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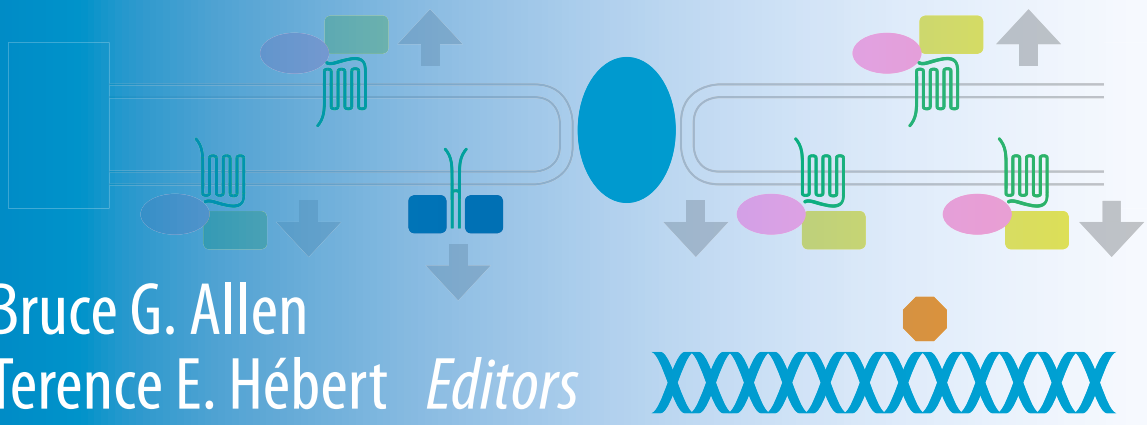

Bruce G. Allen
Terence E. Hébert *Editors*

Nuclear G-Protein Coupled Receptors

Methods and Protocols

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Edited by

Bruce G. Allen

Montreal Heart Institute & Université de Montréal, Montreal, QC, Canada

Terence E. Hébert

Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada

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Editors
Bruce G. Allen
Montreal Heart Institute & Université de Montréal
Montreal, QC, Canada

Terence E. Hébert
Pharmacology and Therapeutics
McGill University
Montreal, QC, Canada

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Preface

Signaling via Nuclear-Localized Transmembrane Receptors: New Homes and New Tasks for Cell Surface Proteins

Growth factor receptors and G protein-coupled receptors (GPCRs) are now known to be present on nuclear or perinuclear membranes, challenging a long-held paradigm regarding their sites of action being restricted to the cell surface membranes (reviewed in refs. 1–4). These observations have led to the concept of intracrine signaling. Intracrine signaling refers to processes whereby ligands, originating within a target cell or taken up from the extracellular milieu, act upon intracellular receptors (reviewed in refs. 3–11). Studies to date have localized Ang II type 1 (AT1R) and type 2 (AT2R) [12–14], bradykinin B2 (15), α- [16, 17] and β-adrenergic [18–20], type B endothelin (ETB) [21, 22], epidermal growth factor (EGF-R) [23], c-erbB-4 [24], insulin [25, 26], interferon β [27], muscarinic cholinergic [28], nerve growth factor [29], prostaglandin E_2 (PGE_2) [30, 31], lysophosphatidic acid type-1 [32], urotensin II [33, 34], and opioid [35] receptors to the nuclear membrane. Consistent with a nuclear location, several GPCRs have now been shown to contain a nuclear localization sequence (NLS) [17, 36–38]. At present, little is known concerning the function of nuclear growth factor receptors or GPCRs: potential roles include regulation of nuclear transport, transcription, and nuclear envelope formation.

