 16 μ U/ml at 26 hours from a basal level about 0.5 or less [70, 71]. This spontaneous increase was blocked by cycloheximide and actinomycin D, supporting the conclusion that it required new mRNA and protein synthesis, like our results on decidua and chorion. We also showed that the spontaneous release could be amplified by treatment with relaxin [72]. Further evidence of the increase in synthesis of prorenin was found when we measured the tissue content of superfused placental minces after superfusion for 24 hours with or without the adenylyl-cyclase stimulator forskolin. This model was useful for rapid kinetic measurements, but the disadvantage was interruption of much cellular connections.

Another model that we used was the dually perfused human cotyledon which allowed nutrients and drugs to reach cells through vascular channels and permitted assessment of vascular reactivity. It was known that AI and AII produced dose-dependent pressor responses which were blocked by the angiotensin antagonist saralasin and the response to AI was blocked by captopril [73]. We showed in this preparation that there was no renin released into the fetal circulation but there was consistent release into the maternal circuit [74]. It was all prorenin.

This preparation suggested that renin in fetal circulation *in vivo* was not coming directly from the maternal vasculature. The advantage of this model was the greater integrity of the *in vitro* system, but it was restricted by logistical considerations to the number of different preparations that could be set up at one time.

The most useful model that we employed retained much cellular connections and could be used over longer periods of time. That was based on an early model of placental explants, sometimes called organ culture [75]. We found the optimal conditions by putting the explants on top of wire-mesh platforms and keeping the fluid level at the surface of the tissue. With this model we examined potential primary and secondary signals in regulation of the placental RAS.

8.3 Prorenin secretion: primary messengers and modulators

It was known in humans that renal renin secretion was stimulated by catecholamines and selectively by β -1 adrenergic agonists [76] and that the villous placenta had both β -1 and β -2 adrenergic receptors [77]. When we studied the effects of epinephrine and beta-adrenergic agonists on placental renin secretion from placental explants, we found that both β -1 and β -2 adrenergic agonists elicited renin secretion, associated with an increase in synthesis [78]. Again, this was about 95% trypsin activatable and presumably prorenin. This is consistent with the view that extrarenal renin in the human reproductive track is almost exclusively prorenin [54, 79]. We discussed the likelihood that beta-agonist-induced renin secretion would be regulated by activators produced by the fetus [78]. At the same time, we found that hCG secretion was selectively stimulated by the β -2 adrenergic agonist terbutaline and that its stimulant action was blocked by a selective antagonist. We showed that the stimulant effects of beta-adrenergic agonists on both renin and hCG secretion were potentiated by selective inhibitors of phosphodiesterase types III and IV [78]. The differences in agonist selectivity between renin and hCG secretion was consistent with findings on their respective localization in term placenta, with renin in cytotrophoblasts and hCG in syncytiotrophoblasts [80].

The likelihood that renin and hCG are released in close proximity to one another suggests that there might be some paracrine regulation involved. With this model system we provided evidence that hCG stimulates renin secretion and tissue levels [81]. Furthermore, the stimulation was potentiated by phosphodiesterase inhibitors, just like renin secretion, and was accompanied by an increase in media cAMP. The effect of hCG was markedly attenuated by the protein kinase A inhibitor H-89. These results suggested that placental renin secretion may be regulated in part by hCG and mediated by cAMP transduction mechanisms [81]. Further support for the influence of cAMP on renin secretion is presented in Section 8.4.

A possible negative regulator on renin release, based on what has been found in studies on cultured juxtaglomerular cells (JG), is angiotensin II, where a purported negative feedback loop reveals an inhibitory action [82]. We found that a stable analog of angiotensin II inhibited the spontaneous release of renin from placental explants during the 72 hour incubation [71, 83]. This paralleled a study where transfected JG cells released prorenin and not renin [82].

Other negative regulators of placental renin secretion that we identified included LPS and the glucocorticoid dexamethasone. They both inhibited spontaneous and stimulated renin release [71]. These agents act at many different sites, including macrophages, so their influence on renin secretion is complicated.

