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REVIEW ARTICLE

Phosphoproteomics for studying signaling pathways evoked by hormones of the renin-angiotensin system: A source of untapped potential

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Abstract

The Renin-Angiotensin System (RAS) is a complex neuroendocrine system consisting of a single precursor protein, angiotensinogen (AGT), which is processed into various peptide hormones, including the angiotensins [Ang I, Ang II, Ang III, Ang IV, Ang-(1–9), Ang-(1–7), Ang-(1–5), etc] and Alamandine-related peptides [Ang A, Alamandine, Ala-(1–5)], through intricate enzymatic pathways. Functionally, the RAS is divided into two axes with opposing effects: the classical axis, primarily consisting of Ang II acting through the AT_1 receptor (AT_1R), and in contrast the protective axis, which includes the receptors Mas, AT_2R and MrgD and their respective ligands. A key area of RAS research is to gain a better understanding how signaling cascades elicited by these receptors lead to either "classical" or "protective" effects, as imbalances between the two axes can contribute to disease. On the other hand, therapeutic benefits can be achieved by selectively activating protective receptors and their associated signaling pathways. Traditionally, robust "hypothesis-driven" methods like Western blotting have built a solid knowledge foundation on RAS signaling. In this review, we introduce untargeted mass spectrometry-based phosphoproteomics, a "hypothesis-generating approach", to explore RAS signaling pathways. This technology enables the unbiased discovery of phosphorylation events, offering insights into previously unknown signaling mechanisms. We review the existing studies which used phosphoproteomics to study RAS signaling

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and discuss potential future applications of phosphoproteomics in RAS research including advantages and limitations. Ultimately, phosphoproteomics represents a so far underused tool for deepening our understanding of RAS signaling and unveiling novel therapeutic targets.

KEYWORDS

cellular signaling, phosphoproteome, phosphoproteomics, renin-angiotensin system

1 | INTRODUCTION

The Renin-Angiotensin System (RAS) is a complex neuroendocrine system composed of the protein angiotensinogen (AGT), peptide hormones derived from AGT after limited proteolysis, and several receptors (Figure 1).[1] RAS components are found in the vast majority of tissues, controlling a large variety of processes including arterial blood pressure and extracellular fluid volume, learning/memory, metabolism, inflammation, fibrosis, reproduction, cell proliferation etc. Disturbances in the RAS are involved in several diseases such as hypertension and related organ damage, kidney disease, cancer, fibrotic disease, ischemic brain damage, among others.[2–4] Understanding the function of the RAS is, therefore, paramount for preventing and treating RAS-associated disorders.

Knowledge of signaling mechanisms elicited by RAS effectors is essential for a deeper understanding of the molecular mechanisms underlying RAS functions. RAS-related signaling mechanisms have been investigated by classical methods (e.g., Western blotting) for many decades and created a solid foundation of knowledge. However, antibody-based methods have limitations such as availability of commercial antibodies with high specificity and sufficient sensitivity. Another limitation is the slow throughput due to the "one protein at a time" approach. Therefore, the investigation of changes in abundance or phosphorylation of proteins within signaling

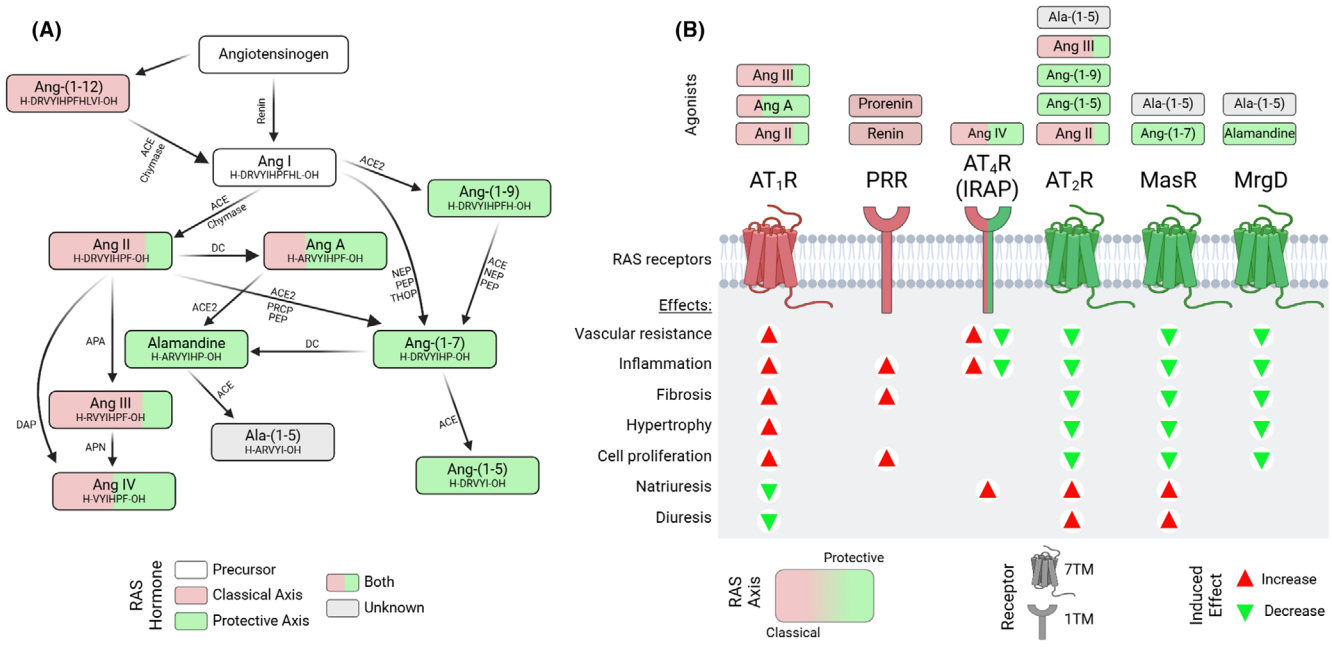

FIGURE 1 An overview of the Renin-Angiotensin System. (A) RAS peptide hormones are formed by the limited proteolysis of the protein precursor angiotensinogen. The amino acid sequence (one letter code) of each peptide is represented below its name. (B) RAS receptors and their ligands. Downstream effects associated with receptor activation is also shown. 1TM, single-pass transmembrane protein; 7TM, seven transmembrane protein; ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme type 2; Ang, angiotensin; APA, aminopeptidase A; APN, aminopeptidase N; AT_1R, angiotensin AT_1 receptor; AT_2R, angiotensin AT_2 receptor DAP, dipeptidyl aminopeptidases; DC, decarboxylase; IRAP, insulin-regulated aminopeptidase; MrgD, Mas-related G-protein-coupled receptor member D; NEP, neprilysin; PEP, prolyl endopeptidase; PRCP, prolyl carboxypeptidase; PRR, prorenin receptor; RAS, Renin-Angiotensin System; THOP, thimet oligopeptidase.

cascades by antibody-based methods is limited to a quite restricted number of target proteins.

Only recently, mass spectrometry (MS)-based "antibody-free" approaches have been added to the armamentarium for studying the RAS. Generally, MS-based techniques have the advantage of very high sensitivity and of the possibility to determine changes in abundance of thousands of proteins at the same time. Importantly, MS-based techniques are also suitable for measuring agonist-induced post-translational modifications (PTMs) such as changes in protein phosphorylation, methylation or glycosylation within the entire cell/tissue proteome. Since PTMs, in particular phosphorylations, are often responsible for changing the activation status of a protein, particularly enzymes, information on PTMs and the respective bioinformatical analysis of such data allows inferences on the activation/inhibition of signaling cascades or other relevant biological processes. This is an important advantage over studies on protein abundance or mRNA expression only, since data on expression do not allow conclusions on protein activity.

This article reviews existing studies which applied MS-based techniques for studying RAS signaling. It focuses on studies applying phosphoproteomics as this technique allows monitoring protein phosphorylation/dephosphorylation events associated with signal transduction. In addition, our article provides an overview over signaling pathways that are shared by different receptors of the protective arm of the RAS as identified by phosphoproteomics. Finally, we discuss knowledge gaps which could be addressed in the future using MS-based approaches.

2 | RAS LIGANDS, ENZYMES, AND RECEPTORS

The discovery of the RAS began in 1898, when Tigerstedt and his assistant Bergman working at the Karolinska Institute in Sweden reported that a protein (renin) extracted from rabbit kidney induced pressor effects when injected into another rabbit.[5] Forty years later, two independent research groups identified the octapeptide angiotensin (Ang) II (H-DRVYIHPF-OH) to be the active hormone responsible for this pressor effect (refer to[6] for an Ang II historical review). Ang II is produced from AGT in a two-step enzymatic process involving renin and angiotensin-converting enzyme (ACE) (Figure 1A).

In the 1970s, Ang III (H-RVYIHPF-OH) was identified as a product of the enzymatic removal of the aspartate residue from the N-terminal of Ang II.[7,8] During the 1970–80s, studies involving Ang II analogues such as Sar^1-Ala^8-Ang II, Sar^1-$Cys(Me)^8$-Ang II or Ang III revealed considerable variability in the responses elicited by these agonists across different tissues indicating the involvement of two or more receptors in mediating the responses of RAS effectors.[9,10] This assumption was finally proven in 1989, when ligands specific for certain receptor subtypes became available such as the non-peptide compounds DuP 753/Ex89 (losartan, AT_1 antagonist), PD123319 (AT_2 antagonist), and the Ang II-peptide analogue CGP42112A (AT_2 agonist). Using these new tools, two independent research groups observed differential displacement of Ang II by these compounds in various tissue preparations, which led to the identification of two distinct receptor subtypes termed the AT_1 receptor (AT_1R) and the AT_2 receptor (AT_2R).[10,11] Existence of these two receptor subtypes was finally proven in the early 1990s with the cloning of the respective cDNA sequences.[12,13]

The first reports on Ang IV (H-VYIHPF-OH) were published in the 1960–70s and were based on structure-to-function studies using Ang II N-terminal fragments. At that time, however, Ang IV was deemed to be biologically inactive.[14] Only from the 1980s, biological effects associated with Ang IV were unveiled, mainly showing modulation of animal behavior such as improvement of learning and memory recall.[15] Ang IV exerts some of its effects by low-affinity binding to the AT_1R and the AT_2R. However, the main endogenous target for Ang IV is the insulin-regulated aminopeptidase (IRAP), also referred to as AT_4R, as only identified in 2001.[16] IRAP has enzymatic activity which is inhibited by Ang IV upon binding. Ang IV can be formed directly from Ang II by dipeptidyl aminopeptidases (DAP) or as an end-product of Ang II N-terminal processing by aminopeptidases (AP) with Ang III as an intermediate of this process (Figure 1A).