Of course, growth factor receptors and GPCRs require the presence of a host of effector molecules in order to transduce signals. Key components of various signaling pathways are also present at the nuclear envelope or within the nucleus itself. These include heterotrimeric G proteins [13, 18, 39, 40] (reviewed in ref. 4), adenylyl cyclase [41], phosphodiesterase [42], diacylglycerol kinase-ζ [43], endothelial nitric oxide synthase (eNOS) [20], phospholipase A_2 [44], phospholipase C-β1 [45, 46], phospholipase D [47–49], phosphatidylinositol 3-kinase C2α [50], RGS proteins (reviewed in ref. 51), β-arrestin1 [52, 53], and G protein-coupled receptor kinases [54–56]. Nuclear membranes also contain sarco/endoplasmic Ca^{2+}-ATPase (SERCA) pumps [57, 58] as well as ryanodine-sensitive (RyR) [58, 59] and inositol trisphosphate-sensitive (IP_3R) [59, 60] Ca^{2+} channels. Hence, the nuclear cisternae are able to accumulate and release Ca^{2+} (reviewed in ref. 61). Furthermore, numerous protein kinases localize to the nucleus, or translocate into or out of the nucleus upon activation [62–66] and inhibition of ERK, p38α/β, PKB, PKG, or JNK inhibited both basal and/or isoproterenol-stimulated transcription in isolated nuclei [19, 20]. Thus, there is evidence supporting the presence and physiological relevance of GPCR signaling at the nuclear membrane.

For intracellular GPCRs to be of functional relevance, barring constitutive receptor activity, there must be a source of the intracellular ligand, whether it be taken up from the extracellular milieu or synthesized within the target cell (reviewed in ref. 3). Ligand-mediated receptor internalization and translocation to the nucleus have been demonstrated for AT1R [37] and EGF-R [67]. In contrast, PGE_2 and catecholamines are taken up by specific transporters [16, 68, 69]. When fluorescent ET-1 or ET-3 analogs were applied extracellularly, they were endocytosed and subsequently trafficked to lysosomes [22, 70], suggesting that endogenous endothelins may serve as ligands for nuclear ETB.

The nuclear envelope (NE) is a double-lipid bilayer structure, comprising inner (INM) and outer (ONM) nuclear membranes, that separates the nucleoplasm from the cytoplasm. The INM and ONM only meet at the nuclear pore: a large complex (>1,000 subunits) that facilitates the regulated exchange of macromolecules and RNA between the nucleoplasm and cytoplasm. The space between the INM and ONM is known as the nuclear cisternae or perinuclear space [71]. The ONM is contiguous with the sarco/endoplasmic reticulum whereas the INM is associated with the nucleoskeleton, a structure analogous to the cytoskeleton, that lines the inner surface of the INM. Furthermore, in adult cardiac myocytes, the sarcoplasmic reticulum and perinuclear space are interconnected [72]. By virtue of the linker of nucleoskeleton and cytoskeleton (LINC) complex, the nucleus is mechanically coupled to the cell surface and extracellular matrix (reviewed in ref. [73]). The LINC complex, comprising the SUN proteins (Sad1p/UNC-84) in the INM and the nesprins at the ONM, has been implicated both in nuclear morphology, positioning, and integrity and in mediating the effects of biomechanical load on gene expression [74]. Lamins A/C and B make up the nucleoskeleton and are coupled to the LINC complex via a direct interaction with SUN proteins. Furthermore, even isolated nuclei are capable of altering their stiffness in response to changes in mechanical force, or tension, and this is thought to involve a phosphorylation-dependent increase in coupling between lamins and the LINC complex [75]. However, it is also becoming increasingly apparent that the nuclear envelope itself is not just a barrier, but plays a role in signaling that may be as complex as that of the cell membrane. The objective of this installment in the series *Methods in Molecular Biology* is to highlight some of the methodologies used to identify and study signal process located in the nuclear membranes.

During their biosynthesis, as a result of an integral N-terminal signal motif, GPCRs orient within membranes with their effector binding site oriented towards the cytoplasm. Hence, whether a receptor is present on either the INM or the ONM, the ligand-binding site will likely be oriented towards the nuclear cisternae. It remains to be determined whether these receptors signal into either the cytoplasm, the nucleoplasm, or both. Hence, whether the receptor is on the INM, the ONM, or both, a significant issue remains—How would an intracrine ligand reach its binding site within the nuclear cisternae? The choices include (1) passive or active transit across the INM or ONM or (2) direct delivery to the nuclear cisternae during biosynthesis. This latter mechanism could include transport from the ER lumen to the nuclear cisternae: the SR and nuclear are interconnected in cardiac myocytes [72] and perhaps this connectivity permits protein trafficking. Alternatively, peptide ligands may arrive at the nucleus by microsomal transport. The nuclear envelope was recently shown to undergo budding [76]; hence, microsomes may also be able to fuse with the ONM and thus deliver their cargo into the perinuclear space.

