Implications of intracrine hormone action for physiology and medicine

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PEPTIDE HORMONES and growth factors are known to act by binding to cell surface receptors and subsequently activating intracellular mediators of signal transduction. Over the last three decades evidence has accumulated to indicate that at least some peptide hormones and growth factors also bind and act in the cellular interior either after internalization by target cells or retention in their cells of synthesis (1–67). Several years ago our laboratory (48, 53) proposed the term intracrine for such intracellularly acting peptides hormones irrespective of whether their binding/action followed internalization or occurred in the cell that synthesized them. In fact, many intracrines act in both fashions. Intracrines not infrequently are synthesized as isoforms, and in some cases, one isoform binds to an intracellular site in its cell of synthesis while another is secreted (47, 51, 52). In some cases, intracellular intracrine binding can be associated with clear biological effect. For example, the angiogenic protein angioten- gen stimulates endothelial cell proliferation, either directly or indirectly, only after translocation to the nucleus of target endothelial cells (34, 47, 51, 52). A similar dependence on nuclear translocation for the stimulation of proliferation is displayed by fibroblast growth factor-2 (FGF-2) (3). In some cases, the intracellular action of an intracrine factor is similar to the action the factor exerts after cell membrane binding, but in some cases it is not. For example, parathyroid hormone-related protein inhibits mitogenesis of vascular smooth muscle cells following cell surface binding but stimulates mitosis following nuclear binding (1, 2, 47, 51, 52). Although in many cases biological action has been shown in association with intracellular intracrine binding, insufficient work has been done to date on the possible intracellular actions of peptide growth factors/hormones to make demonstrated intracellular biological function a requirement for ascribing intracrine status to a protein. Rather, the binding of a signaling protein to an intracellular organelle not associated with the secretory or degradatory pathways, or the demonstration of an intracellular action of bound hormone, will be taken here as evidence of intracrine status. Although intracrine binding need not be restricted to any one intracellular organelle (for example, angiotensin II binds to both nuclei and mitochondria), the great majority of intracrines reported to date traffic to nucleus and often to nucleolus.

Implicit in the definition of intracrine employed here is the fact that intracrine status is defined functionally (i.e., by intracellular binding/action of a peptide factor that is active outside the cell) rather than structurally (except insofar as all intracrines are here required to be peptides). As it happens, a great structural diversity is to be found among intracrines. Cytokines such as interleuken-6, G protein-coupled receptor activating hormones such as angiotensin, growth factors such as insulin, as well as homeoproteins such as Engrailed 1 and 2 and Hoxa 5, and enzymes such as platelet-derived endothelial cell growth factor (PDECGF-thymidine phosphorylase) all are putative intracrines (Table 1) (6, 32, 43, 47, 51, 52, 65, 67). With the exception of renin-prorenin, all the factors listed in Table 1 have been reported in the nucleus. Against this background, we have recently made proposals regarding the origin and actions of intracrines. These notions suggested 1) that higher order functionalities could result from the establishment of intracellular intracrine regulatory feedback loops, and 2) that some intracellular regulatory proteins may in fact be intracrines with extracellular functions (47, 50–52). Here the actions of several intracrines will be reviewed to begin to explore the potential relevance of intracrine functionality for physiology and medicine.

Intracrine Renin Angiotensin System

The renin angiotensin system (RAS) is one of the more intensively studied physiological systems in higher organisms. Appreciated as an important regulator of cardiovascular homeostasis, recent evidence...
has indicated that local RAS, complete or partial, exist and operate in a variety of tissues under various circumstances (49). One is then lead to ask whether the RAS could also operate in the intracellular arena; i.e., in an intracrine mode (iRAS). More than three decades ago it was reported (47, 48, 50) that binding of angiotensin to isolated nuclei resulted in increased gene transcription was later provided by several investigators, initially in the brain (which have angiotensin receptors), it is likely that renin, as opposed to prorenin. Also, adrenal mitochon.