Studies from the late 1980s reported biological effects of Ang-(1–7) (H-DRVYIHP-OH),[17,18] a peptide previously considered as an inactive product of Ang II degradation. However, only in 2003 the receptor Mas (MasR) was identified as the receptor for Ang-(1–7).[19] In the second half of the 2000s and the first half of the 2010s, two more RAS peptides were discovered: Ang A (H-ARVYIHPF-OH)[20] acting via the AT_1R to elicit similar effects as Ang II / AT_1R, and Alamandine (H-ARVYIHP-OH) and its receptor MrgD.[21] Alamandine and Ang-(1–7) are both 7-mer peptides differing only at position 1; Ala^1 in Alamandine versus Asp^1 in Ang-(1–7) (Figure 1A). It is believed that an enzyme with a carboxylase activity is responsible for producing Alamandine by removing a CO_2 group from the side chain of Asp^1 to produce Ala^1, thus transforming Ang-(1–7) into Alamandine (Figure 1A). However, to this date, such enzyme is yet to be identified.

Figure 1 represents a most up-to-date view of the RAS including its two functional arms: the classical (canonical) axis and the protective (non-canonical) axis.[3,22] The main receptor of the classical axis is the AT_1R, whereas

the main receptors of the protective axis include the AT_2R, MasR, and MrgD (Figure 1B).

Most recently, Ang-(1–5), a degradation product of Ang-(1–7), was shown to be another biologically active hormone of the RAS.[23–25] Thorough characterization of the peptide revealed that it is an endogenous AT_2R agonist, which elicits effects typical for AT_2R activation such as nitric oxide (NO) synthesis via protein kinase B (Akt)/endothelial nitric oxide synthase (eNOS) signaling, relaxation of mouse and human resistance arteries and lowering of blood pressure in male and female normotensive mice.[25] Another recent addition to the RAS peptide family was the endogenous peptide Alamandine-(1–5) [Ala-(1–5)] (H-ARVYI-OH).[26] Ala-(1–5) seems to signal through the protective RAS receptors: MasR, MrgD and AT_2R. However, only some effects of Ala-(1–5) are typical for MasR, MrgD or AT_2R mediated actions (e.g., increased NO production and reduction of blood pressure in normotensive (Wistar) and hypertensive (SHR) rats), whereas others are not (e.g., constriction of mouse aortic rings and reduced contractility of cardiac myocytes).[26] The unconventional effects elicited by Ala-(1–5) suggest that it potentially binds to different receptor sites and/or elicits G-protein-independent signaling pathways.

Effects evoked by the two RAS arms are usually counter-regulatory. For example, while the activation of the classical axis leads to vasoconstriction, inflammation, fibrosis, and proliferation, activation of the protective axis leads to vasodilation, anti-inflammatory, anti-fibrotic, and antiproliferative effects (Figure 1B).

3 | PHOSPHOPROTEOMICS FOR THE STUDY OF CELL SIGNALING WITHIN THE RAS AND BEYOND

Proteomics encompasses the investigation of a specific proteome, which is defined as a set of proteins being synthesized or degraded within a particular cell or tissue within a specific time. The development of proteomics as we know it today took place in the 1990s,[27] but its rapid advancement accelerated from the 2000s onwards. This progress was primarily propelled by the introduction of novel sample preparation techniques,[28–32] by more sophisticated mass spectrometers,[33–39] and by the development of new bioinformatic tools.[40–48]

From the early days of proteomics, it was evident that novel strategies were required to extend the application of this technique to the study of protein phosphorylation, a PTM that typically occurs at low abundance and therefore cannot be identified through conventional proteomics approaches.[49] To overcome this issue, phosphopeptide enrichment techniques were developed, enabling the identification, localization and quantification of phosphorylation sites. Therefore, in contrast to proteomics, which serves to quantify protein abundances (proteome quantification), phosphoproteomics quantifies protein phosphorylation levels (phosphoproteome quantification) thus allowing conclusions on the activation level of certain proteins such as kinases/phosphatases within signaling cascades. When applying phosphoproteomics it is important to run proteomics as well on the same samples so that phosphorylation levels can be normalized to protein abundances.[50]

Proteomics and phosphoproteomics can be applied in two distinct manners: the targeted and the untargeted approaches. Untargeted proteomics and phosphoproteomics are a hypothesis-generating approach and do not require the pre-definition of certain proteins of interest. Instead, it maps the global proteome or phosphoproteome of a cell or tissue for changes in protein expression or phosphorylation in response to a certain intervention, thereby potentially identifying so far unknown biological processes. In contrast, targeted proteomics/phosphoproteomics is a hypothesis-driven approach that quantifies pre-defined proteins and phosphoproteins (targets) to be assessed in a similar way as the antibody-based methods (i.e. Western blotting) but without the need for antibodies and without the restrictions regarding the number of investigated proteins per experiment. While the untargeted approach is typically favored during the discovery phase of a research project, the targeted approach can be employed to validate findings obtained during the discovery phase.

Figure 2 illustrates a typical workflow for investigating a specific proteome and phosphoproteome within the context of cellular signaling.

Since changes in the phosphorylation status of certain proteins at specific residues is a most common feature of cell signaling cascades, detection of such events (phosphorylation / dephosphorylation) by MS-based phosphoproteomics represents a potent tool for unbiased exploration of cell signaling pathways. Nevertheless, so far only a few studies have investigated signaling mechanisms within the RAS by phosphoproteomics meaning that the power of this technique has not yet been fully taken advantage of in RAS research. Since 2010, the year of the first two studies on RAS signaling using phosphoproteomics,[51,52] only 22 articles have been published which in some way or the other had to do with signaling and RAS components. This contrasts sharply with the more than 17000 studies on RAS signaling since 2010 using other techniques or the more than 3500 publications that have employed phosphoproteomics to study cellular signaling networks unrelated to RAS in the same timeframe (PubMed searches made in November 2024