Nuclear localization has been reported for many peptide ligands (e.g., Ang II, epidermal growth factor, insulin, platelet-derived growth factor, nerve growth factor, parathyroid hormone-related protein, prolactin, interleukin 1, somatostatin, fibroblast growth factors 1 and 2, and TGFα). Hence, the actual source of the intracrine ligand as well as the means whereby it is trafficked to its site of action within the nuclear cisternae may be distinct for each ligand, or class of ligands. Hence, intracrine signaling represents a potentially novel target for therapeutic intervention.

In this volume, we bring together a number of conceptual and methodological aspects important for the validation and characterization of intracrine signaling systems. To date, the best characterized intracrine signaling system is that of angiotensin II (Ang II). AT1R and AT2R have been demonstrated on the nuclear membranes in cardiomyocytes [3],

hepatocytes, and vascular smooth muscle cell lines [13, 77]. In the first chapter of this book, Re and Cook recount the history of Ang II intracrine signaling. How to target such receptors to understand their physiology is the focus of the next several chapters. Internal receptors can be targeted by direct ligand injection into cells (Merlen and Ledoux, Chapter 2), and the use of caged ligands that can be released by UV irradiation to study nuclear GPCR signaling in an intact cell context (Chatenet et al., Chapter 3 and Merlen et al., Chapter 4). Exploring potential sources of ligands and measuring their uptake and delivery to the nucleus are the subject of Dahl et al., Chapter 5. Another interesting possibility for intracrine signaling is the association of the enzyme responsible for ligand synthesis with the receptor for the ligand in question as recently demonstrated for the prostaglandin D_2 DP1 receptor and the intracellular l-prostaglandin D synthase [78]. In this volume, Binda and Parent provide guidance as to how to identify and characterize such interactions with endogenous proteins (Chapter 6).

Methodology to study the subcellular localization and function of GPCRs and other signaling systems is provided in several chapters. Both Tadevosyan et al. (Chapter 7) and Bhosle et al. (Chapter 8) describe techniques to isolate nuclei and localize signaling molecules to this compartment. GPCRs, as discussed above, are not the only receptor family expressed on the nuclear membrane and biochemical techniques to further fractionate inner and outer nuclear membranes are critical tools to understand their trafficking and function (Wang et al., Chapter 9). Another critical issue for the study of intracrine signaling is the requirement to study these systems in native cells to avoid artifacts associated with overexpression. Jong and O'Malley describe methods to study nuclear GPCR signaling in neurons (Chapter 10) and the intact cardiomyocyte is the focus of a number of chapters (Merlin and Allen, Chapter 5; Ryall and Saucerman, Chapter 11; Ljuboyevic and Bers, Chapter 12; and Bossuyt and Bers, Chapter 13).

In addition, numerous chapters focus on methods designed to understand signaling mediated by nuclear and other internal GPCRs. Ryall and Saucerman (Chapter 5) use a high-content microscopy approach to examine phenotypic changes in neonatal cardiomyocytes, an approach that will be generally amenable to studying nuclear GPCR and RTK signaling in the intact cell context. Studying signaling in isolated nuclei has been the focus of many of the studies cited above. It has become clear that nuclear GPCRs control gene expression, and Vaniotis et al. (Chapter 14) describe methods to assess and validate how these receptors modulate transcription using transcription initiation assays, gene arrays, and qPCR. The connections between nuclear GPCRs, G proteins, and their effectors remain incompletely understood. It may be that signals from surface GPCRs and their associated G protein-dependent pathways may integrate with those from nuclear-localized signaling complexes as well as with receptor-independent G protein signaling. Thus, Campden et al. (Chapter 15) demonstrate an unbiased method of identifying nuclear G protein-interacting proteins in order to begin to characterize these integrated networks. As discussed, the nuclear membrane is not the only endomembrane site where GPCR signaling occurs, as described by Calebiro et al. (Chapter 16). Finally, methods are described to study the formation of second messengers such as cAMP and to study the trafficking of receptors from the cell surface (Calebiro et al., Chapter 16). Together, we incorporate a number of state-of-the-art approaches to characterize what is becoming a common theme in cellular signaling.