Adrenal Intracrine RAS

A local tissue RAS is known to be present in the adrenal, and the likely existence of an adrenal intracellular RAS has recently been reported (8, 40). The activity of this intracellular renin system is unmasked by nephrectomy, which all but eliminates plasma renin activity, but upregulates adrenal renin with the result that aldosterone secretion is increased by local renin and hyperkalemia. Both full-length renin destined for secretion and so-called renin exon 1A, encoded by a transcript lacking the sequences for the secretory signal and therefore expected to remain intracellular, are found in adrenal cells (8, 28, 40, 60). Transcripts identical or similar to renin exon 1A have been reported by several investigators, initially in the brain and subsequently in other tissues (8, 28, 60). Of note, renin exon 1A appears to be synthesized as an active renin, as opposed to prorenin. Also, adrenal mitochondrial renin granules increase following nephrectomy (40). Because renin exon 1A traffics to mitochondria (which have angiotensin receptors), it is likely that renin exon 1A is upregulated by nephrectomy, traffics to mitochondria, generates angiotensin, and stimulates aldosterone secretion. Thus in rats maintained long term by dialysis after nephrectomy, renin synthesis by adrenal cells increases and aldosterone production is maintained (40). This aldosterone secretion is inhibited by the angiotensin receptor blocker losartan. However, when angiotensin is infused into these animals, aldosterone synthesis actually falls, likely because of the suppression of local renin. These results suggest that intracellular angiotensin may be more potent in the stimulation of aldosterone secretion than extracellular angiotensin and also suggest that losartan can be internalized to inhibit intracellular angiotensin action (40).

Table 1. Representative intracines

<table>
<thead>
<tr>
<th>Intracine</th>
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<th>Intracine</th>
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<tbody>
<tr>
<td>Insulin</td>
<td>Heregulin/neuregulins</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>Angiotensin</td>
<td>Homeoproteins</td>
<td>Dynorphin B</td>
</tr>
<tr>
<td>FGF-1 (aFGF)</td>
<td>Lactoferrin</td>
<td>TRH</td>
</tr>
<tr>
<td>FGF-2 (bFGF)</td>
<td>VIP</td>
<td>Maspin</td>
</tr>
<tr>
<td>FGF-3</td>
<td>INF-α, INF-β</td>
<td>Renin/prorenin (aspartyl protease)</td>
</tr>
<tr>
<td>EGF</td>
<td>VEGF</td>
<td>Leptin</td>
</tr>
<tr>
<td>NGF</td>
<td>PTHrP</td>
<td>Amphoterin (HMGB1)</td>
</tr>
<tr>
<td>PDGF</td>
<td>Angiogenin (an Rnase)</td>
<td>PDECGF</td>
</tr>
<tr>
<td>Growth hormone</td>
<td>Somatostatin</td>
<td>TGF-α</td>
</tr>
<tr>
<td>Prolactin</td>
<td>Proenkephalin</td>
<td>IGFBP 3, 5</td>
</tr>
<tr>
<td>Interleukins</td>
<td>Defensins</td>
<td>Granzyme A, B</td>
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<tr>
<td>Insulin-like growth factor I</td>
<td>Factor J</td>
<td>Hepatopoitin</td>
</tr>
<tr>
<td>Pigmented epithelium-derived factor</td>
<td>Tat</td>
<td>ES/kine/CCL 27</td>
</tr>
<tr>
<td>Brain-derived neurotrophic factor</td>
<td>Midkine</td>
<td>Thioredoxin</td>
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<tr>
<td>Gonadotropin</td>
<td>Phospholipase A2-I</td>
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<tr>
<td>Gonadotropin releasing hormone</td>
<td>Macrophage colony stimulating factor</td>
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<tr>
<td>Hepatoma-derived growth factor</td>
<td>Chorionic gonadotropin</td>
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<td>Schwannoma-derived growth factor</td>
<td>Pleiotrophin (HBGAM)</td>
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Maspin and pigmented epithelium-derived factor are members of the serine protease inhibitor family (serpin). FGF, fibroblast growth factor; EGF, epidermal growth factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; VIP, vasoactive intestinal peptide; INF, interferon; VEGF, vascular endothelial growth factor; PTHrP, parathyroid hormone-related peptide; TRH, thyrotropin-releasing hormone; HMGGB1, high mobility group protein-1; PDECGF, platelet-derived endothelial cell growth factor (thymidine phosphorylase); TGF-α, transforming growth factor; IGFBP 3, 5, insulin-like growth factor binding proteins 3 and 5; HBGAM, heparin-binding-growth-associated molecule. (See Refs. 4, 15, 17, 18, 21, 23, 25, 32, 41, 47, 51, 52, 55–57, 65).
Renin-Prorenin Internalization