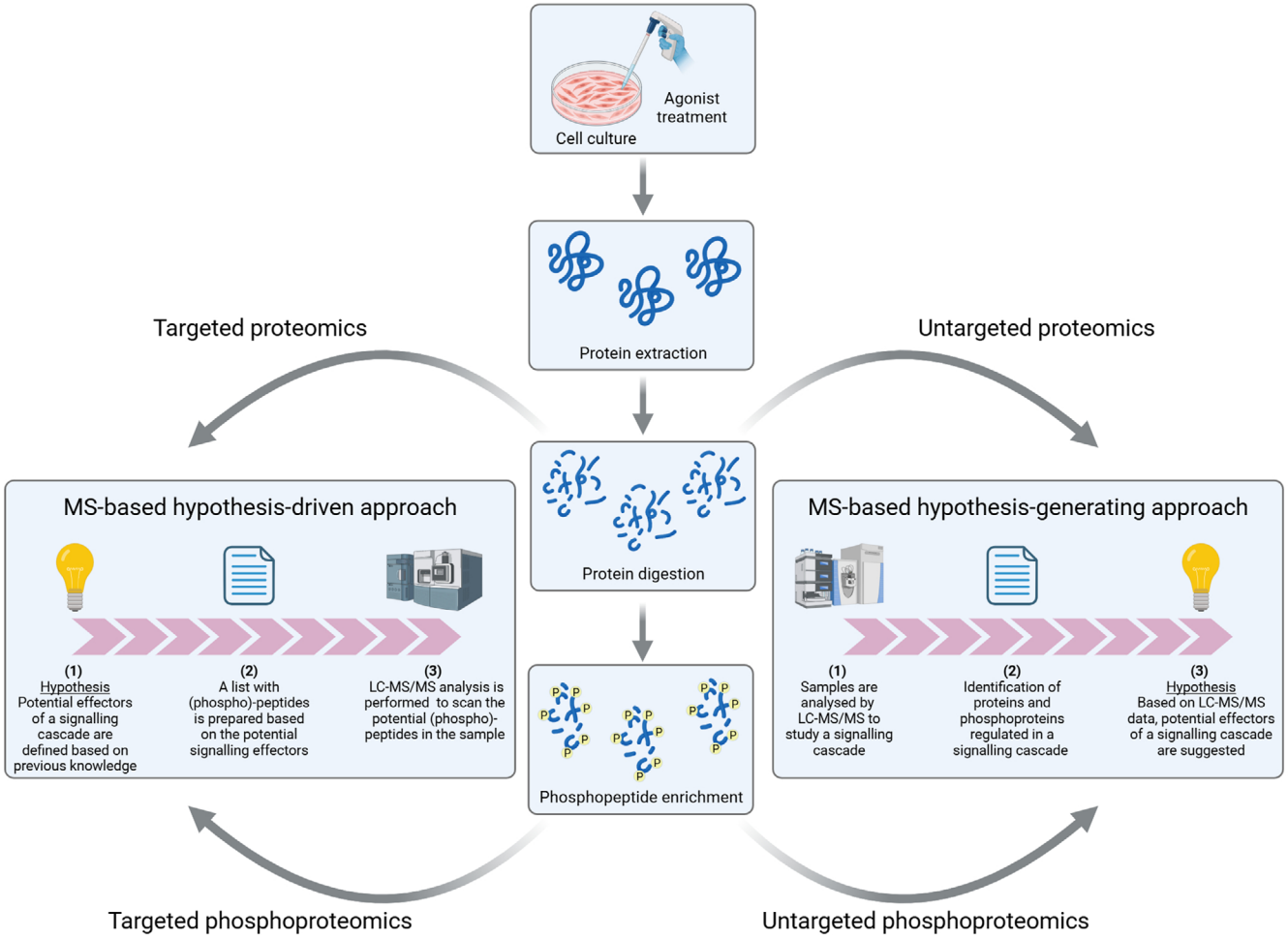

FIGURE 2 Typical proteomics and phosphoproteomics workflow to study cell signaling. Cells are treated with a suitable agonist to trigger the signaling cascades of interest. Cells are then lysed, and proteins and phosphoproteins extracted. Proteins' thiol groups are reduced (e.g., with dithiothreitol) and alkylated (e.g., with iodoacetamide). Subsequently, proteins are digested with specific proteases (e.g., trypsin) followed by an enrichment step to increase the phosphopeptide content in the sample. At this point, the protocol can be continued either as untargeted (hypothesis-generating approach) or targeted (hypothesis-driven approach) proteomics/phosphoproteomics. In the former approach (right panel), peptides/phosphopeptides are analyzed by LC–MS to obtain a global map of the cell's proteome/phosphoproteome. After bioinformatic analysis, lists of regulated proteins and phosphorylation sites are used to infer signaling cascades activated by the respective agonist (hypothesis-generation). In the latter approach (left panel), potential effectors of a given signaling cascade are selected (pre-defined target list) and specifically analyzed by MS for changes in protein abundances and phosphorylation status (hypothesis-driven).

using the following search terms: "angiotensin AND signalling"; "signalling AND phosphoproteome"; "angiotensin AND signalling AND phosphoproteome").

Therefore, in the following sections we will highlight the power of phosphoproteomics for the investigation of RAS-related intracellular signaling, aiming to spark the interest in phosphoproteomics by providing a critical assessment of the utilization of this technology and by reviewing those studies which have applied phosphoproteomics in RAS research so far. Table 1 summarizes the key publications discussed in this review, which helped defined what is now known related to RAS signaling.

4 | THE AT_1 RECEPTOR

The AT_1R is a classical class A G-protein-coupled receptor (GPCR) which signals through G_q and $G_{11/12}$ pathways and through β-arrestin. AT_1R signaling mechanisms have been well characterized by conventional methods and include activation of phospholipase C, IP_3-triggered calcium release, protein kinase C mediated cell proliferation and smooth muscle contraction, as well as activation of the Rho kinase, MAPK/ERK (mitogen-activated protein kinases/extracellular signal-regulated kinases), JAK/STAT (tyrosine-protein kinases JAK/signal transducer and activator of transcription),

TABLE 1 Phosphoproteomics studies targeting the RAS.

Target receptor	Agonist	Agonist type	Models	Proteomic technique used	Reference
AT_1R	Ang II	Unbiased	AT_1R-AB8 3F	SILAC LC-MS/MS	[53]
	Ang II	Unbiased	AT_1R-HEK	SILAC LC-MS/MS	[2]
	$[Sar^1,Ile^4,Ile^8]$Ang II	β-arrestin			
	$[Sar^1,Ile^4,Ile^8]$Ang II	β-arrestin	AT_1R-HEK	SILAC LC-MS/MS	[51]
	$[Sar^1,Ile^4,Ile^8]$Ang II	β-arrestin	AT_1R-HEK	2DGE MALDI-TOF/TOF	[54]
	Telmisartan	Antagonist	Purified AT_1R	LC-MS/MS	[55]
	Ang II	Unbiased			
	TRV023	β-arrestin			
AT_2R	C21	N.A.	HAEC	TMT LC-MS/MS	[56]
	Ang-(1–5)	N.A.	HAEC	TMT LC-MS/MS	[25]
Mas	Ang-(1–7)	N.A.	HAEC	iTRAQ LC-MS/MS	[57]
MrgD	Alamandine	N.A.	MrgD-CHO	iTRAQ LC-MS/MS	[113]
			Mia PaCa-2		
			A549		
AT_4R	Ang IV	N.A.	N2A	SILAC LC-MS/MS	[59]

Abbreviations: 2DGE, two-dimensional gel electrophoresis; iTRAQ, isobaric tags for relative and absolute quantitation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; N.A., not available; SILAC, stable isotope labelling by amino acids in cell culture; TMT, tandem mass tag.

NF-κB (nuclear factor kappa-light-chain-enhancer in B cells), TGF-β (transforming growth factor-beta), Src family (proto-oncogene tyrosine-protein kinase Src), PI3K (phosphatidylinositol 4-phosphate 3-kinases)/Akt, and CaMK (calcium/calmodulin-dependent protein kinases) pathways.[1]

According to our literature search, five studies have been published applying phosphoproteomics for studying AT_1R signaling,[51–55] four of which investigated signaling mechanisms of biased agonists. Generally, depending on the agonist applied, stimulation of GPCRs can result in activation of either the entire signalosome or only of a subset of signaling mechanisms. This phenomenon is known as biased agonism and was initially observed for the PACAP type I (PAC1) receptor[60] and the muscarinic M1 receptor,[61] and subsequently, also for several other GPCRs including the AT_1R.[62,63] In case of the AT_1R, biased ligands selectively activate (with different efficacy profiles) either G-protein-dependent pathways or β-arrestin signaling.

Before reviewing those phosphoproteomics studies which investigated AT_1R biased signaling, we would like to review those two studies first, which looked at AT_1R signaling in a general way.

One of these studies examined AT_1R signaling in AT_1R-transfected immortalized podocytes (AB8 3F- AT_1R).[53] Treatment with Ang II (100 nM, 15 mins) led to changes in the phosphorylation status of 6323 protein fragments that could be assigned to 2081 distinct proteins. As expected for a classical class A GPCR, phosphorylation events were more frequent than dephosphorylation events.[53] Within the phosphorylated sites, the authors observed that the MAPK motif (proline at position +1) was enriched. This is consistent with substantial evidence in the literature that the MAPK pathway is involved in AT_1R signaling.[64,65] Other proteins found to undergo large changes in their phosphorylation status were tenascin, integrin-β6, neuroblast differentiation-associated protein, LCP1 (L-plastin), optineurin, plasminogen activator inhibitor 1, serine/threonine protein kinase D2, protein bicaudal C homolog 1, phalladin, and ephrin type-A receptor. Gene ontology analysis of Ang II-treated AB8 3F-AT_1R phosphoproteomics data revealed an enrichment of terms related to actin cytoskeleton and lamellipodia, among them the protein LCP1 (phosphorylated at Ser^5) which is a member of the α-actinin family and important for actin assembly.[66] Ang II-induced phosphorylation of LCP1 at Ser^5 was validated by Western blot analysis and shown to be indeed AT_1R-mediated, since it was inhibited by the AT_1R-antagonist losartan. In further experiments using specific kinase inhibitors, the authors could show that Ang II-induced phosphorylation of LCP1 was dependent on activation of ERK, RSK (ribosomal S6 kinase), PKC (protein kinase C) and PKA (cAMP-dependent protein kinase), Finally, functional experiments demonstrated Ang II-induced trafficking of LCP1 together with actin to the cell margins as well as Ang II-induced formation of filopodia and cell–cell contacts that was dependent on Ser^5-LCP1 phosphorylation.[53]

The authors compared the outcome of their phosphoproteomic study with a study from Jakob L Hansen's group, which investigated AT_1R signaling by phosphoproteomics applying a (widely) identical protocol (100 nM Ang II for 3 and 15 mins) but in a different

renal cell line, AT_1R-transfected human embryonic kidney (HEK)-293 cells.[52] The comparison revealed that 121 proteins which had increased phosphorylation levels in response to Ang II, were identical in both studies, whereas there were 323 phosphoproteins only detected in podocytes and 406 phosphoproteins only detected in AT_1R-HEK-293 cells upon AT_1R activation. Some of the HEK-293 specific phosphoproteins may be attributable to the 3 mins stimulation, since apparently proteins from both stimulations (3 and 15 mins) were analyzed together, whereas in podocytes, only the 15-min time-point was investigated. Nevertheless, the important lesson from this comparison is that it is not possible to get a general picture of the AT_1R-coupled signaling network from a single study, since results will always be cell/tissue specific and differ from other cells/tissues. It should also be noted that both studies used transfected cells with an artificially high expression level of AT_1Rs. This may have an impact on the results meaning that AT_1R-mediated signaling in primary cells with endogenous receptor expression may be different from signaling in overexpressing cell lines.