8.4 Prorenin secretion: second messengers and nuclear signals

We had already identified cAMP as a second messenger for chorion and decid-
ual renin release and we examined if similar mechanisms existed in placental

prorenin secretion. Evidence for cAMP mediation was supported by our finding that renin release from explants was stimulated by several orders of magnitude by forskolin and by cholera toxin (CTX) [83]. The effects were potentiated by a cAMP phosphodiesterase inhibitor. The enhanced release of renin was accompanied by an increase in hCG found in the media. It is important to note that prolactin was not detected in the media, thus excluding decidual contamination. This was also supported by the fact that the phosphodiesterase inhibitor did not cause an increase in renin release from similarly-treated decidual explants [84]. Not only did the tissue levels of renin increase indicating new synthesis, but there was a decrease in LDH leakage demonstrating cellular integrity [83]. An interesting finding was that an angiotensin II agonist inhibited both the spontaneous and the CTX-enhanced release of renin. This effect was blocked by an angiotensin receptor antagonist. Further evidence on the role of cAMP came from our studies on cAMP-dependent protein kinase (cAPK) [85]. We found that the dobutamine-stimulated secretion of renin and cAMP was accompanied by an increase in tissue cAPK. We used substituted analogues of cAMP, selective for binding sites on cAPK, and found that site B analogues which bound to catalytic or regulatory sites were stimulants of renin secretion but that site A analogs were not [85]. Strengthening the case for the role of cAPK in dobutamine-induced renin secretion, we found that the specific cAPK inhibitor H-89 blocked secretion and an activator SP-cAMPS stimulated secretion [85]. We then used molecular biology techniques to assess the role of mRNA synthesis in the stimulation of renin secretion, using the β -1 agonist dobutamine and the β -2 adrenergic agonist terbutaline. These agents both increased renin mRNA in a dose-dependent manner which paralleled their effects on renin secretion and tissue levels [86]. The effects on renin secretion and tissue levels were blocked by cycloheximide (a translational inhibitor in protein synthesis) and actinomycin D (a transcriptional inhibitor which acts directly on DNA). Actinomycin D blocked the increase in renin mRNA but cycloheximide did not, thus showing the specificity of these agents and the importance of gene regulation in adrenergic stimulation of placental prorenin secretion [86].

Other second messengers that have been studied in a wide variety of tissues including the placenta include eicosanoids, protein kinase C and calcium. Prostaglandins have been known to influence renal secretion of renin and are actively secreted by utero-placental tissues. We reported that meclofenamate, a relatively selective inhibitor of cyclooxygenase, inhibited the release of renin from placental cells and (unpublished studies) that it also inhibited the ET-1 induced renin release from decidua [71]. As with the decidua, the protein kinase C agonist PMA increased renin release from placental explants and the enzyme inhibitor staurosporine was inhibitory [71]. The influence of calcium was contrary to many, but not all, studies on the kidney where calcium is considered an inhibitory messenger on renin secretion. Extracellular calcium caused a dose-dependent increase in short-term renin release between 1.0 and 3.6 mM in contrast to the kidney. It should be noted that there are some studies on renal tissues in special situations where calcium is a positive regulator [87–89] and other studies on extra-renal renin secretion where calcium also has a positive influence on renin secretion [90, 91]. Similarly, angiotensin was shown to have inhibitory effects on the synthesis and release of renin from placental explants but was stimulatory during short term exposure of superfused chorion [48].

These studies on different anatomical portions of the utero-placental complex suggest that local conditions and times of gestation can modulate the regulation of the RAS and that studies on single cells may be missing the complex interactions that exist *in vivo*.

