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Invited Review

Highly sensitive myosin phosphorylation analysis in the renal afferent arteriole

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Abstract

The regulation of smooth muscle contraction and relaxation involves phosphorylation and dephosphorylation of regulatory proteins, particularly myosin. To elucidate the regulatory mechanisms, analyzing the phosphorylation signal transduction is crucial. Although a pharmacological approach with selective inhibitors is sensitive and a useful technique, it leads to speculation regarding a signaling pathway but does not provide direct evidence of changes at a molecular level. We developed a highly sensitive biochemical technique to analyze phosphorylation by adapting Phos-tag SDS-PAGE. With this technique, we successfully analyzed myosin light chain (LC_{20}) phosphorylation in tiny renal afferent arterioles. In the rat afferent arterioles, endothelin-1 (ET-1) induced diphosphorylation of LC_{20} at Ser19 and Thr18 as well as monophosphorylation at Ser19 via ET_B receptor activation. Considering that LC_{20} diphosphorylation can decrease the rate of dephosphorylation and thus relaxation, we concluded that LC_{20} diphosphorylation contributes, at least in part, to the prolonged contraction induced by ET-1 in the renal afferent arteriole.

Key words: smooth muscle, renal arteriole, phosphorylation, Phos-tag, endothelin-1

Introduction

Smooth muscle is found everywhere in the body — the walls of blood vessels, lymphatic vessels and hollow organs, and the ciliary muscle and iris of the eye. Although they basically have the same contractile and regulatory apparatuses in common, they respond differently to various stimuli to achieve specific effects at individual times.

The regulation of smooth muscle contraction and relaxation involves phosphorylation of regulatory proteins, including myosin (1–3), which generates contractile force. To study the regulatory signal transduction pathways, a variety of physiological and pharmacological techniques have been employed. Although pharmacological observations with the use of selective inhibitors can lead to speculation about the signal

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transduction pathways, they will not provide conclusive evidence due to their general lack of specificity.

By combining biochemical and pharmacological techniques, changes at a molecular level will be measurable, and thus conclusive evidence will be attainable. In this review, I will give examples of phosphorylation analysis, especially in small smooth muscles, which had been difficult to study biochemically. And I will summarize our recent studies on a tiny blood vessel, the renal afferent arteriole.

Phosphorylation analysis in smooth muscles

It is widely accepted that the contraction of smooth muscle is primarily regulated by phosphorylation of myosin regulatory light chains (see reviews in Refs. (1–3)). Actin-activated myosin ATPase and motor activities increase upon phosphorylation of its 20-kDa regulatory light chain (LC_{20}) at Ser19, resulting in contraction. These activities decrease when LC_{20} is dephosphorylated, resulting in relaxation.

The level of LC_{20} phosphorylation is determined by the balance between kinase and phosphatase activities. When smooth muscle receives contractile stimuli, intracellular Ca^{2+} concentration increases, and the Ca^{2+}/calmodulin complex is formed (4). The Ca^{2+}/calmodulin complex activates myosin light chain kinase (MLCK), resulting in accumulation of phosphorylated LC_{20} and contraction (5). Some contractile stimuli also activate another pathway whereby myosin phosphatase activity is decreased by phosphorylation of its myosin targeting subunit (MYPT1) (6) or by direct binding of phosphorylated 17-kDa PKC-potentiated inhibitory protein of PP1 (CPI-17) (7). This decrease in myosin phosphatase activity changes the balance between kinase and phosphatase activities, resulting in further accumulation of phosphorylated LC_{20} and thus stronger contraction. This mechanism is called "Ca^{2+}-sensitization" (1, 2).

To elucidate the regulatory mechanisms of smooth muscle contraction, analyzing the phosphorylation signaling pathway is crucial. A variety of physiological and pharmacological techniques have been employed to this end. Pharmacological techniques exploiting selective inhibitors lead to speculation about the signaling pathways, but do not provide direct molecular evidence. For example, a Rho-associated protein kinase (ROCK) inhibitor, H1152, inhibited rabbit urethral smooth muscle contraction, suggesting that ROCK would phosphorylate MYPT1 and thus increase accumulation of phosphorylated LC_{20} (8). H1152, however, did not alter the level of LC_{20} phosphorylation in the rabbit urethrae. Things would become even more complicated if a less selective inhibitor was employed.