Montreal, QC, Canada

Bruce G. Allen
Terence E. Hébert

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Contents

Contributors

Bruce G. Allen • *Montreal Heart Institute, Montréal, QC, Canada; Department of Medicine, Université de Montréal, Montréal, QC, Canada; Department of Biochemistry and Molecular Medicine, Université de Montréal, Montréal, QC, Canada; Department of Pharmacology and Therapeutics, McGill University, Montréal, QC, Canada*

Stéphane Angers • *Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, ON, Canada*

Nicolas Audet • *Department of Pharmacology and Therapeutics, McGill University, Montréal, QC, Canada*

Donald M. Bers • *Department of Pharmacology, University of California, Davis, CA, USA*

Vikrant K. Bhosle • *Department of Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada; CHU Sainte-Justine Hospital Research Centre, Montreal, QC, Canada; Research Centre of Maisonneuve-Rosemont Hospital, Montreal, QC, Canada*

Chantal Binda • *Department of Medicine, Université de Sherbrooke, Sherbrooke, QC, Canada*

Julie Bossuyt • *Department of Pharmacology, University of California, Davis, CA, USA*

Steve Bourgault • *Department of Chemistry, University of Québec in Montreal, Montreal, QC, Canada*

Davide Calebiro • *Bio-Imaging Center/Rudolf Virchow Center for Experimental Biomedicine, and Institute of Pharmacology and Toxicology, University of Würzburg, Würzburg, Germany*

Rhiannon Campden • *Department of Pharmacology and Therapeutics, McGill University, Montréal, QC, Canada*

David Chatenet • *INRS – Institut Armand-Frappier, Institut National de la Recherche Scientifique, QC, Canada*

Sylvain Chemtob • *Department of Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada; Departments of Pediatrics, Ophthalmology and Pharmacology, CHU Sainte-Justine Hospital Research Centre, Montreal, QC, Canada; Research Centre of Maisonneuve-Rosemont Hospital, Montreal, QC, Canada*

Julia L. Cook • *Department of Cardiology, Ochsner Health System, Tulane University School of Medicine, New Orleans, LA, USA; Ochsner Health System, Institute for Clinical Research, Louisiana State University Health Sciences Center, New Orleans, LA, USA; Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, New Orleans, LA, USA; Neuroscience Center of Excellence, Louisiana State University Health Sciences Center, New Orleans, LA, USA*

Erika F. Dahl • *Department of Pharmacology, University of Minnesota, Minneapolis, MN, USA*

Alain Fournier • *INRS – Institut Armand-Frappier, Institut National de la Recherche Scientifique, QC, Canada*

Fernand Gobeil Jr • *Department of Pharmacology, Université de Sherbrooke, Sherbrooke, QC, Canada*

Amod Godbole • *Bio-Imaging Center/Rudolf Virchow Center for Experimental Biomedicine, and Institute of Pharmacology and Toxicology, University of Würzburg, Würzburg, Germany*