Receptors for renin and renin exist on a variety of cell types (36, 37, 39, 54, 58). Prorenin internalization via the mannose/insulin-like growth factor-II (IGF-II) receptor with subsequent intracellular activation has been reported (54). A high-affinity renin receptor has also been reported that binds prorenin, is coupled to an intracellular signaling system, and produces a biological effect (36, 37). The presence of this receptor in the mesangium, as well as the vascular smooth muscles cells of coronary arteries, suggests the possibility that renin exerts a direct biological effect in the vasculature. Binding of renin to this receptor is also associated with an enhanced ability of the enzyme to generate angiotensin I in the extracellular space, thereby providing a second mechanism by which ligand binding to the receptor produces a biological action (36, 37). Also, recent studies in transgenic animals have indicated that prorenin can be internalized by cardiac and vascular cells by a mechanism different from the mannose-IGF-II receptor pathway and can then generate angiotensin II after internalization and activation (39). This internalized, activated prorenin causes both hypertension and cardiac injury. These results suggest that the mannose-IGF-II pathway may be degradative, whereas the second internalization pathway results in functional intracellular renin. In any case, the multiple reports of renin activity in the heart (for the most part under pathological conditions or in normal development), the upregulation of renin exon 1A in the ventricles following myocardial infarction, and the ability of prorenin to be internalized/activated by cardiac cells all point to the existence of functional intracellular renin (8, 39, 49).

Intracellular Angiotensin

Our group recently studied rat hepatoma cells expressing renin and angiotensin-converting enzyme. Transfection of these cells with an angiotensinogen construct lacking the signal sequence encoding region, and therefore likely to produce a nonsecreted angiotensinogen, led to enhanced proliferation, which was blocked by the At-1 blocker losartan and phenylarsine oxide (an inhibitor of receptor internalization) but not by the insurmountable At-1 blocker candesartan CV-11974 (the active candesartan moiety) or anti-angiotensin II antibodies (9, 10). PDGF gene transcription was upregulated in the transfected cells, and their enhanced proliferation was partially blocked by anti-PDGF antibodies. The most likely explanation for these findings is that the angiotensinogen construct was cleaved to release angiotensin II in the intracellular space and thereafter upregulated a series of genes (including PDGF), which stimulated proliferation. Losartan blocked the action of intracellular angiotensin after receptor-dependent internalization. In particular, we have shown that the large isoform of PDGF was upregulated in the transformed cells, and this effect was blocked by losartan but not candesartan CV-11974. Although it frequently has been assumed that nonpeptide angiotensin II antagonists are not internalized after receptor binding, this has not actually been shown for losartan (9, 10, 33, 52). Indeed, a recent study employing confocal microscopy demonstrated clear internalization of angiotensin II receptors following the administration of losartan (33). It is also noteworthy that these hepatoma cells expressed so-called exon-1A renin—a transcript predicted to produce an active renin (as opposed to prorenin) and to remain intracellular because it lacks a secretory signal sequence (8, 49).