Biochemical approaches provide molecular evidence to test inconclusive speculations. By combining biochemical analysis with pharmacological techniques, we can measure changes in signal transduction pathways at a molecular level, and thus acquire conclusive evidence.

A variety of biochemical techniques have been applied to measure phosphorylation of myosin and other regulatory proteins in smooth muscle. For example, isoelectric focusing (9, 10), 2D-electrophoresis (11, 12), and urea/glycerol PAGE (13–15) have been used to measure myosin LC_{20} phosphorylation. In these electrophoreses, LC_{20} is separated into discrete bands or spots based on its phosphorylation state, and the ratio of the phosphorylated to unphosphorylated form(s) is quantified densitometrically. These conventional electrophoretic techniques, however, are relatively insensitive (> ~300 pg LC_{20} are required) (16), and their application is, therefore, limited to relatively large smooth muscle samples.

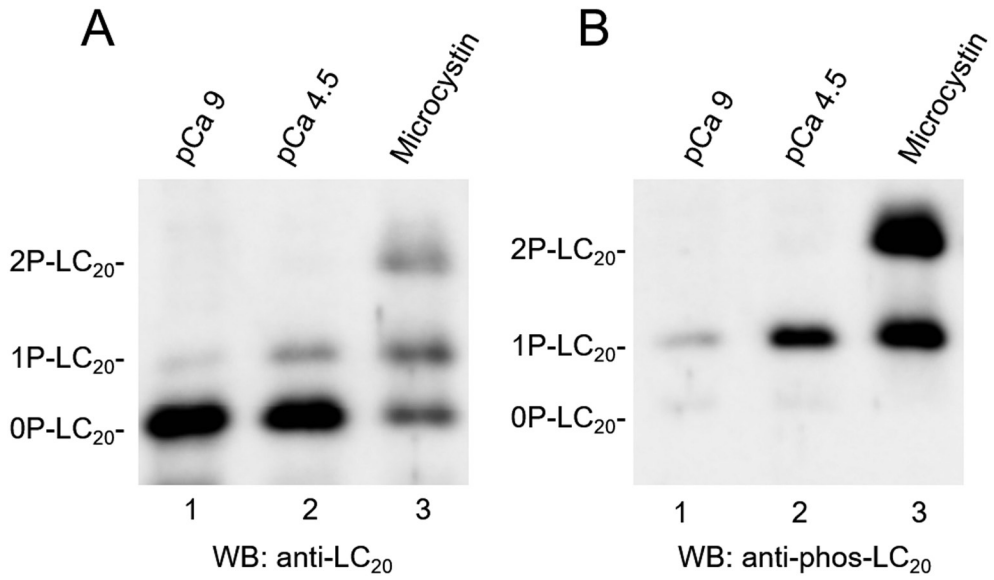

Fig. 1. Phosphorylation-based LC_{20} separation by Phos-tag SDS-PAGE. Skinned rat tail artery strips were treated with pCa 9 (lane 1), pCa 4.5 (lane 2), or 1 μM microcystin (lane 3). (A) All forms of LC_{20}s, regardless of the phosphorylation state, were detected by western blotting with pan anti-LC_{20} antibody. In a Phos-tag gel, phosphorylated LC_{20}s (monophosphorylated, 1P-LC_{20}; diphosphorylated, 2P-LC_{20}) migrated more slowly than the unphosphorylated form (0P-LC_{20}). (B) Phosphorylation-based LC_{20} separation was confirmed by using phospho-specific antibody against pSer19-LC_{20}, which also recognizes diphosphorylated-(pThr18, pSer19)-LC_{20}. This figure was reproduced from ref. (17).