Sarah Gora • *Department of Pharmacology and Therapeutics, McGill University, Montréal, QC, Canada*
Terence E. Hébert • *Department of Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada*
Jennifer L. Hsu • *Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; Center for Molecular Medicine and Graduate Institute of Cancer Biology, China Medical University, Taichung, Taiwan*
Mien-Chie Hung • *Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; Center for Molecular Medicine and Graduate Institute of Cancer Biology, China Medical University, Taichung, Taiwan*
Longfei Huo • *Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA*
Yuh-Jiin I. Jong • *Department of Anatomy and Neurobiology, Washington University School of Medicine, Saint Louis, MO, USA*
Jonathan Ledoux • *Montreal Heart Institute, Montreal, QC, Canada; Department of Medicine, Université de Montréal, Montreal, QC, Canada; Department of Molecular and Integrative Physiology, Université de Montréal, Montreal, QC, Canada*
Senka Ljubojević • *Department of Pharmacology, University of California Davis, Davis, CA, USA; Department of Cardiology, Medical University of Graz, Graz, Austria*
Martin J. Lohse • *Bio-Imaging Center/Rudolf Virchow Center for Experimental Biomedicine, and Institute of Pharmacology and Toxicology,, University of Würzburg, Würzburg, Germany*
Sandra Lyga • *Bio-Imaging Center/Rudolf Virchow Center for Experimental Biomedicine, and Institute of Pharmacology and Toxicology,, University of Würzburg, Würzburg, Germany*
Clémence Merlen • *Montreal Heart Institute, Montreal, QC, Canada; Department of Biochemistry and Molecular MedicineUniversité de Montréal, Montreal, QC, Canada*
André Nantel • *National Research Council Canada, Montreal, QC, Canada; Department of Anatomy and Cell Biology, McGill University, Montreal, QC, Canada*
Stanley Nattel • *Montreal Heart Institute, Montreal, QC, Canada; Department of Medicine, Université de Montréal, Montreal, QC, Canada; Department of Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada*
Timothy D. O'Connell • *Department of Integrative Biology and Physiology, University of Minnesota, Minneapolis, MN, USA*
Karen L. O'Malley • *Department of Anatomy and Neurobiology, Washington University School of Medicine, Saint Louis, MO, USA*
Jean-Luc Parent • *Department of Medicine, Université de Sherbrooke, Sherbrooke, QC, Canada*
Darlaine Pétrin • *Department of Pharmacology and Therapeutics, McGill University, Montréal, QC, Canada*
Richard N. Re • *Departments of Medicine and Physiology, Tulane University School of Medicine, New Orleans, LA, USA; Department of Cardiology, Ochsner Health System, Tulane University School of Medicine, New Orleans, LA, USA*
Alfredo Ribeiro-da-Silva • *Department of Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada*
Jose Carlos Rivera • *CHU Sainte-Justine Hospital Research Centre, Montreal, QC, Canada; Research Centre of Maisonneuve-Rosemont Hospital, Montreal, QC, Canada*

Mélanie Robitaille • *Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, ON, Canada*
Karen A. Ryall • *Department of Biomedical Engineering, University of Virginia, Charlottesville, VA, USA*
Jeffrey J. Saucerman • *Department of Biomedical Engineering, University of Virginia, Charlottesville, VA, USA*
Artavazd Tadevosyan • *Montreal Heart Institute, Montreal, QC, Canada; Department of Medicine, Université de Montréal, Montreal, QC, Canada*
George Vaniotis • *Montreal Heart Institute, Montreal, QC, Canada; Department of Biochemistry and Molecular Medicine, Université de Montréal, Montreal, QC, Canada*
Louis R. Villeneuve • *Montreal Heart Institute, Montreal, QC, Canada*
Ying-Nai Wang • *Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; Center for Molecular Medicine and Graduate Institute of Cancer Biology, China Medical University, Taichung, Taiwan*
Casey D. Wright • *Department of Integrative Biology and Physiology, University of Minnesota, Minneapolis, MN, USA*

Chapter 1

Studies of Intracellular Angiotensin II

Richard N. Re and Julia L. Cook

Abstract

Many extracellular signaling proteins act within their cells of synthesis and/or in target cells after internalization. This type of action is called intracrine and it plays a role in diverse biological processes. The mechanisms of intracrine intracellular action are becoming clear thanks to the application of modern techniques of molecular biology. Here, progress in this area is reviewed. In particular the intracrine biology of angiotensin II is discussed.