Interestingly, the study by the Hansen group[52] included a comparative phosphoproteomics approach in order to distinguish between G-protein-mediated and β-arrestin-mediated AT_1R signaling by treating AT_1R-HEK-293 either with the unbiased agonist Ang II (100 nM) or with the biased agonist [Sar^1,Ile^4,Ile^8]Ang II (SII Ang II; 18.7 μM) which activates $G\alpha_q$ protein-independent (including β-arrestin) signaling. The authors only included phosphosites with an increase (not a decrease) in phosphorylation level into further analysis. They found 1183 of such regulated phosphosites on 527 phosphoproteins, with 427 (36%) phosphosites regulated in response to SII Ang II meaning they are attributable to $G\alpha_q$ protein-independent AT_1R signaling. Further analysis of the data generally revealed a much more diverse and frequent abundance of $G\alpha_q$ protein-independent AT_1R signaling than previously thought. This included a considerable importance of the AGC/CAM kinase family, which includes for example PKD (protein kinase D), PKC and CaMKII, for both Ang II and SII Ang II-induced signaling. Unexpectedly, it was noted that all PKD proteoforms were enriched in the dataset of AT_1R-HEK treated with SII Ang II coinciding with an increased phosphorylation of peptides with the consensus PKD phosphorylation motif. In further experiments using pharmacological inhibitors, the authors found that PKD activation by SII Ang II in AT_1-HEK (i.e., $G\alpha_q$ protein-independent) involved the Ras/ROCK (Rho-associated protein kinase)/PKCδ pathway, whereas PKD activation by Ang II ($G\alpha_q$ protein-dependent and -independent) also involved other PKCs.[52] Other findings comprised $G\alpha_q$ protein-dependence of activation of transcription factors such as c-JUN (transcription factor Jun), HOXA3 (homeobox protein HOX-A3), and EP400 (E1A-binding protein p400), phosphorylation of proteins promoting migration and phosphorylation of other membrane receptors such as the insulin receptor, the insulin-like growth factor 2 receptor or the β2-adrenergic receptor, whereas $G\alpha_q$ protein-independent signaling included reduced transcriptional activity in the nucleus and phosphorylation of CXC chemokine receptor 4 or fibroblast growth factor receptor 3 (among others). Phosphorylation of proteins involved in receptor endocytosis, anti-apoptosis, cytoskeletal rearrangement and cell cycle control were found for both signaling mechanisms, although the exact proteins in each pathway were not identical.[52]

In the year of publication of the study by the Hansen group (2010),[52] the group of Robert Lefkowitz also applied phosphoproteomics for the study of AT_1R signaling using the exact same cell type (AT_1R-HEK-293; the Lefkowitz group provided these cells to the Hansen group), but with a focus on $G\alpha_q$ protein-independent/β-arrestin-dependent AT_1R-signaling by treating cells with SII Ang II only.[51] The incubation time was 5 min and, therefore, similar to but not identical with the incubation times in the study by the Hansen group, which were 3 and 15 min. The dose of SII Ang II was slightly higher in the Lefkowitz study (30 μM) than in the Hansen study (18.7 μM).[52]

Using this approach, the authors identified 4552 phosphopeptides from 1555 phosphoproteins, of which 288 phosphopeptides met their rigorous definition of significance. In 222 phosphopeptides (from 171 phosphoproteins), phosphorylation levels were increased, and in 66 phosphopeptides (from 53 phosphoproteins), phosphorylation levels were decreased in response to the biased agonist SII Ang II.

For verifying their experimental approach, the authors successfully confirmed 5 of the identified phosphoproteins by Western blotting. They further noted an overproportional abundance of kinases among the phosphopeptides (38 protein kinases) as, for example, ERK1, c-Src, Akt, mTOR (mammalian target of rapamycin), and CAMK2, which they could (partly) confirm by additional bioinformatic analysis (Motif-X, Kinase Enrichment Analysis KEA). In a further approach for analyzing the entire dataset, the authors applied a combination of bioinformatic tools including gene ontology (GO) analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) canonical pathway analysis, and Ingenuity Network Analysis and found an enrichment of terms related to actin cytoskeleton reorganization. Together with data from a previous study, which identified an β-arrestin interactome by a global proteomics approach,[67] the authors outlined an AT_1R-coupled, β-arrestin-dependent cytoskeletal reorganization subnetwork. A central role in this network played the slingshot phosphatase, which was found to be significantly

dephosphorylated at Ser^{937} and Ser^{940} by SII Ang II treatment, which is an activation mechanism.[68] Knockdown of β-arrestin 1 and 2 by siRNA prevented SII Ang II-induced slingshot activation thus showing β-arrestin-dependence of the effect. In a series of further, elegant experiments, the authors showed that slingshot dephosphorylates cofilin at Ser^{3}, which is a mechanism related to activation of actin reorganization and lamellipodia formation.[69] This AT_1R-induced effect seems to involve the formation of a β-arrestin-slingshot-cofilin complex that may additionally contain the phosphatase PP2A (protein phosphatase 2), which is able to dephosphorylate and thus activate slingshot.[51] Finally, the authors performed yet another series of bioinformatic analyses of their dataset, this time applying an inference algorithm and a literature-based kinome network combined with known β-arrestin-regulated proteins and the results from the kinase prediction part of their study to construct an interconnecting network of AT_1R- β-arrestin mediated signaling events. This way they found that major areas of AT_1R- β-arrestin actions are the regulation of cell proliferation and cell cycle dynamics, cytoskeletal reorganization, adhesion and inter-cellular communication.[51]

Although the two studies by the Hansen and the Lefkowitz groups had very similar objectives and designs, their results are only partially congruent with only ≈30% identical hits. One reason may be the different methods for phosphopeptide enrichment in the two studies, another the stricter criteria for significance in the Lefkowitz study. However, the difference is also an expression of the fact that there is a risk of false-positive or false-negative hits in the (phospho)-proteomics datasets. Nevertheless, and importantly, the major functional areas, which were predicted to be modulated by AT_1R- β-arrestin signaling in the Hansen and Lefkowitz studies, were widely identical.

A third study by Louis Luttrell's group also investigated SII Ang II-induced AT_1R- β-arrestin signaling and Ang II-induced global AT_1R signaling by phosphoproteomics.[54] As the Hansen/Lefkowitz studies, the authors used AT_1R-HEK-293 cells treated with SII Ang II (50 μM) or with Ang II (100 nM). The incubation time was 5 min. This study revealed much less phospho-modified proteins than the other two for methodological reasons—use of two-dimensional gel electrophoresis (2DGE) and matrix-assisted laser desorption/ionization mass spectrometer (MALDI-MS) instead of liquid chromatography coupled to electrospray ionization mass spectrometer (LC-ESI-MS). The authors identified 36 phosphoproteins, of which 16 were only modified after SII Ang II meaning they are part of the AT_1R- β-arrestin axis. Two peptide inhibitors of protein phosphatase 2A (I1PP2A/I2PP2A) and prostaglandin E synthase 3 (PGES3) were selected for further validation. Additional co-immunoprecipitation studies suggested the existence of I2PP2A/PP2A/Akt-β-arrestin and PGSE3-β-arrestin complexes.[54] Phosphorylation of I2PP2A within the β-arrestin/I2PP2A/PP2A/Akt complex led to inhibition of PP2A activity and subsequently to activation of Akt through Thr^{308} dephosphorylation.

Furthermore, the authors reported formation of a β-arrestin-PGSE3 complex in response to SII Ang II which was responsible for increased PGE_2 production. This effect could be abolished by knocking down β-arrestin.[54]

The study of the Luttrell group was of particular importance because some of the findings (SII Ang II-induced I2PP2A phosphorylation and PGE_2 synthesis) in the AT_1R-HEK-293 cell line were confirmed in primary cells of the cardiovascular system, namely in vascular smooth muscle cells, whereas the other two studies were entirely performed in the artificial system of AT_1R-overexpressing HEK-293 cells. None of the studies investigated any functional (cardiovascular) effects in ex vivo or in vivo experiments such as SII Ang II-induced vasorelaxation through PGE_2[70] or through Akt-mediated eNOS activation.[71] However, increased PGE_2 production in response to the AT_1R-β-arrestin-biased agonist $Des\text{-}Asp^{1}$-Ang I was shown in human umbilical vein endothelial cells in a subsequent study by another group thus pointing to induction of a vasorelaxant mechanism by AT_1R-β-arrestin signaling.[72]

In addition to the above studies, which looked at the entire AT_1R-coupled signaling network, a study by Gareri and co-authors took a more targeted approach and specifically looked at changes in phosphorylation of the C-terminal tail of the AT_1R in response to biased (TRV023) and unbiased (Ang II) agonists.[55] For this purpose, FLAG-tagged human AT_1Rs were enriched from HEK-293 cell lysates using FLAG-tag affinity chromatography and, subsequently, phosphoproteomics performed on the purified receptor. Applying this unique approach, the authors indeed identified different phosphorylation patterns (so-called barcodes) of the AT_1R C-terminal tail in response to the biased or unbiased agonist, respectively. A major finding of the study was that for full β-arrestin recruitment, phosphorylation of a certain cluster of serine and threonine residues in the proximal and middle portions of the tail was necessary. The authors concluded that binding of biased or unbiased agonists triggers different receptor conformations thus inducing divergent phosphorylation patterns at the C-terminus of the receptor.

Interestingly, a few years after the above-reviewed phosphoproteomics studies on AT_1R-β-arrestin-biased signaling, the Lefkowitz group was able to show that biased or unbiased AT_1R agonists stabilize the AT_1R in distinct receptor conformations, which explains the different types of signaling mechanisms elicited by G-protein- or -β-arrestin-coupled receptor activation.[73] Figure 3 illustrates the main findings of AT1R signaling using phosphoproteomics.

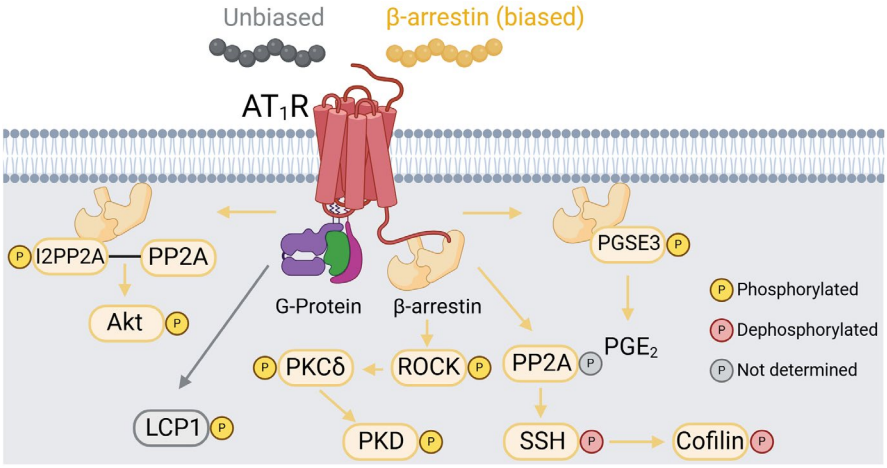

FIGURE 3 AT_1R signaling. Summary of the knowledge acquired by MS-based phosphoproteomics regarding AT_1R signaling using unbiased (Ang II) or β-arrestin-biased (TRV023, TRV027) agonists. Novel components of β-arrestin-biased pathway (depicted in yellow) were identified by several research groups, providing further insights into AT_1R signaling. LCP1, PKC, and PKD (depicted in gray) were observed to be activated by Ang II treatment and, while it is tempting to assume these proteins are related to G-Protein-biased pathways, confirmation with G-Protein-biased agonists (such as TRV055 or TRV056) is much warranted.