Highly sensitive phosphorylation analysis by Phos-tag SDS PAGE and 3-step western blotting

Recently, we successfully improved the sensitivity of the LC_{20} phosphorylation analysis by combining Phos-tag SDS-PAGE with 3-step western blotting (17).

Phos-tag SDS-PAGE provides separation of phosphorylated proteins from their unphosphorylated forms based on the number and position of the phosphorylated sites (18, 19). Immobilized phosphate-affinity ligand (Phos-tag reagent) in a Laemmli SDS gel slows the migration of phosphorylated proteins due to binding to the ligand. Thus, the higher the stoichiometry of phosphorylation, the slower the migration rate through the gel (Fig. 1) (13, 17).

Three-step western blotting with the use of biotin-avidin binding significantly improved the sensitivity (17). By optimizing the conditions of western blotting, we are able to detect as little as 0.5 pg LC_{20} (Fig. 2). This is sufficiently sensitive to quantify LC_{20} in tiny smooth muscle tissues. For example, we were able to quantify LC_{20} phosphorylation in rat cerebral artery (20, 21) and renal arterioles (see below and Refs. (17, 22)).

Renal microvasculature

The renal afferent and efferent arterioles regulate the inflow and outflow resistance of the glomerulus, thereby controlling the pressure within the intervening glomerular capillaries (P_{GC}). P_{GC} is a primary determinant of glomerular filtration rate (GFR) and must be maintained within precise limits for normal renal function and to protect against hypertensive injury.

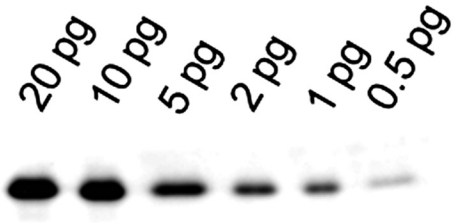

Fig. 2. Highly sensitive three-step western blotting. Purified LC_{20} (0.5–20 pg) was electrophoresed in a Laemmli SDS-gel and detected by 3-step western blotting with primary anti-LC_{20} antibody, secondary biotin-conjugated anti-IgG antibody and tertiary HRP-conjugated NeutrAvidin. As little as 0.5 pg of LC_{20} was detectable.

Angiotensin II (Ang II) is a renal vasoconstrictor that contributes to renal vascular resistance under normal physiological conditions and thus plays an important role in modulating renal hemodynamics (23). Bolus administration of Ang II elicits a transient constriction of the afferent arteriole (22, 24).

Endothelin-1 (ET-1) is also a potent renal vasoconstrictor. Unlike Ang II, ET-1 does not contribute to renal vascular resistance under normal physiologic conditions, but rather is implicated in abnormal renal vasoconstriction in a wide variety of pathologic states (25–27). Bolus administration of ET-1 elicits long-lasting constriction of the afferent arteriole (22, 24).

Although both Ang II and ET-1 are potent renal vasoconstrictors, the nature of renal vascular tone induced by these two agents is qualitatively different (22). In order to address the molecular determinants underlying these differences, biochemical analysis is essential.

The renal afferent arteriole is too small to detect molecular changes with conventional biochemical techniques. The isolated afferent arteriole is approximately one-tenth the size of a human eyelash (10–20 μm in diameter) and consists of < 100 smooth muscle cells on average. It contains ~50 pg (2.5 fmol) of LC_{20}, well below the limit of detection of conventional assays (300 pg or 15 fmol) (16).

By utilizing the newly developed Phos-tag electrophoresis and highly sensitive 3-step western blotting as described above, we successfully measured LC_{20} phosphorylation in isolated afferent arterioles and were able to address the question whether Ang II and ET-1 activate distinct signaling pathways, resulting in different contractile responses (22).

Myosin LC_{20} phosphorylation in the renal microvasculature

We isolated afferent arterioles from agarose-supported rat kidney (17, 22). In brief, the left kidney of anesthetized rats was perfused *in vivo* with warmed Ca^{2+}-free medium containing agarose. After the kidney was excised, it was chilled to solidify the agarose. The solidified agarose mimics the intraluminal pressure and thus allows the arterioles to maintain their physiological functions. Cortical slices were then treated with collagenase and dispase to separate microvessels from tubules. Individual arterioles were isolated and collected by using a dual-pipette micromanipulator. With this technique, we were able to measure molecular changes in afferent arterioles without contamination with tubules and other types of vessels.