Collectively, these findings suggest the existence of intracrine angiotensin action and also again raise the possibility that a complete iRAS exists in some cells (53). An additional point can be made. Renin and prorenin are secreted and circulate in the blood. Cellular receptors have been reported for prorenin and renin, and binding of renin and prorenin to some of these receptors produces a biological action either as a result of the generation of angiotensin in the extracellular space, prorenin internalization, and activation, or as a direct result of receptor binding (36, 37, 39, 49, 54, 58). Moreover, as noted above, intracellular angiotensin and renin are biologically active. Thus not only angiotensin II, but renin and prorenin, can be considered intracriners. The existence of renin exon-1A indicates that renin, like other intracriners, is synthesized in more than one form with one or more isoforms acting intracellularly. It is noteworthy that des-angiotensin-I-angiotensinogen is a member of the serpin family of proteins (like the intracriners pigment epithelium-derived factor and maspin) and is anti-angiogenic, but as yet, no intracellular binding/action of this protein has been described (5).

Clinical implications. The existence of intracrine renin-angiotensin action could be clinically important. Angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) that enter cells could have different pharmacological activities than those that do not. Whereas no such differences have as yet been reported, it would be worthwhile to look for them, although they would be expected to be more likely related to long-term effects on growth and differentiation than to blood pressure or hemodynamic alterations. Because renin exon-1A has been reported to be the only renin upregulated in the ventricles of rats following myocardial infarction, ACEIs and ARBs that act in cells may exert therapeutic effects different from those that act only outside the cell in patients who have suffered myocardial infarction. The observation that prorenin can be taken up into cardiac cells, activated, and produce pathology raises the possibility that ACEIs and ARBs that act in the intracellular space could prevent other forms of cardiovascular disease, possibly including some forms of arrhythmia given the effects of intracellular angiotensin on intracellular conductance. From the role played by angiotensin in the genesis of left ventricular hypertrophy, cardiac fibrosis, and cardiac aldosterone synthesis, it appears reasonable to consider the possible participation of the iRAS in these processes as well (49). The contention that
renin is not simply a circulating aspartyl protease but is a hormone and an intracrine in its own right may be startling. Nonetheless, the available evidence indicates that renin and angiotensin are active within cells. A fuller understanding of the iRAS could open new avenues of research and therapy and lead to a search for intracrine analogs of other established physiological systems.

Angiogenesis

Many intracrines are angiogenic (e.g., angiogenin, FGF-2, and angiotensin) or anti-angiogenic (e.g., pigment epithelium-derived factor maspin) either directly or through the stimulation of an angiogenic factor such as vascular endothelial growth factor (VEGF), which itself has recently been shown to be an intracrine (29, 47, 51, 52). As already noted, angiogenin, an RNase, must be internalized by target endothelial cells and traffic to nucleolus to stimulate, either directly or indirectly, the cellular proliferation essential for angiogenesis (34, 47, 51, 52). A similar requirement for nuclear translocation in stimulating proliferation is seen in the case of some other intracrines such as FGF-2 (3, 47). Most identified intracrines have been shown to traffic to nucleus (renin and prorenin are exceptions so far) as well as in some cases to other intracellular organelles (47, 51, 52). It has been suggested that nuclear translocation of angiogenic factors was an essential feature of their action (34). Indeed, it is the case that virtually every intracrine that traffics to nucleolus is either directly or indirectly angiogenic or anti-angiogenic. Nuclear but not nucleolar binding has been reported in the case of other angiogenic intracrines (e.g., angiotensin and VEGF) (29, 47, 51, 52). Another link between intracrine action and the nucleolus is the fact that the pleurifunctional nucleolar protein nucleolin, which regulates rRNA transcription and participates in ribosomal synthesis, also is a cell surface protein, which shuttles the angiogenic intracrines pleiotrophin and midkine from the cell surface to nucleus (47, 51, 52). Given the likelihood that the nucleolus is an ancient functional unit of the eukaryotic cell and given the binding of many intracrines to the nucleolus, it can be hypothesized that intracrines first acted at the nucleolus and thereafter assumed additional roles in the cell and in extracellular signaling (38, 47, 51, 52). An additional implication of the frequent association of nucleolar binding and angiogenesis is the possibility that the endothelial cell continues to use ancient regulatory mechanisms and in that sense may be more primitive or pleurifunctional than other cells (52).