5 | THE AT_2 RECEPTOR

As the AT_1R, the AT_2R is categorized as a class A G-protein-coupled receptor. However, signaling of the AT_2R as determined by conventional methods and phosphoproteomics (the latter reviewed in detail in the following) is fundamentally different from classical GPCRs such as the AT_1R, which made some researchers conclude that the AT_2R may represent a distinct subclass of class A GPCRs.[74]

For example, the AT_2R does not signal through G_q and $G_{11/12}$ pathways, it does not recruit or signal through β-arrestin and it is not internalized.[75,76] Instead, it signals through coupling to $G\alpha_{i/o}$—which, however, does not lead to a decrease in cAMP formation as usual for other GPCRs[77]—or it signals through G-proteinindependent mechanisms such as coupling to the AT_2R-interacting protein (ATIP).[4,78] Studies on AT_2R signaling by low-throughput techniques consistently showed that upon agonist binding, the AT_2R activates protein phosphatases such as SHP-1 [Src homology region 2 (SH-2) domain-containing phosphatase 1], PP2A and MKP-1 (MAPK phosphatase-1).[76,79,80] These activated protein phosphatases interfere with other kinase-driven signaling pathways in an inhibitory way. For example, PP2A- and $G\alpha_i$-dependent dephosphorylation of ERK-2 leads to inhibition of insulin-induced ERK1/2 signaling.[81] AT_2R signaling can also involve kinase activation like for example Akt, which is phosphorylated at the activating residue Ser^{473} in response to AT_2R stimulation.[71,82] Akt promotes eNOS activation through phosphorylation of eNOS-Ser^{1177}, which ultimately increases NO release by endothelial cells.[71] In addition to eNOS-Ser^{1177} phosphorylation, eNOS activation by the AT_2R also involves dephosphorylation of eNOS by phosphatases.[56] The above-reviewed signaling pathways—and others reviewed elsewhere[4]—promote the classical effects of AT_2R activation such as natriuresis,[83] vasodilation,[84,85] anti-inflammation,[86] and antiproliferation,[78,87] as illustrated in Figure 1B.

The first study deploying time-resolved, quantitative phosphoproteomics for the study of AT_2R signaling used an untargeted approach for investigating early changes in the phosphorylation pattern of primary human aortic endothelial cells (HAEC) in response to short-term (up to 20 min) AT_2R activation by the small molecule agonist compound 21 (C21).[56] Unexpectedly, the study revealed that in contrast to the prevailing notion that AT_2R signaling is mainly driven by phosphatase activation, the frequency of kinase-driven phosphorylation events was slightly higher. Kinase prediction identified the involvement of Akt in these phosphorylations, and also kinases that are known to activate phosphatases.[56] In order to identify novel AT_2R-coupled signaling pathways with this hypothesis-generating approach, proteins with modified phosphorylation levels were first analyzed by gene ontology (GO), a bioinformatic method for categorizing genes/proteins according to their molecular function, cell compartments or biological processes, followed by STRING analysis for identification of functional protein networks. These analyses unveiled an enrichment of terms related to cell proliferation and apoptosis. Within these terms, the authors selected, HDAC1 (histone deacetylase-1), which was dephosphorylated following C21 treatment at $Ser^{421/423}$ (as subsequently confirmed by Western blotting) and which took a central position in the STRING-analysis cluster related to proliferation/apoptosis.[56] The authors used this result derived from the untargeted approach to further explore a potential, novel, AT_2R-induced signaling

pathway that is initiated by AT_2R-induced $Ser^{421/423}$-HDAC1 dephosphorylation in a targeted approach. They could eventually show that AT_2R-induced HDAC1 dephosphorylation attenuates its deacetylase activity leading to lessened deacetylation of the tumor suppressor p53, which is an activation mechanism that leads to nuclear translocation of p53 and culminates in antiproliferative and anti-apoptotic effects of AT_2R activation—functionally shown in this study in HAEC and in PC9, a non-small lung cancer cell line.[56]

In a second study with a similar protocol (up to 20 min AT_2R stimulation in HAEC) but an improved MS methodology with higher sensitivity, the same authors used the newly identified endogenous AT_2R agonist Ang-(1–5) for receptor activation.[25] In this analysis and in contrast to the study with C21 reviewed above, dephosphorylations were slightly prevailing over phosphorylations. This difference may be due to the improved methodology in the 2nd study, which allowed the detection of many more sites with changes in phosphorylation status than the 1st study—including tyrosine phosphorylations, which could not be detected by the methodology of the 1st study, but which play an important role in AT_2R signaling as was already detected by conventional methods years ago.[88] Another reason for the slightly different result of the two studies in terms of the phosphorylation/dephosphorylation ratio may be that C21 and Ang-(1–5) act as biased agonists and do not elicit the exact same array of signaling cascades. Importantly, despite these differences in the phosphorylation pattern, both phosphoproteomic studies clearly point to tissue protective, antiproliferative actions of the AT_2R. In the study with Ang-(1–5) as AT_2R agonist, this was evident from performing a KEGG pathway analysis of the data, which detects enrichment of phospho-modified proteins within defined signaling pathways pointing to activation or inhibition of these pathways by the applied agonist. In case of AT_2R activation by Ang-(1–5), KEGG pathway analysis revealed inhibition of VEGF (vascular endothelial growth factor) and HIF-1 (hypoxia-inducible factor-1) signaling, inhibition of leucocyte transendothelial migration as well as effects on the actin cytoskeleton and on adhesion. These results still await confirmation by a 2nd method and by functional tests in future studies.

6 | THE Mas RECEPTOR

As the AT_2R, the MasR, which is the main receptor for Ang-(1–7), is a class A GPCR with unconventional signaling mechanism as defined by conventional methods. Interestingly, MasR and AT_2R signaling mechanisms have a lot of similarities.

For example, as described for the AT_2R in the preceding section, MasR-mediated vasodilation induced by Ang-(1–7) is resulting from an increase in NO release.[19,89,90] Studies using classical approaches have shown that Ang-(1–7)-induced NO release involves a rapid and long-lasting phosphorylation of eNOS at Ser^{1177} after 5 to 30 min of treatment resulting in eNOS activation and NO production as shown in HAEC and MasR-transfected CHO cells.[91,92] Western blotting further revealed that Akt, a kinase that phosphorylates eNOS at Ser^{1177},[91] was phosphorylated at its activation site (Ser^{473}) following 5 min of Ang-(1–7) treatment via the PI3K-Akt pathway.[92] The role of MasR in this process was confirmed using the selective MasR-antagonist A779, and by the absence of the effect in non-transfected CHO cells.[92]

A crosstalk has been described between Ang-(1–7)/MasR signaling and insulin/insulin receptor (IR) signaling.[93] In brief, Ang-(1–7)/MasR increases the expression of insulin, and induces beneficial outcomes in insulin resistance and metabolic syndrome experimental models.[94–98] Furthermore, Ang-(1–7)/MasR signaling and Insulin/IR signaling share important effectors like PI3K, Akt, GSK-3β (glycogen synthase kinase-3 beta), IRS-1 (insulin receptor substrate-1) and JAK2.[99–101]

Another important aspect of Ang-(1–7)/MasR signaling is the inhibition of pathways activated by Ang II/AT_1R explaining, at least in part, the counter-regulatory effects of Ang-(1–7) against Ang II effects (Figure 1B). It has been shown in different models that Ang-(1–7)/MasR induces the dephosphorylation and inhibition of key effectors of Ang II/AT_1R signaling including ERK1/2, c-Src, p38 MAPK, JNK (jun N-terminal kinase), NF-κB, STAT3, Akt, PKC-α, GSK-3β, and NADPH (nicotinamide-adenine dinucleotide phosphate).[92,102] The dephosphorylation of components of the MAPK/ERK pathway by Ang-(1–7)/MasR involves activation of the phosphatases SHP-2 and MKP-1.[103,104]

A work published in 2012[57] was the first publication and the only one thus far applying phosphoproteomics to study Ang-(1–7)/MasR signaling. The study focused on early phosphorylation events in HAEC (up to 20 min after Ang-(1–7) stimulation). A total of 1288 unique phosphorylation sites on 699 proteins were identified. Of these, the phosphorylation levels of 121 sites on 79 proteins were reported to change significantly in response to the treatment, thus identifying potential components of Ang-(1–7)/MasR signaling pathways in HAEC. This study supports the potential interplay between Ang-(1–7)/MasR signaling and insulin/IR signaling as eight of the identified phosphoproteins are also components of insulin/IR signaling: Akt, AKTS1 (proline-rich AKT1 substrate 1), CAV1 (caveolin-1), FOXO-1 (forkhead box protein O1), MAPK1, PXN (paxillin), PIK3C2A (phosphatidylinositol

4-phosphate 3-kinase C2 domain-containing subunit alpha), and VIM (vimentin). The shared phosphoproteins represent approximately 10% of all proteins identified as differentially phosphorylated/dephosphorylated in response to Ang-(1–7) treatment. In this study, FOXO-1 was selected for further confirmatory experiments. FOXO-1 is a transcription factor that undergoes Akt-induced phosphorylation at Thr^{24}, Ser^{256}, and Ser^{319}. Phospho-FOXO-1 is localized in the cytoplasm and is translationally inactive.[105] However, upon its dephosphorylation, FOXO-1 is translocated into the nucleus and becomes transcriptionally active. Following 5 min of Ang-(1–7)/MasR stimulation, a significant dephosphorylation of FOXO-1-Ser^{256} was revealed by phosphoproteomics. Functional validation by confocal microscopy confirmed that Ang-(1–7) led to nuclear accumulation of FOXO-1 in HAEC.