Ang II induced exclusively monophosphorylation of LC_{20} at Ser19. ET-1, on the other hand, induced not only monophosphorylation of LC_{20}, but also diphosphorylation of LC_{20} (Fig. 3). The second phosphorylation site in ET-1-treated afferent arterioles was identified as Thr18 by western blotting with a diphosphorylation-specific antibody that recognizes LC_{20} only when phosphorylated at both Ser19 and Thr18 (22).

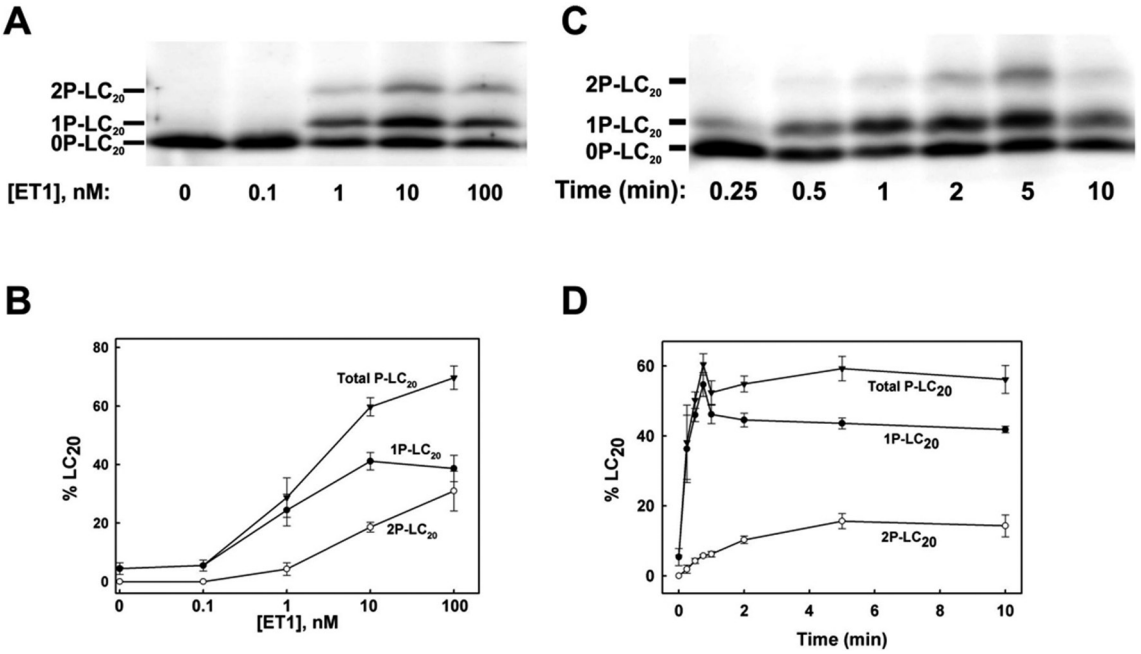

Fig. 3. ET-1-induced LC_{20} phosphorylation in renal afferent arterioles of Wistar rats. (A) and (B) Isolated afferent arterioles were treated with the indicated concentrations of ET-1 for 5 min. Phosphorylated and unphosphorylated forms of LC_{20} were separated by Phos-tag SDS-PAGE and detected by a 3-step western blotting procedure with anti-LC_{20}. A representative western blot is shown in (A) with cumulative quantitative data in (B). Data indicate the mean ± S.E.M. (n = 5 except for 10 nmol/L ET-1 where n = 7). (C) and (D) Time-courses of LC_{20} mono- and diphosphorylation in response to ET-1 (10 nmol/L). A representative western blot is shown in (C) with cumulative quantitative data in (D). Data indicate the mean ± S.E.M. (n = 4 except for 15 s, 45 s and 5 min where n = 3, 2 and 8, respectively). Percent phosphorylation was calculated from the following equations: % 1P-LC_{20} = [1P/(0P + 1P + 2P)] × 100%; % 2P-LC_{20} = [2P/(0P + 1P + 2P)] × 100%; % total P-LC_{20} = [(1P + 2P)/(0P + 1P + 2P)] × 100%. This work was originally published in Kidney International. Takeya K et al. Endothelin-1, but not angiotensin II, induces afferent arteriolar myosin diphosphorylation as a potential contributor to prolonged vasoconstriction. 2015; 87(2): 370–81. © International Society of Nephrology.