Key words Angiotensin II, Intracrine, Growth factors

1 Introduction

The earliest studies of nuclear angiotensin binding to nuclei involved the injection of tritiated angiotensin II into animals, followed by electron microscopic autoradiography. These studies detected angiotensin II trafficking to nuclei and led to a wide range of follow-up studies [2–26]. Binding studies were conducted on isolated nuclei, and transcriptional runoff studies defined the positive effect of nuclear angiotensin II binding on transcription. These techniques were refined over time to the point that both AT-1 and AT-2 receptors (AT-1R and AT-2R) could be shown to be on the nuclear membrane and AT-1R-like receptors were associated with chromatin. Direct binding of angiotensin II to isolated nuclei was shown to up-regulate genes such as renin, angiotensinogen, and platelet-derived growth factor (PDGF). Collectively, these early studies revealed the existence of physiologically relevant angiotensin II action at nucleus, although the full scope of that activity was, and remains, only partially understood [1–9, 16, 20, 25, 26].

In time, the development of more precise techniques confirmed and expanded these early observations. For example, improved electron microscopy immuno-staining techniques confirmed angiotensin II binding to euchromatin. The transfection of cells with AT-1R fluorescent fusion proteins, coupled with confocal or

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3D deconvolution microscopy, demonstrated the trafficking of the receptor from cell membrane to nucleus after exposure to extracellular ligand. Transfection of cells with a variety of constructs designed to generate angiotensin II solely in the intracellular space informed our view of the actions of the peptide within the cell and the establishment of novel transgenic mouse models now offers the prospect of new breakthroughs in the near future [8–25]. Below, we describe some of the models we have developed and review their implications.

2 Intracellular Angiotensin II

In order to further explore the actions of intracellular angiotensin II (iAII), we developed a construct designed to lead to the generation of iAII. Specifically, we mutated angiotensinogen cDNA by removing the region that encodes the signal-sequence [Ang(-S) Exp]. This was intended to produce an angiotensinogen which remained in the intracellular space. A corresponding control construct was also developed. Of note, both constructs contained a Kozak consensus sequence to enhance ribosome association and therefore translation. Rat hepatoma cells were then stably transfected with [Ang(-S)EXP] and control expression plasmids. These cells expressed renin as well as angiotensin-converting enzyme. We were able to show that these cells produced a previously described non-secreted form of renin which was expected to remain in the intracellular space in an active form, as opposed to pro-renin. Expression of [Ang(-S)Exp], but not control expression plasmid, resulted in increased proliferation. Anti-angiotensin II antibodies delivered to the culture medium did not affect this stimulation of mitosis. Proliferation was blocked by renin antisense and the angiotensin receptor blocker (ARB) losartan which had been shown to be internalized after binding to the cell surface AT-1R. The ARB candesartan, which fixes AT-1R at the cell surface, did not block the proliferative effect of [Ang(-S)EXP]. Phenylarsine oxide, which blocks receptor internalization, prevented the antiproliferative effect of losartan. We also found that [Ang(-S)Exp] up-regulated platelet-derived growth factor expression and the addition of anti-PDGF antibody to the culture medium partially blocked proliferation. These results argue for a direct effect of iAII on proliferation and PDGF up-regulation. Inhibition of intracellular iAII synthesis or blockade of intracellular AT-1R blocked this proliferation. Collectively, these results argued that the intracellular generation of iAII can occur with physiologically relevant results and that at least some of the effects of iAII are AT-1R mediated [8, 9].

In order to more directly investigate the trafficking of iAII, AT1R, and the effects of their interaction, we studied A10 rat

vascular smooth muscle cells transfected with both a plasmid encoding a fluorescent AII fusion protein (pAII/ECFP encoding AII fused downstream of enhanced cyan fluorescent protein) and a plasmid encoding a rat AT-1Ra fluorescent fusion protein (pAT1R/EYFP, encoding the AT-1Ra receptor upstream of enhanced yellow fluorescent protein). The AII fusion protein did not contain a secretion signal and 3D deconvolution microscopy revealed its localization in the cell nuclei. AII was not detected in the media of transfected cells. AT-1Ra, when expressed in the absence of AII/ECFP, was localized to cytoplasm and cell surface and was not found in cell nucleus. However, when AII was introduced into the culture medium the receptor fusion protein localized to nucleus, just as it did when the cells were simultaneously transfected with pAT1R/EYFP and pAII/ECFP. In doubly transfected cells, both AT-1Ra and AII fusion proteins were found throughout the nucleus, although the receptor localized in nucleoli to a larger extent than did the AII fusion protein Transfection of the cells with pAII/ECFP alone resulted in increased proliferation as did the introduction of AII into the culture medium. However, while anti-angiotensin II antibody, delivered to the culture media, prevented the proliferation induced by extracellular AII, it had no effect on the proliferation stimulated by iAII. Collectively, these studies confirmed a role for iAII in the regulation of cellular proliferation and the interaction of iAII with AT1R in the intracellular space. Co-trafficking of AT1Ra and either extracellular or intracellular AII to nucleus was consistent with earlier autoradiographic studies of AII trafficking from the extracellular space to nucleus and with studies of isolated nuclei demonstrating widely dispersed nuclear receptors and effects of nuclear AII on gene regulation [10, 11].