The identification of FOXO-1 as an important downstream component of Ang-(1–7)/MasR signaling is an example of the potential of untargeted phosphoproteomics in generating new hypotheses. As mentioned before in this review, Ang-(1–7) induces the activation of PI3K-Akt signaling in HAEC (Figure 4A). Since PI3K-Akt signaling has been reported to lead to the phosphorylation of FOXO-1, resulting in its inactivation and cytoplasmic accumulation,[105] the observed dephosphorylation and nuclear accumulation[57] was against expectations and would probably not have been found with a targeted approach (Figure 4B). The finding of Ang-(1–7)/MasR induced FOXO-1 activation by this study initiated a number of follow-up studies investigating the role of FOXO-1 for Ang-(1–7)/MasR signaling and actions by hypothesis-driven approaches.[106]

Another example of the use of MS-based technologies for studying Ang-(1–7)/MasR signaling is an interesting study by Hoffmann et al.[107] in rat microvascular endothelial cells (RMVECs), which employed a combination of immunoprecipitation of MasR in native conditions to co-precipitate its interacting proteins before and after stimulation with Ang-(1–7) followed by the MS-based identification of the MasR interacting proteins. A total of 50 proteins co-precipitated with MasR including AT_1R, mTOR, PRKD1 (serine/threonine protein kinase D1), RASGRF1 (ras-specific guanine nucleotide-releasing factor 1), TRPM6 (transient receptor potential cation channel subfamily M member 6), and GRIP1 (glutamate receptor-interacting protein 1). In addition to identifying

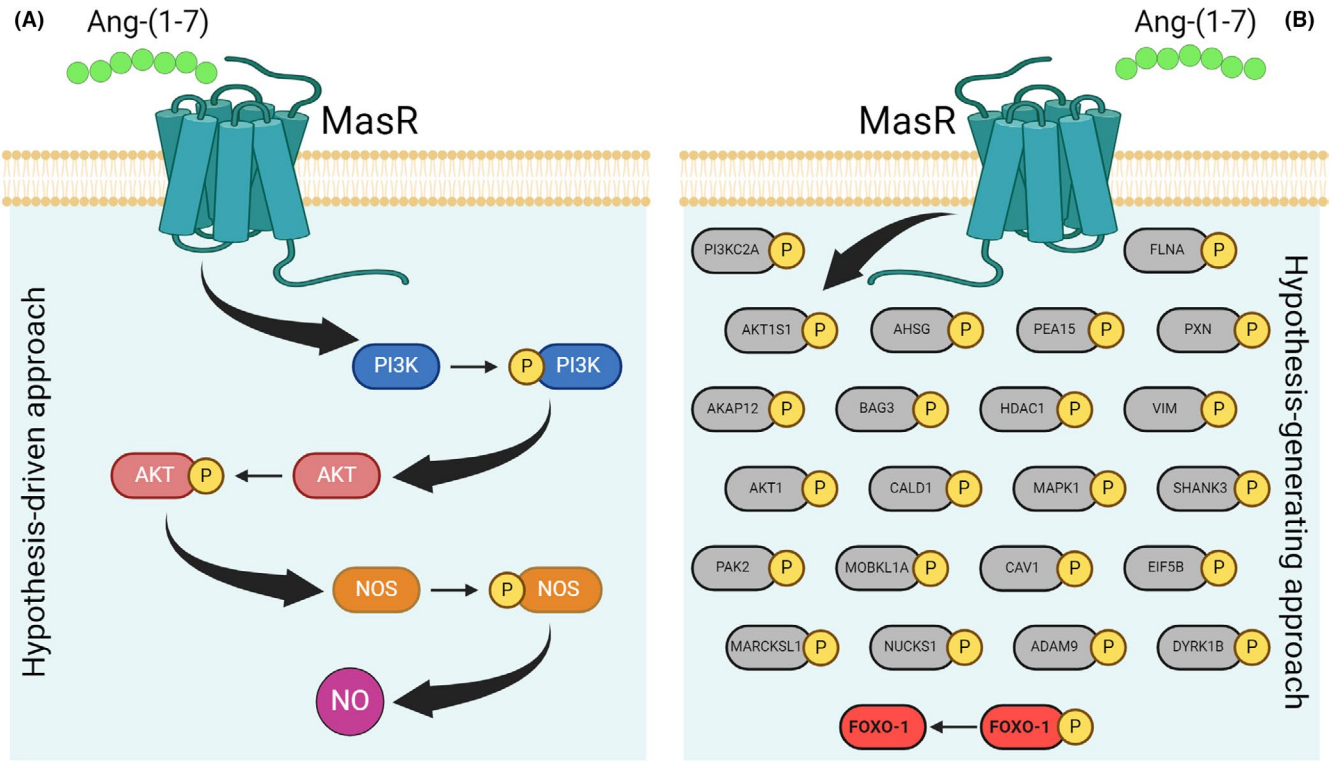

FIGURE 4 Ang-(1–7)/MasR signaling. Comparison of the classical Western blot-based hypothesis-driven approach (A) and MS-based hypothesis-generating approach (B) in the study of Ang-(1–7)/MasR signaling. A solid knowledge about the Ang-(1–7)/MasR signaling was built using Western blotting resulting for example, in the identification of PI3K-Akt pathway activation by Ang-(1–7) to induce NO generation in endothelial cells. The use of a MS-based shotgun phosphoproteomics method (hypothesis-generating approach) allowed the identification of 79 potential new downstream effectors of Ang-(1–7)/MasR signaling (some represented in the figure), including the validated new effector FOXO-1.

new interaction partners of the MasR, the study also confirmed heterodimerization of the MasR with the AT_1R, which is one of several heterodimers described for RAS receptors.[108] MasR/AT_1R heteromerization negatively modulates Ang II/AT_1R signaling, for example by inhibiting AT_1R-induced inositol phosphate generation and intracellular Ca^{2+} increase.[109]

7 | THE MrgD RECEPTOR

MrgD is a member of the Mas-related G-protein-coupled receptor family and of the protective axis of the RAS with Alamandine as its primary ligand.[21] β-alanine and GABA have been described as MrgD ligands too, though GABA is a low-affinity MrgD agonist.[110]

A structural study of MrgD complexed with β-alanine was recently published using cryo-electron microscopy (Cryo-EM). β-alanine binds to a shallow pocket close to the extracellular loop 2 (ECL2), surrounded by TM3, TM4, TM5, and TM6 transmembrane (TM) domains. The β-alanine/MrgD complex is stabilized by electrostatic interactions between the β-alanine carbonyl group (C=O) with Arg^{103} (TM3) and Asp^{179} (TM5). Hydrogen bounds stabilize interactions of β-alanine with Cys^{164} (TM5) and Trp^{241} (TM6).[111]

It is possible that Alamandine binds to the same site as β-alanine because effects of Alamandine are abolished by a pre-treatment with β-alanine,[21] suggesting that both ligands compete for the same site. However, it cannot be ruled out that Alamandine binds to a different site and that the observed β-alanine "antagonistic" effect is due to an allosteric conformational change rather than a site competition or that Alamandine binds to the same site but with different interaction partners within the receptor pocket. Thus, an investigation of MrgD complexed with Alamandine is still warranted.

As the other protective RAS receptors, AT_2R and MasR, MrgD mediates the induction of NO production. However, at least in cardiomyocytes, the signaling mechanism leading to Alamandine/MrgD-induced NO synthesis seems different and includes the activation of the LKB1 (serine–threonine liver kinase B1)/AMPK (AMP-activated protein kinase) pathway in a PI3K/Akt-independent fashion.[112] The LKB1/AMPK pathway seems also crucial for the MrgD-mediated prevention of the hypertrophic effect induced by Ang II/AT_1R in neonatal rat cardiomyocytes.[112] This observation was confirmed in an in vivo transverse aortic constriction (TAC) model of cardiac hypertrophy in mice. TAC led to the dephosphorylation of AMPK-Thr^{172}, but Alamandine via MrgD restored AMPK-Thr^{172} phosphorylation, which is consistent with AMPK activation.[58]

Other signaling pathways and cellular events associated with the cardioprotective effect induced by Alamandine/MrgD in the TAC model, as identified by conventional methods, included the dephosphorylation and consequent inhibition of ERK1/2-Thr^{202}/Tyr^{204}, phosphorylation of PLN (cardiac phospholamban)-Thr^{17}, and reduced expression of MMP-2 (matrix metallopeptidase 2). Regarding TAC-induced ROS production, Alamandine/MrgD decreased the expression of a subunit of NADPH oxidase (gp91phox) and increased the expression of SOD2 (superoxide dismutase 2, mitochondrial) and CAT (catalase).[58]

The MrgD-coupled signaling network induced by Alamandine was explored by untargeted phosphoproteomics complemented with antibody-based approaches in the context of a study that investigated a potential MrgD-dependent antiproliferative and anti-cancer effect in the human pancreatic cancer cell lines Mia PaCa-2 and A549 and in MrgD-transfected CHO cells (MrgD-CHO).[113]

Phosphoproteomics of CHO-MrgD stimulated by Alamandine (up to 20 min) identified similar signaling pathways with potential tissue protective outcomes as the phosphoproteomics studies for the AT_2R[25,56] and MasR,[57] comprising the inhibition of the pathways PI3K/Akt/mTOR and BRAF/MKK/ERK1/2, as well as the activation of FOXO-1 and p53.[113]