As seen in Fig. 3, ET-1 treatment increased LC_{20} diphosphorylation as well as monophosphorylation in a concentration- and time-dependent manner. The level of LC_{20} monophosphorylation reached a plateau of ~40% at 10 nM ET-1. The second phosphorylation at Thr18 increased the total phosphorylation level to ~60% at 10 nM ET-1 and to ~70% at 100 nM ET-1. Monophosphorylation of LC_{20} increased rapidly following the application of 10 nM ET-1, reaching a steady-state level within 60 sec. Diphosphorylation of LC_{20}, on the other hand, increased more slowly, reaching a maximum level within 5 min.

In contrast to ET-1, Ang II induced only monophosphorylation of LC_{20} even at a high concentration (100 nM), suggesting that only MLCK is involved in LC_{20} monophosphorylation in response to AngII (22).

The diphosphorylation of LC_{20} at Ser19 and Thr18 in response to ET-1 treatment was confirmed by proximity ligation assay (Fig. 4), which provides higher sensitivity, specificity, and signal-to-noise ratio than regular immunostaining (28). When stained with pan anti-LC_{20} and anti-pT18, pS19-LC_{20} antibodies, strong fluorescent signals were observed in the smooth muscle cells in ET-1 treated afferent arteriole, but not in untreated control or AngII-treated vessels.

Biochemical phosphorylation analysis also revealed that 1) inhibitors of MLCK and ROCK reduced ET-1-induced LC_{20} monophosphorylation as well as diphosphorylation, while they failed to abolish LC_{20}