Further studies of the actions of iAII in this model revealed that extracellular AII treatment of A10 cells activated cAMP response element-binding protein (CREB) as assessed by one-hybrid assays and immunoblotting. Expression of iAII similarly increased CREB but via different signaling pathways. Western blot and one-hybrid assays revealed that exogenous AII activated p38 MAPK and ERK 1/2. This finding was confirmed by demonstrating that the p38MAPK inhibitor SB203580 and the MEK inhibitor PD98059 each partially blocked the activation of CREB by exogenous AII. However, intracellular AII/ECFP activated p38MAPK in these cells but not ERK 1/2. Possibly the shared signaling pathway represented signaling by the internalized membrane receptor or AII binding to intracellular receptors, while the pathway exclusively activated by extracellular AII represents signaling by receptor on the cell surface [10, 11]. The details of intracellular AII action remain to be completely worked out [22].

3 Transgenic Mice

In order to shed light on the physiological effects of iAII we elected to employ transgenic technology. With our colleagues at the University of Iowa, we developed a line carrying the AII/ECFP construct regulated by the metallothionine promoter [12, 13]. Although the expression of the construct was widely distributed in tissues (and could be easily assessed using the attendant fluorescence), plasma angiotensin II concentrations were not elevated.

Although two independent mouse lines widely expressed the transgene (both RNA and protein), the prominent phenotypic change occurred in the kidney. By 2 months of age these mice developed systolic and diastolic hypertension. Some mice then developed glomerular thrombotic microangiopathy (TMA) involving microthrombosis of the glomerular capillaries and small vessels. This pathological picture has been reported in other transgenic models that over-express components of the renin-angiotensin-aldosterone system (RAAS). Blood pressure rose by 2 months of age while TMA developed at 4–6 months to varying degrees in individual animals. We found similar changes in males and females which contrast with some reports of sexually dimorphic blood pressure changes following the infusion of angiotensin II.

When isolated mouse embryonic fibroblasts (MEFs) from the transgenic animals were studied, ECFP/AII was detected in cytoplasm and in 30–40 % of nuclei. The cytoplasmic fluorescence was punctate and localized with mitochondrial markers. Multiple studies, including ours, have shown AII and/or AII receptors to be associated with mitochondria in adrenal, kidney, and brain. Moreover, mitochondrial dysfunction has been reported to play a role in RAAS-mediated disease and specifically in vascular endothelial dysfunction and hypertension, likely as the result of increasing levels of reactive oxygen species (ROS). In this regard, yeast two-hybrid studies followed by glutathione S-transferase (GST) pull-down studies revealed that AII associated with two mitochondrial electron transport chain proteins and increased ROS generation. Direct binding of AII to these proteins would preclude AII binding to integral membrane AT-1R. This raises the possibility that AII acts in the mitochondria in what we have termed a noncanonical fashion—that is, independent of membrane-associated receptor [12–14]. Recently, others using immunogold staining have reported AT-1R and AT-2R associated with mitochondria. The density of AT-1R per mitochondria increased with age while the AT-2R number fell. These authors further reported that mitochondrial AT-2R was coupled with nitric oxide production, thereby suggesting a canonical role for AII in mitochondria, that is, a function mediated by membrane-associated receptor [15]. It should be noted that a more recent study failed to identify AT-1aR in rat