Of note, the phosphoproteomic experiments exploring the antitumoral effect of Alamandine in Mia PaCa-2 cells focused on later time points (up to 48 h) than all other RAS receptor phosphoproteomics studies. These incubation times were chosen because the antiproliferative effects elicited by Alamandine were only observed after 2 days of treatment. The authors reported that Alamandine induced a significant change in the phosphorylation of proteins associated with cytoskeleton regulation, potentially reducing their capability of cellular migration. It was also reported that Alamandine/MrgD activation led to dephosphorylation and consequent inhibition of key proteins associated with cell division, such as EIF3B (eukaryotic translation initiation factor 3 subunit B) at Ser^{85}/Ser^{119} and EIF4B at Ser^{422}/Ser^{498}/Thr^{500}/Ser^{504}.[113]

8 | THE AT_4 RECEPTOR/IRAP

Unlike the other RAS receptors AT_1R, AT_2R, MasR, and MrgD, which are seven-transmembrane (7TM) G-protein-coupled receptors (GPCRs), the AT_4R/IRAP is a transmembrane M1 zinc aminopeptidase (1TM).[114,115] The receptor has a broad tissue distribution including expression in the brain, heart, kidneys, adrenal glands, and blood vessels. Ang IV binds to the IRAP catalytic site with high affinity reducing its ability to degrade neuropeptides

like vasopressin, oxytocin, kallidin, somatostatin, among others.[1,16]

Classical experiments have shown that Ang IV modulates different signaling pathways depending on cell type or tissue, some of which could be inhibited by AT_1R or AT_2R antagonists and thereby attributed to activation of these receptors.

However, the important beneficial effects of Ang IV on cognition (and others) seem AT_1R/ AT_2R-independent, but AT_4R/IRAP-dependent. Signaling of Ang IV through IRAP is still not entirely understood and may involve effects of the accumulated IRAP substrates or direct signaling effects of IRAP.[115,116]

To gain more insights into potential signaling pathways elicited by Ang IV/AT_4R/IRAP, Wang et al.[59] employed phosphoproteomics on N2A cells (mouse neuroblasts) treated or not with Ang IV for 30 min. In their publication, the authors focus the analysis of their data entirely on the dephosphorylation of the alpha catalytic subunit of the phosphoprotein phosphatase 1 (PP1α-Thr^{320}), which is an activation mechanism. In line with that, PP1α downstream substrates were found dephosphorylated, suggesting its important role in signaling in neuronal cells. Finally, the authors observed Ang IV-induced G1/S cell arrest, which they attributed to the increased activity of PPP1α.[59]

9 | COMMON RAS SIGNALING COMPONENTS

Even though the number of phosphoproteome studies investigating RAS receptor signaling is still limited, it is, nevertheless, striking that the studies using untargeted approaches looking at receptors of the protective axis of the RAS identified widely similar signaling pathways thus creating a kind of a "déjà vu" experience. Analyzing four different phosphoproteome datasets from AT_2R,[25,56] MasR,[57] and MrgD,[113] we observed a remarkable overlap of regulated phosphorylation events in response to short-term agonist stimulation.

Figure 5 illustrates some key signaling effectors shared by MasR, AT_2R and MrgD according to the phosphoproteomics studies. For example, activation of all three receptors induced: FOXO-1 dephosphorylation and consequent activation, p53 dephosphorylation and consequent activation, HDAC dephosphorylation and consequent inhibition, and ERK1/2 dephosphorylation and consequent

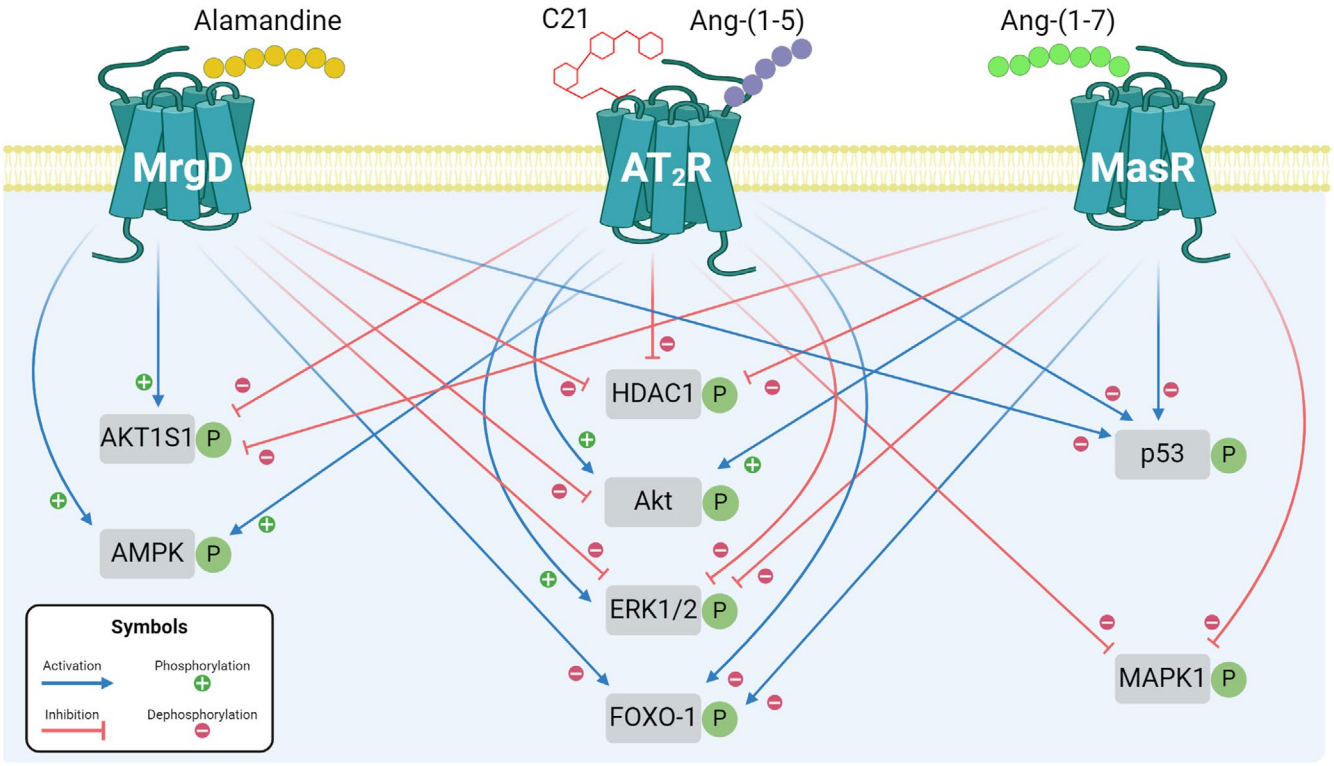

FIGURE 5 Common signaling pathways. Non-exhaustive list of signaling effectors and phosphorylation events shared by receptors of the protective axis of the RAS as determined by phosphoproteomics. Akt, serine/threonine protein kinase (protein kinase B); AKT1S1, proline-rich AKT1 substrate 1; AMPK, AMP-activated protein kinase; C21, Compound 21; ERK1/2, extracellular signal-regulated kinase 1/2; FOXO-1, forkhead box protein O1; HDAC1, histone deacetylase 1; MAPK1, mitogen-activated protein kinase 1; p53, tumor protein p53 (tumor suppressor protein).

inhibition. Akt and AKT1S1 (proline-rich Akt1 substrate 1) were phospho-modified in the same way by the MasR and the AT_2R (phosphorylation/activation of Akt; dephosphorylation / inhibition of AKT1S1) whereas MrgD activation induced opposing effects (dephosphorylation / inhibition of Akt; phosphorylation/activation of AKT1S1). AMPK phosphorylation / activation was only observed for MrgD and AT_2R (but not MasR) signaling, whereas MAPK1 dephosphorylation/inhibition was only detected for AT_2R and MasR signaling.

Surprisingly, C21-induced AT_2R activation led to ERK1/2 phosphorylation and consequent activation,[56] while Ang-(1–5)-induced AT_2R activation led to ERK1/2 dephosphorylation and consequent inhibition.[25] However, C21-induced ERK1/2 activation happened very early (after 1 min), whereas Ang-(1–5)-induced ERK1/2 inhibition occurred only after 20 min, which may indicate that these events are not part of the same signaling pathway and biological process. ERK1/2 activation can mediate a multitude of different biological effects such as phosphatase activation (a potentially protective mechanism) at very early time points or promotion of pro-inflammatory and pro-fibrotic pathways at later time points.

10 | WHAT TO CONSIDER WHEN DOING PHOSPHOPROTEOMICS

10.1 | Cell lines and animal models

Untargeted phosphoproteomics relies on protein databases to identify (phospho)-proteins in samples. There are two main types of protein databases: those containing unreviewed proteins (e.g., UniProtKB/TrEMBL) and those with reviewed proteins (e.g., UniProtKB/Swiss-Prot). Unreviewed proteins are "computationally annotated", while reviewed proteins are "manually annotated", which is preferable since the results are more reliable. As of September 2024, the UniProtKB/Swiss-Prot database included 26821 reviewed proteins from *Homo sapiens* (human), 17823 from *Mus musculus* (mouse), 8304 from *Rattus norvegicus* (rat), and 247 from *Cricetulus griseus* (Chinese hamster). Thus, the choice of cell lines and animal models can significantly impact (phospho)-proteomics results, since the size of reference databases differs between species. Therefore, the choice of species is critical, and samples from humans or mice are generally preferred over other species for (phospho)-proteomic studies.