Clinical implications. Angiogenesis and vasculogenesis play important roles not only in the development but also in a variety of pathological conditions such as ischemic revascularization and tumor angiogenesis. These processes are complex and are under the influence of competing angiogenic/anti-angiogenic inputs. For example, angiotensin is angiogenic, and this effect appears to be mediated by the At-1 receptor; angiotensin binding to the At-2 receptor produces apoptosis and can be anti-angiogenic (59). Many intracrines are proapoptotic or anti-angiogenic, and it appears likely that understanding the nature of the interaction of pro- and anti-angiogenic intracrine factors will be important to the design of effective revascularization and anti-tumor strategies, be they gene therapy based like VEGF gene therapy or based on more traditional pharmaceuticals (50). For example, it has been shown (34, 47, 51, 52) that the angiogenic effect of angiogenin is dependent on not only the nucleolar trafficking and RNase activity of the protein, but also on its ability to stimulate rRNA synthesis. Drugs designed to inhibit this action of the protein could be expected to inhibit tumor angiogenesis produced by angiogenin and perhaps by other intracrines as well (34, 66).

Homeoproteins

Homeodomain transcription factors, or homeoproteins, are regulators of gene expression and play an important role in a variety of processes, most notably development. They are large complex molecules possessing DNA binding capacity and interact with other transcription factors and homeoproteins to coordinate embryonic tissue patterning and development. Homeoproteins contain a 60-amino acid DNA-binding domain known as the homeodomain composed of three α-helices, the third of which is important for cognate DNA binding. In the context of this essay they are of particular interest because there is a body of evidence to suggest these proteins may function in an intracrine mode (6, 26, 31, 42, 44, 52). In the early 1990s it was discovered that the homeodomain of the Drosophila transcription factor Antennapedia is internalized by cultured cells. The internalization does not depend on traditional endocytosis and involves translocation of the protein into the cytoplasm and then to the nucleus. It was subsequently reported that the homeodomains of Fushi tarazu, Hoxa 5, Hoxa 8, and Engrailed are internalized. Moreover, the entire mammalian homeoproteins Engrailed and Hoxa 5 are internalized, and the latter has been shown to traffic to the nucleus. The third helix of the homeodomain was shown to be important for internalization. Subsequent studies demonstrated that transfected COS-7 cells can secrete Engrailed 2 with subsequent uptake of the protein by embryonic neurons in coculture. The secretion of Engrailed does not involve a traditional secretory signal but rather is dependent on a sequence located between the second and third helices of the homeodomain (6, 26, 31, 42, 44). Collectively, these studies indicate that at least some homeotranscription factors are intracrines. This conclusion is consistent with the concept of the intracrine, which as described above, is based on functionality rather than structure. Indeed, the variety of intracrine structures suggests an ancient origin for intracrine functionality. To be sure, a recent report (31) raises the possibility that the apparent internalization of positively charged DNA binding proteins may be the result of fixation artifacts. Studies of homeoprotein
internalization in living (nonfixed) cells therefore must be performed. However, there exists considerable functional data supporting the internalization of these proteins (as exemplified by the penetratins; see below) (6, 26, 44). In any case, the view of intracrine functionality developed here is not dependent on the intracrine action of homeodomain and other DNA binding proteins, but rather is consistent with it. Moreover, the notion that intracrines and transcription factors are related derives support from observations related to plant transcription factors and viral proteins such as Tat (24, 30, 47, 51, 52, 67). This laboratory has previously suggested that this phenomenon derives from the origins of intracrines as regulators of cellular memory and differentiation on the one hand and of metazoan development on the other (47, 51, 52).