9. Role of the RAS in pulmonary fat embolism

9.1 Fat embolism model in rats

After 40 years in academia and an enormous expansion of information in the RAS field, I retired. It was short lived because I still wanted to see new developments and help in obtaining information. That is why I was eager to join (and help revitalize) a project on fat embolism that had been dormant for 40 years. Dr. Federico Adler, a retired orthopedic surgeon, asked for my help in restarting a study of fat embolism in rats that he had worked on in the 1960s. My focus was gaining evidence on the potential role of the RAS in fat embolism syndrome, a sometime fatal consequence of long bone fracture (and some other conditions). This brought me back to *in vivo* studies with its advantages and disadvantages. We initially did time- and dose-related studies with intravenous dosing of the triglyceride triolein and used histochemical methods to evaluate pulmonary pathology. We found that there was an early phase beginning very early and peaking at 48 hours [92] with inflammatory, fibrotic and vasoconstrictive effects.

9.2 Effect of RAS drugs on pulmonary fat embolism

Since there were reports that some RAS drugs had beneficial effects in other types of pulmonary injury, we examined the effects of the angiotensin converting enzyme inhibitor (ACEI) captopril and the type 1 antagonist (AT1) losartan when given 1 hour after the triolein. Both agents provided significant protection against the histopathological effects when viewed at 48 hours [93] and provided strong evidence that the acute effects of fat embolism involved the production of angiotensin II and actions on the AT1 receptor. In a later study we found that the pulmonary injury was also ameliorated by the renin inhibitor aliskiren [94].

We subsequently determined that the initial acute phase after fat embolism was followed by a slowly developing smaller inflammatory response and this was associated with an increase in the presence of angiotensin peptides [95]. Since there were still some fat particles present at this later time period, we suggested that one mechanism could be the continued activation of macrophages that were engulfing the fat and signaling mast cells (and perhaps other cells) to release renin and then local angiotensin release. Some support for this view came from two further studies. In one, we gave the AT1 blocker losartan 6 weeks after the triolein injection and examined the rats 4 weeks later. In this experiment the protective effect of losartan was still demonstrated at this late stage, supporting the view of continued activation of the RAS [96]. In another study we found that 24 and 48 hours after triolein there was an increase in renin staining in lungs that diminished but was still present at 3 and 6 weeks [97]. The renin staining increased again at this late stage when the rats were treated with lipopolysaccharide (LPS) [97] which was known to interact with the RAS [98]. Since we had suggested that some of this renin could be in mast cells, we examined the presence of mast cells in triolein-treated rats and the influence of losartan. We found that 10 weeks after triolein there was an increase of mast cells and this was attenuated by losartan [99]. Addition of LPS at 6 weeks caused slightly more mast cells and this was also blocked by losartan. We also considered that macrophages could be a source of renin.

In several of our papers, we have suggested that the RAS drugs could be useful in the treatment or prevention of fat embolism syndrome and there are some other findings of potential clinical interest. The lungs from a pregnant patient who had succumbed to a pulmonary fat embolism were examined at our affiliated hospital

and showed the same kind of histopathological changes that we had observed on our rat experiments [100]. Another point of possible clinical interest was our finding that 6 weeks after fat embolism when the animals appeared normal and had grown as well as the saline-treated controls, they were especially sensitive to a “second hit” with LPS [101]. The potential clinical relevance of these findings is that patients who have severe respiratory distress more than would be expected from their presenting diagnosis could be suffering from a “second hit” a long time after a forgotten trauma which has left a smoldering low-grade inflammatory process continuing in the lung. A recent review of our studies on fat embolism syndrome has been published online [102] that implicates the RAS as a key component of this condition.

10. Role of the RAS in homeostasis and lessons for the future

10.1 Historical developments as seen through my research journey

The latest part of my journey related to the RAS (which is still ongoing) comes 53 years after my first paper mentioning angiotensin [2] and 59 years after my first scientific paper as a medical student [103]. Although I have enjoyed working in many areas of biomedical research, it has been very gratifying to see this major part of my career get closer to the long-range goal of improving health care for people in need. An ironic and maybe not surprising development is that my early and long-standing studies on chromaffin granules and their ATPase [17, 20, 104–107] have come full circle with the finding that the granules contain renin and prorenin [15] and their membranes contain the prorenin receptor [108, 109]; and it is now known, but not in the 1960s, that there is a receptor for renin/prorenin (P)RR that can act on second messengers independent of the RAS [110, 111]. It was also not appreciated that there was an opposing arm of the RAS that could antagonize many of the deleterious effects of the angiotensin-ACE-AT1 receptor axis, and it is ubiquitous in distribution outside and inside the cell [112–115]. My studies and the current literature suggest that it will be difficult to find any extra-renal system that does not have some components of an endogenous RAS.