However, samples from less commonly used species can still be valuable under certain circumstances. For example, the CHO cell line originating from Chinese hamster (*C. griseus*) is often used for transfection and expression of RAS receptors (MasR, AT_1R, AT_2R, or MrgD) because it does not constitutively express these receptors, which means that non-transfected cells can serve as perfect negative controls. Rat models such as spontaneous hypertensive rats (SHR) and transgenic rats are also widely employed in RAS research and often the optimal model for studying certain diseases. For species with a limited number of annotated proteins in a reviewed database, researchers may use the UniProtKB/TrEMBL database of unreviewed proteins. As of September 2024, it contained 83438 proteins for *C. griseus* and 100383 for *R. norvegicus*. However, the fact that these proteins are only computationally annotated needs to be kept in mind, conclusions done with more caution and where possible validated by additional experiments.

For phosphoproteome studies, availability of data about the role of phosphorylation/dephosphorylation of certain residues (e.g., whether phosphorylation leads to activation or inactivation of a protein) is even more limited, though there are specific databases like the PhosphoSitePlus database (https://www.phosphosite.org) that can be used to interrogate specific phosphorylation sites. There are also algorithms that use experimental datasets to predict active kinases (e.g., KSTAR[117]) and active signaling pathways (e.g., phuEGO[118]). Nevertheless, interpretation of untargeted phosphoproteomic data can be difficult, and it may be necessary to limit follow-up studies to only those identified phosphoproteins for which information is available in databases.

10.2 | Selectivity of ligands

Phosphoproteomics as reviewed in this article serves to unravel signaling mechanisms induced by the activation of a receptor by a respective agonist. Since phosphoproteomics is a highly sensitive technique, it is crucial to verify in advance whether the agonist to be used is highly selective for the targeted receptor. Since ligand selectivity is also a matter of dosing (every ligand loses selectivity at some point when increasing the dose/concentration), it is also essential to choose a dose/concentration for the agonist at which the agonist binds to and activates exclusively the target of interest. Data on selectivity of a certain ligand often only exist for a restricted number of potential off-targets—if at all. Therefore, there will always be some remaining uncertainty whether all observed effects can really be attributed to the interaction of the agonist with the target of interest. Thus, control experiments, for example with antagonists or in cells/animals, which do not express the receptor of interest, are essential to control for off-target effects.

11 | REMAINING KNOWLEDGE GAPS

Although the above-reviewed MS-based phosphoproteome studies provided major insights into RAS-associated signaling mechanisms, some "puzzle stones" are still missing for a global understanding of the RAS signaling networks.

For AT_1R signaling, for example, none of the phosphoproteomic studies used cells which endogenously express AT_1R. However, AT_1R signaling patterns have been thoroughly characterized by low-throughput techniques (reviewed elsewhere ref [119]) using cells or tissues endogenously expressing the receptor, and most of the findings from phosphoproteomics in transfected cells are in concordance with findings from these low-throughput studies. Whether, and to which extent, additional signaling mechanisms identified in the phosphoproteomics studies using transfected cells, which are not "backed up" by conventional studies, are also relevant in models endogenously expressing AT_1Rs remains to be investigated.

To date, phosphoproteome-based studies of RAS signaling have primarily relied on simplified systems such as primary cells (e.g., HAEC) or transfected cell lines expressing specific receptors (e.g., CHO-MrgD, CHO-AT_2R). While these models provide a controlled environment to dissect receptor-specific pathways and downstream effectors, they lack the physiological complexity. Investigating RAS signaling in more complex systems, such as whole organisms or tissue-specific models would provide critical insights into the biological relevance of these signaling pathways. Such studies could determine whether the effectors identified in vitro are similarly modulated in vivo, where the interplay of multiple cell types, tissue environments, and systemic factors could influence the signaling dynamics. Moreover, in vivo phosphoproteomics could reveal novel effectors and pathway regulations that are not evident in isolated cell models, advancing our understanding of RAS biology and its role in health and disease.

Phosphoproteomics are a potential tool for comparing "shared" versus "unique" signaling patterns in different cell types/conditions. For example, Schenk and coworkers[53] reported for AT_1Rs substantial differences between Ang II-induced signaling in HEK versus AB8/13 cells, both with exogenous AT_1R expression. The same approach could also be used in cells/organisms with endogenous AT_1R expression to unveil system bias (differences in signaling between different cells/tissues) or differences in signaling between normal and diseased conditions. Furthermore, the use of biased AT_1R agonists in this setup would allow distinguishing between G-protein- and β-arrestin-dependent signaling patterns involved in physiological processes in different cells and/or in the progression of diseased states.

What is indeed still much warranted is the characterization of the signaling pathways elicited either by G-protein- or by β-arrestin-biased ligands in systems endogenously expressing the AT_1R. Such research has been hampered in the past by the unavailability of the respective biased AT_1R agonists. The G-protein-biased AT_1 agonist TRV055 became only recently available (first publication in 2019[73]). β-arrestin-biased AT_1R agonists have been available for longer with the first, [[Sar^1,Ile^4,Ile^8]Ang II (SII Ang II)], published in 2003.[63] Therefore, the initial approach to study G-protein-coupled versus β-arrestin-coupled AT_1R signaling was a comparison of signaling cascades elicited by the balanced full agonist Ang II with those elicited by the β-arrestin-biased partial agonist SII Ang II.[52,54] In this approach, the overlapping signaling components represent β-arrestin-dependent signaling pathways, whereas signaling components activated by Ang II only (but not by SII Ang II) constitute G-protein-dependent signaling pathways. As SII Ang II is a low-affinity, partial β-arrestin-biased AT_1R agonist with some residual G-protein activation capability that becomes apparent particularly in AT_1R overexpressing cells,[120] and since AT_1R overexpressing cells have been the standard model for studies on biased AT_1R signaling so far, it is likely that existing data on AT_1R β-arrestin-dependent signaling have some inaccuracies. Thus, a systematic phosphoproteomic investigation of cells with endogenous AT_1R expression treated with the now available optimized biased AT_1R agonists such as TRV055 (for G-protein-biased signaling) and TRV027 (for β-arrestin-biased signaling) would accurately characterize AT_1R signaling through the two major receptor activation mechanisms.

Another area which has hardly been investigated is the characterization of signaling pathways elicited by RAS receptor heterodimers. RAS receptors form heterodimers with other receptors of the RAS (e.g., AT_1R-AT_2R, AT_2R-Mas) or with non-RAS receptors (e.g., AT_1R-B_2 bradykinin B2 receptor), AT_1R-β-adrenergic receptors.[4] This is important because heterodimerization can change receptor conformations and, thereby, receptor signaling.[108] This has potential clinical relevance, for example due to the phenomenon of cross-inhibition, which means that one antagonist (e.g., an ARB) inhibits signaling of the dimerized other receptor (e.g., a β1-adrenergic receptor).[121]

The AT_2R, MasR and MrgD have been described to be constitutively active, i.e., they elicit intracellular signaling on a low level without agonist binding. A further potential area of phosphoproteomics could be to determine whether constitutive signaling patterns differ from agonist-induced signaling.

Phosphoproteomic-based studies on the signaling mechanisms elicited by several RAS components including (pro-)renin/PRR, Ang-(1–12), Ang-(1–9), Ang A, and

Ala-(1–5) have not been performed yet. For some of these components, detailed knowledge of the signaling mechanisms may also help to identify the responsible receptor. Such studies may also clarify whether biased agonism only exists for the AT_1R, that is, the classical arm of the RAS, or whether it can be found in receptors of the protective RAS as well.

Finally, our understanding of RAS signaling could be significantly advanced through the integration of multi-omics approaches. For instance, while phosphoproteomics offers critical insights into phosphorylation events and their roles in signaling cascades, examining other PTMs (e.g., glycosylation, methylation, and acetylation) could provide a more comprehensive view of the molecular mechanisms underlying RAS activity (e.g., regulation of gene expression and epigenetics). Furthermore, combining phosphoproteomics with metabolomics and lipidomics could reveal how RAS signaling pathways interact with cellular metabolism. From an in vivo perspective, recent advancements in single-cell transcriptomics and single-cell proteomics offer unprecedented opportunities to study RAS signaling at the resolution of individual cells. These techniques enable the characterization of cell-type-specific signaling dynamics and the identification of heterogeneous responses to RAS stimuli within complex tissues.

12 | CONCLUSIONS

Phosphoproteomics is a powerful technique for quantifying phosphorylation events in an unbiased manner and has proven invaluable for studying signaling pathways across numerous receptor systems. However, its application in the context of RAS-related signaling pathways remains surprisingly underexplored. There is significant potential to utilize phosphoproteomics for investigating the signaling cascades of emerging RAS components, such as Ang-(1–5) and Ala-(1–5), to study biased agonism within the RAS, and to explore how heterodimerization of RAS receptors impacts cellular signaling networks. With recent advancements enabling the identification of tens of thousands of phosphorylation sites per experiment, a comprehensive re-examination of RAS receptor signaling is warranted, as new effectors and regulatory mechanisms are likely to emerge.

Moreover, extensive datasets containing thousands of phosphorylated proteins modulated by RAS hormones are available in public repositories (e.g., PRIDE, Peptide Atlas, MassIVE, iProX) through the ProteomeXchange Consortium (https://www.proteomexchange.org). These datasets are often only partially analyzed in the original studies and, therefore, can be regarded as "goldmines" which offer opportunities for re-analysis or meta-analysis to identify signaling effectors which were previously overlooked or not explored in detail in the original studies. By revisiting these datasets with focused questions, researchers can extract valuable new insights from the data, broadening our understanding of RAS biology and potentially uncovering novel therapeutic targets.

AUTHOR CONTRIBUTIONS

Igor Maciel Souza-Silva: Conceptualization; writing – original draft; writing – review and editing. **Victor Corasolla Carregari:** Writing – original draft. **U. Muscha Steckelings:** Conceptualization; funding acquisition; writing – original draft; writing – review and editing; supervision. **Thiago Verano-Braga:** Conceptualization; funding acquisition; writing – review and editing; writing – original draft; supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing does not apply to this work as no new data is reported.

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