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ORIGINAL PAPER

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Immunolocalization of phospho-Arg-directed protein kinase-substrate in hypoxic kidneys using in vivo cryotechnique

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Abstract Protein kinases (PKs) phosphorylate proteins at active regions for signal transduction. In this study, normal and hypoxic mouse kidneys were prepared using an "in vivo cryotechnique" (IVCT) and examined immunohistochemically with specific antibodies against phospho-(Ser/Thr) PKA/C substrate (P-PK-S) and phospho-(Ser/Thr) Akt substrate (P-Akt-S) to capture their time-dependent regulation in vivo. Left kidneys were cryofixed with IVCT under normal blood circulation and after varying hypoxic intervals, followed by freeze-substitution with acetone containing paraformaldehyde. Deparaffinized sections were immunostained for P-PK-S, Na^+/HCO_3^- cotransporter NBC1, and a membrane skeletal protein, 4.1B. The P-PK-S was diffusely immunolocalized in the cytoplasm of the proximal tubules in normal kidneys, whereas NBC1 and 4.1B were detected at the basal striations of S1 and S2 segments of the proximal tubule. After 10 or 30 s hypoxia, P-PK-S was still immunolocalized in the cytoplasm of kidneys, but it was detected at the basal striations after 1 or 2 min hypoxia. The immunolocalization of P-Akt-S was the same as P-PK-S in the normal and hypoxic kidneys. Immunoblotting analyses of the kidney tissues under normal or hypoxic condition clearly identified the same 40-kDa bands. The IVCT is useful for time-dependent analysis of the immunodistribution of P-PK-S and P-Akt-S.

Key words In vivo cryotechnique · Freeze-substitution · Hypoxic kidneys · Phospho-(Ser/Thr) PKA/C substrate · Phospho-(Ser/Thr) Akt substrate · Na^+/HCO_3^- cotransporter 1 · 4.1B

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Introduction

Immunohistochemistry (IHC) using many antiphosphorylation-specific antibodies has been applied to examining molecular signal transduction, cell growth, and cytokinesis in animal cells, which depend on energy consumption. Phosphorylation of platelet-derived growth factor (PDGF) receptor was biochemically demonstrated in BALB/c 3T3 cells in response to binding of its ligand, PDGF, with a phosphotyrosine-specific antibody.[1] However, IHC, which is often used to analyze immunolocalization of various proteins in cells and tissues, has been routinely performed with chemical fixation and alcohol dehydration, which can cause technical artifacts, such as tissue shrinkage, diffusion artifacts, and antigen masking.[2–5] In addition, the preparation steps following resection of tissues or perfusion- or immersion-fixation always cause anoxia and ischemia.[6] Therefore, it has been difficult to accurately maintain the phosphorylation conditions in dynamically functioning cells from living animal organs using conventional preparation techniques.[7,8] In contrast, the recently developed "in vivo cryotechnique" (IVCT) is a cryofixation method that directly freezes living animal organs under normal or experimental conditions.[9–11] The IVCT has been used for time-dependent immunohistochemical analysis of rhodopsin phosphorylation in the living mouse retina under dark and light exposure, and the photoreceptor cells were examined with a time resolution of the order of seconds.[12] The results suggest that the IVCT-prepared tissue samples can reflect the physiological and pathological significance of cells and tissues in living animals without the conventional technical artifacts.

Arg-directed kinases, such as cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), Ca^{2+}/phospholipid-dependent protein kinase C (PKC), Akt/Protein kinase B (PKB), and ribosomal s6 kinase (RSK)[13] usually share a motif characterized by an Arg at position 3 relative to the phosphorylated Ser or Thr. The representative Arg-directed kinase, PKC, has been also reported to play a major role in transmembrane signal transduction.[14]

The PKC family has many proteins in various animal organs, including the brain, liver, kidney, spleen, lung, heart, and testis, but their intracellular PKC distribution is likely dependent on the proliferation state of cells and tissues in the living animals.[15,16] The present study used IVCT to arrest some transient physiological processes of P-PK-S in living mouse kidneys under normal blood circulation and with time-dependent hypoxia. The immunolocalization of P-PK-S in the IVCT-prepared tissue samples was different from that observed with the conventional resection of fresh kidney tissues and quick-freezing followed by freeze-substitution fixation (RF-QF-FS). The different immunolocalization of P-PK-S observed in association with the time-dependent hypoxia indicate that the discrepancy of immunodistribution was primarily caused by the transient effects of hypoxia during the common preparation steps.

Materials and methods

Tissue preparation under normal or hypoxic condition

The present animal experiment was approved by the University of Yamanashi Animal Care Use Committee. Twenty-one C57BL/6 mice, aged 4–8 weeks, were divided into three experimental groups. Two mice were used for the resection of fresh kidney tissues and quick-freezing (RF-QF) followed by freeze-substitution fixation (FS). Fifteen mice were used to obtain living mouse kidneys under normal blood circulation and hypoxia, which were prepared with IVCT and followed by FS. The kidneys of 4 mice prepared with IVCT were used for electrophoresis and immunoblotting analysis.