10.2 Lessons that I have learned about biomedical research

I will conclude with my views on ways of approaching biomedical research based on my experience and lessons that I have learned.

10.2.1 Pharmacological lessons

Many of these lessons were not appreciated when I was a medical student.

- a. Low doses of drugs may have opposite effects of higher doses, the hormetic effect. Sometimes this is because receptors of different sensitivity are activated as the dose is increased. Other times this may be due to non-receptor mediated effects, such as enzyme inhibition. An example in the RAS is the activation of AT2 receptors by angiotensin II opposing the actions on AT1 receptors and the activation of Ang (1–7) receptors as angiotensin II is converted. This is further discussed below under moonlighting (10.2.d.).
- b. Species differences: It has long been clear that other species may have differences in metabolism, pharmacokinetics, morphology and a host of features

that make direct extrapolation to humans problematic. Also, mice and rats are not equivalent when compared to human biology.

- c. Short term vs. long term experiments: Many experiments have shown biphasic response to drugs with opposite effects seen depending on when the observations were made. This is an argument for examining time-response curves in addition to dose-response curves. This has led in the past to some studies missing a response by looking at a single time-point.
- d. Response of young animals (organs) may not be the same as that of older ones. This has been obvious for a long time since the changes during maturation in animal biology has long been appreciated. A striking change during development in a single organ has been found in the human placenta as we have noted in our studies on the RAS. Of course, the placenta is a unique organ and has features of many other organ systems, including the liver, kidney and endocrine and nervous systems among others.

10.2.2 Biochemical lessons

Another lesson that was not appreciated is that of moonlighting. This is the area pioneered by the ground-breaking research of Constance Jeffery from 1999 [116] and still expanding in 2018 [117]. It appears that prorenin and the prorenin receptor are archetypes of multi-functional proteins. Prorenin (a) serves as the zymogen precursor of renin that follows cleavage of the prosegment; (b) becomes a catalytically active enzyme without cleavage when bound to its receptor, (c) activates a surface membrane receptor coupled to the generation of intracellular kinases, and (d) likely serves in several capacities intracellularly [118]. This complexity was not imagined at the time we were calling this 'inactive renin'. The prorenin receptor ((P)RR) also has multiple functions, some not related to angiotensin peptides [119].

10.2.3 Experimental lessons from genes to molecules to intact humans

I have carried out experiments using extreme reductionist approaches, such as studies on isolated proteins and on gene expression. Some of our studies were on isolated organelles and on isolated cells. These types of experiments removed many cells from their natural environment and from potential neuronal, paracrine, or endocrine modulation. The next step up in complexity were studies on isolated perfused glands or tissue slices where there was some contact between different cells as in the intact animal but still not complete signaling from the entire organism. Finally, I have studied the effects of drugs on whole animals *in vivo*, including humans. The result of this wide range of studies has provided me with the following perspective. No one approach will give us a complete picture although bringing us closer to a true view of what is going on in health and disease. Our reductionist approach strives to reduce as many variables as possible and each provides some useful information. However, many interactions at higher levels of complexity may be lost or overlooked. Only experiments on normal humans could come close to a real world understanding but that is beyond ethical consideration. In addition to the reservations listed above, our studies do not take into consideration the epigenetic, environmental and social factors that influence how we interact with internal and external stimuli. Some of this newer point of view includes the field of hormesis [120].

Finally, I have been fortunate to have chosen to study the RAS for much of my research career since this system seems to be ubiquitous throughout biology and has only in the past five decades begun to reveal the many ways we depend upon its proper regulation to maintain our health and suffer when it is out of control.

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