Clinical implications. The implications of homeoprotein intracrine action could be wide ranging. First, slightly modified versions of the third helix of the Antennapedia homeodomain have been employed as carriers for the intracellular transfer of proteins. Known as penetratins, these peptides can be designed to deliver fusion proteins into the cytoplasm only or into the cytoplasm and nucleus (26, 44). They are being used as vehicles for protein transfer in research studies and have the potential to serve as the basis for a new class of pharmaceuticals. Interestingly, other nuclear proteins apparently can be taken up by target cells, and at least one of these is an intracrine (25, 41, 55, 63). This phenomenon of nuclear protein uptake by cells suggests the possibility of an extracellular signaling role for nuclear regulatory proteins early in metazoan development and raises the possibility that from these origins evolved the intracrines of today.

Intracrines are involved in the differentiation of a variety of cells, and this coupled with the observation that homeoproteins can be intracrines suggests the possibility that some intracellular regulatory proteins, and in particular homeoproteins, could be made to function therapeutically as intracrines. One candidate for such application is the islet-cell-inducing homeoprotein PDX1 (52, 62). Much will depend on determining exactly how these DNA binding proteins are taken up from the extracellular space. Similarly, the wide range differentiating actions of other intracrines could well be useful in recruiting and maintaining various stem cell populations for therapeutic use (1, 25, 42, 47, 51, 52).

Future Directions

The view of intracrine action outlined here generates testable predictions based on the proposed intracrine feedback loops and novel functions of intracellular regulatory proteins. These are discussed elsewhere and could have implications for clinical care (47, 50–52). For example, the argument made here suggests the possibility that the tumor suppressor protein p53 could be an intracrine; it traffic to nucleolus, is anti-angiogenic, exists in multiple isoforms, and is a regulator of transcription. If correct, this would imply that p53 (or a p53 fragment or homologue) could be active outside cells (51, 52). The fact that many intracrines regulate cellular development suggests the possibility that these factors will increasingly find applications in the recruitment of stem cells of various sorts for therapeutic purposes. Also, the accumulating evidence for intracellular interactions between intracrines, as well as between intracrines and transcription factors, suggests that the study of these interactions and the regulatory loops they appear to form could yield valuable insights into cellular physiology. For example, the intracrine dynorphin B after nuclear binding upregulates transcription of its precursor preprodynorphin. Nuclear angiotensin binding upregulates renin and angiotensinogen transcription. PDX1 upregulates its own transcription as well as transcription of insulin, itself an intracrine. The intracrine dynorphin B can indirectly upregulate transcription of the intracrine FGF-3 (16, 35, 47, 51, 52). Other examples could be given, and many others undoubtedly remain to be discovered. These interactions, and the feedback loops they potentially produce, provide the substrate for the development of considerable regulatory complexity within the cell and arguably form the basis of a physiology of intracrines with implications for angiogenesis, growth, hormonal responsiveness, and development. It must also be recalled that intracrines bind and act at organelles other than the nucleus such as mitochondria, endocytotic vesicles, and golgi. The exploration of these activities will also be important.

Irrespective of the validity of the expanded view of intracrine action proposed here, intracrine peptide hormone action is a reality. The implications of intracrine action are only now being investigated, but every indication is that intracrine action is physiologically relevant. Indeed, the intracrine concept could influence the direction of physiology research in important ways by 1) stimulating the exploration for the intracellular analogs of well-established homeostatic systems such as the RAS, and 2) suggesting an expanded view of the mechanisms of differentiation and homeostasis in cells and tissues, i.e., by suggesting an expanded view of cellular physiology.

REFERENCES


AJP-Heart Circ Physiol • VOL 284 • MARCH 2003 • www.ajpheart.org