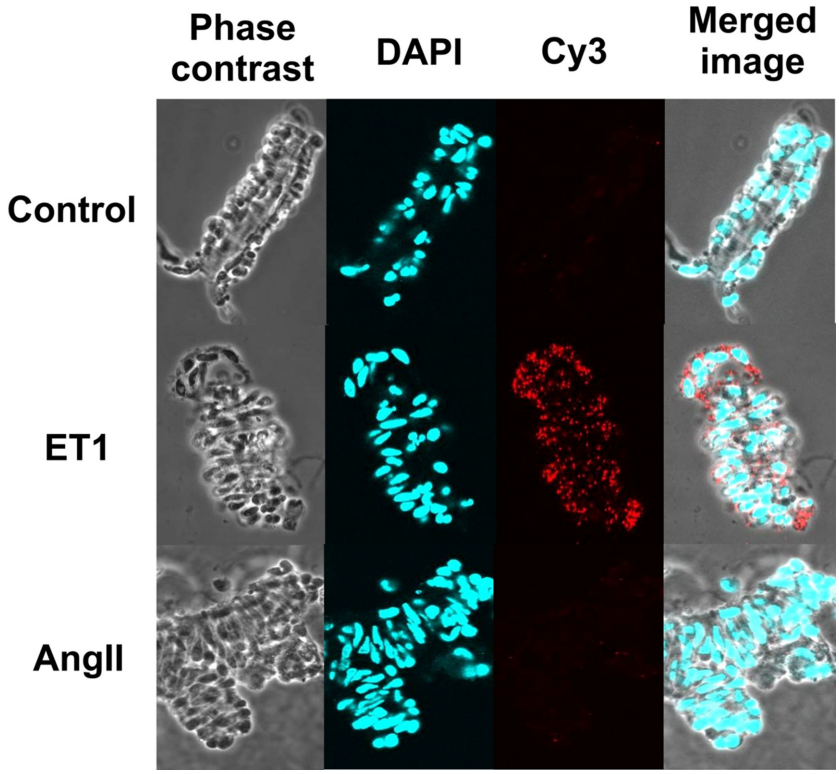

Fig. 4. Proximity ligation assay for LC_{20} diphosphorylation. Untreated Wistar rat afferent arterioles (control) and afferent arterioles treated with ET-1 or Ang II (10 nmol/L for 5 min) were fixed, permeabilized and incubated with pan-LC_{20} antibody and anti-pT18, pS19-LC_{20}. Bound antibodies in close proximity were detected by Cy3 staining. Panels show, from left to right, phase contrast images of the isolated arterioles, nuclear staining with DAPI, Cy3 fluorescence to illustrate LC_{20} diphosphorylation, and merged images. Results are representative of 18 (control), 4 (ET-1) and 4 (Ang II) independent experiments. This work was originally published in Kidney International. Takeya K et al. Endothelin-1, but not angiotensin II, induces afferent arteriolar myosin diphosphorylation as a potential contributor to prolonged vasoconstriction. 2015; 87(2): 370–81. © International Society of Nephrology.

diphosphorylation, suggesting that other kinases (zipper-interacting protein kinase (ZIPK) (29) and/or integrin-linked kinase (ILK) (30)) are involved in the phosphorylation of LC_{20} at Thr18; 2) an ET_B agonist (sarafotoxin 6c) induced LC_{20} diphosphorylation, suggesting that ET_B, but not ET_A receptors mediate the ET-1-induced LC_{20} diphosphorylation (22).

The observation that ET-1-induced constriction was relatively refractory to Ca^{2+} channel blockade (22) is also consistent with the involvement of Ca^{2+}-independent kinases – ZIPK and/or ILK.

Role of LC_{20} diphosphorylation in the renal microvasculature

As it was reviewed previously (31, 32), diphosphorylation of LC_{20} was first demonstrated *in vitro* by high concentrations of MLCK (33). The additional phosphorylation at Thr18 increased actomyosin MgATPase activity, but not actin-filament velocity in the *in vitro* motility assay (33–37). Although LC_{20} diphosphorylation has been associated with pathophysiological conditions involving smooth muscle hypercontractility (38–43), the mechanism whereby LC_{20} diphosphorylation causes abnormal contraction remains controversial.

It has been proposed recently that the functional effect of LC_{20} diphosphorylation is to reduce the dephosphorylation rate and thus to slow smooth muscle relaxation (44). Based on this, we hypothesized that

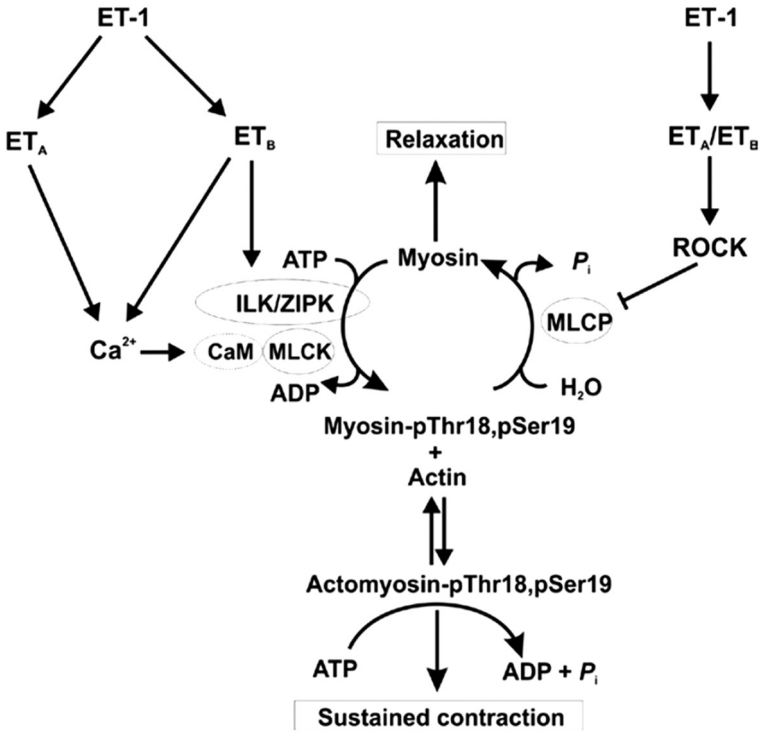

Fig. 5. Proposed signaling pathways leading to ET-1-induced sustained vasoconstriction of the renal afferent arteriole. ET-1, acting via ET_A receptors, triggers an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), largely through Ca^{2+} entry via voltage-gated Ca^{2+} channels (Ca_v), leading to binding of Ca^{2+} to calmodulin (CaM), activation of myosin light chain kinase (MLCK), phosphorylation of LC_{20} at Ser19 and cross-bridge cycling. Activation of ET_A receptors also induces inhibition of myosin light chain phosphatase (MLCP) via activation of the Rho-associated kinase (ROCK) pathway, leading to increased LC_{20} phosphorylation at Ser19 due to the increase in MLCK : MLCP activity ratio. MLCP inhibition unmasks basal activity of integrin-linked kinase (ILK) and/or zipper-interacting protein kinase (ZIPK) that phosphorylate LC_{20} at both Thr18 and Ser19. Activation of ET_B receptors in the vascular smooth muscle cells leads to activation of ILK and/or ZIPK (or possibly other kinase(s) capable of phosphorylating LC_{20} at Thr18 and Ser19). The rapid increase in Ser19 phosphorylation accounts for the initial phase of the contractile response to ET-1, while the slower diphosphorylation at Thr18 and Ser19, associated with reduced rates of LC_{20} dephosphorylation and relaxation (44), can account for the sustained contractile response to ET-1 and prolonged contraction that occurs following removal of the stimulus. This figure was originally published in Kidney International. Takeya K et al. Endothelin-1, but not angiotensin II, induces afferent arteriolar myosin diphosphorylation as a potential contributor to prolonged vasoconstriction. 2015; 87(2): 370–81. © International Society of Nephrology.

the prolonged constriction induced by ET-1 in the afferent arteriole was due to the diphosphorylation of LC_{20} via ET_B-mediated signal transduction. To test this hypothesis, we administered a specific ET_B agonist and antagonist to an *in vitro* perfused afferent arteriole (22).

Vasodilation following washout of the ET_B agonist was slow and comparable to that following washout of ET-1, consistent with delayed vasorelaxation due to LC_{20} diphosphorylation (22). ET_B receptor blockade significantly increased the rate of vasodilation following ET-1-induced vasoconstriction in the presence of the NO synthase inhibitor, L-N^G-nitroarginine methyl ester (L-NAME), further implicating ET_B receptors in the slow vasodilatory response following ET-1 washout (22).

We concluded, therefore, that LC_{20} diphosphorylation via ET_B receptor activation contributes, at least in part, to the prolonged contraction induced by ET-1 in the renal afferent arteriole (Fig. 5). Considering that ET-1

increases in ischemic kidney (45), ET-1-induced LC_{20} diphosphorylation and consequent prolonged contraction of afferent arterioles may contribute to ischemia/reperfusion-induced acute renal failure. Although endothelial ET_B receptors may offer renal protection via NO synthesis (46), the activation of ET_B receptors on the afferent arteriolar myocytes might contribute to abnormal vasoconstriction or vasospasm associated with recovery.

Conclusions

In this review, I have provided an example of highly sensitive phosphorylation analysis that is capable of measuring phosphorylation in tiny tissue samples. By combining this biochemical approach with physiological, pharmacological and immunocytochemical approaches, we will be able to evaluate physiological events at a molecular level. As I showed in this review, Phos-tag based phosphorylation analysis, combined with proximity ligation assay, is suitable for studying micro-samples with high sensitivity.

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Conflict of Interest

The author declares that he has no conflict of interest.

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