Procedure of IVCT followed by FS

Under diethyl ether anesthesia, the IVCT was performed by directly pouring isopentane-propane (IP) cryogen (–193°C) over living mouse kidneys either under normal blood circulation or at various times of hypoxia. Such hypoxia of the anesthetized mice was induced by surgically opening the pleural cavities for 10 s, 30 s, 1 min, and 2 min before cryofixation. The frozen left kidneys were removed with a dental electric drill in liquid nitrogen and commonly processed for FS with 2% paraformaldehyde in acetone at –80°C for 48 h, –30°C for 2 h, –10°C for 2 h, and 4°C for 2 h each, as reported previously.[5] The freeze-substituted kidney tissues were routinely washed twice with acetone and xylene, and finally embedded in paraffin wax.

Resection of fresh tissues and quick-freezing (RF-QF) followed by FS

The anesthetized mouse kidneys were removed, cut into small pieces with razor blades, and quickly plunged into the IP cryogen. The frozen tissues were then processed for FS and paraffin-embedding steps, as described above. The preparation time from tissue removal to the freezing step usually was at least 30 s.

Hematoxylin and eosin (H&E) staining and immunohistochemistry

The paraffin-embedded tissues were cut into 5-μm sections and serially mounted on Matsunami Adhesive Slide (MAS)-coated slides (Matsunami Glass, Osaka, Japan). The cut sections were deparaffinized with xylene and rehydrated with a graded series of ethanol. The frozen tissues prepared by the two cryotechniques, IVCT-FS and RF-QF-FS, were commonly stained with hematoxylin and eosin (H&E) on serial sections. Cryofixation conditions were then checked with light microscopy, and we proceeded to immunohistochemical analyses. For immunohistochemistry, the rehydrated sections were incubated with 1% hydrogen peroxide in PBS and with 5% normal goat serum in PBS for 1 h each. They were incubated with rabbit polyclonal antibodies against phospho-(Ser/Thr)-protein kinase A-substrate (P-PK-S) recognizing the RXXT* and RRXS* motifs (dilution, 1:50; Cell Signaling Technology, Danvers, MA, USA) and phospho-(Ser/Thr) Akt-substrate (P-Akt-S) recognizing the (R/K)X(R/K)XX(T*/S*) motif (dilution, 1:100; Cell Signaling Technology), Na^+/HCO_3^- cotransporter (NBC)1[17] (dilution, 1:1000), and protein 4.1B[18] (dilution, 1:50), at 4°C overnight. The P-PK-S and P-Akt-S anti-bodies recognize phosphoserine or phosphothreonine, respectively, in the motif of RXXT*/S* and (R/K)X(R/K)XX(T*/S*), where X can be replaced by any amino acid. They were incubated with biotinylated goat antirabbit IgG antibody (dilution, 1:500; Vector Laboratories, Burlingame, CA, USA) at room temperature for 1 h. Some sections were incubated with the blocking solutions and also the aforementioned secondary antibody as immunocontrols. Finally, they were incubated with avidin-biotin-conjugated horseradish peroxidase (HRP) complex solution (Vector Laboratories) for 1 h and visualized with cobalt-enhanced diaminobenzidine (DAB) in buffer solution containing hydrogen peroxide (Pierce Chemical, Rockford, IL, USA) for 5 min. The immunostained sections were incubated with 0.04% osmium tetroxide in 0.1 M PB for 30 s to enhance the immunoreaction contrast.

Protein preparation for electrophoresis and immunoblotting

The left kidneys from the two mice under normal blood circulation or hypoxic condition for 2 min, which were cryofixed with IVCT, were removed by a dental electric drill in liquid nitrogen (LN) and then crushed with a chilled iron hammer in LN. Some fragmented tissue powders were directly thawed and solubilized in 200 μl Laemmli sample buffer at 95°C with a pestle (Homogenization Pestle for 1.5 ml Microcentrifuge Tube; Scientific Specialties, Lodi,

CA, USA). The protein concentration was adjusted using the BCA Protein Assay Kit (Pierce Chemical). The tissue homogenates were processed for sodium dodecyl sulfate (SDS) electrophoresis through 5%–20% gradient polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% bovine serum albumin in PBS-Tween (0.1%) (PBS-T) and then were incubated with the same anti-P-PK-S antibody (dilution, 1:500) at 4°C overnight, followed by washing with PBS-T and incubation with HRP-conjugated goat antirabbit IgG (H+L) antibody at a dilution of 1:50 000 (Vector Laboratories) at RT for 60 min. Following their several washes in PBS-T, immunoreactive proteins were routinely visualized using the chemiluminescence method (Super Signal Femto West Maximum Sensitivity Substrate; Pierce Chemical).

Results

Immunolocalization of P-PK-S with IVCT-FS and RF-QF-FS

The morphology obtained with the two preparation methods was compared with H&E staining and immunohistochemistry for P-PK-S in mouse kidney tissues. At low magnification, the P-PK-S was mainly immunolocalized in the cortical renal tubules, but not in the medulla of living mouse kidney tissues prepared with IVCT-FS (Fig. 1a,b). At higher magnification with H&E staining, some Bowman's spaces and the renal proximal tubules in the cortex seemed to maintain their open lumens (Fig. 1c). However, the tissue samples prepared with RF-QF-FS showed collapsed Bowman's spaces and renal tubules in the cortex, probably as a result

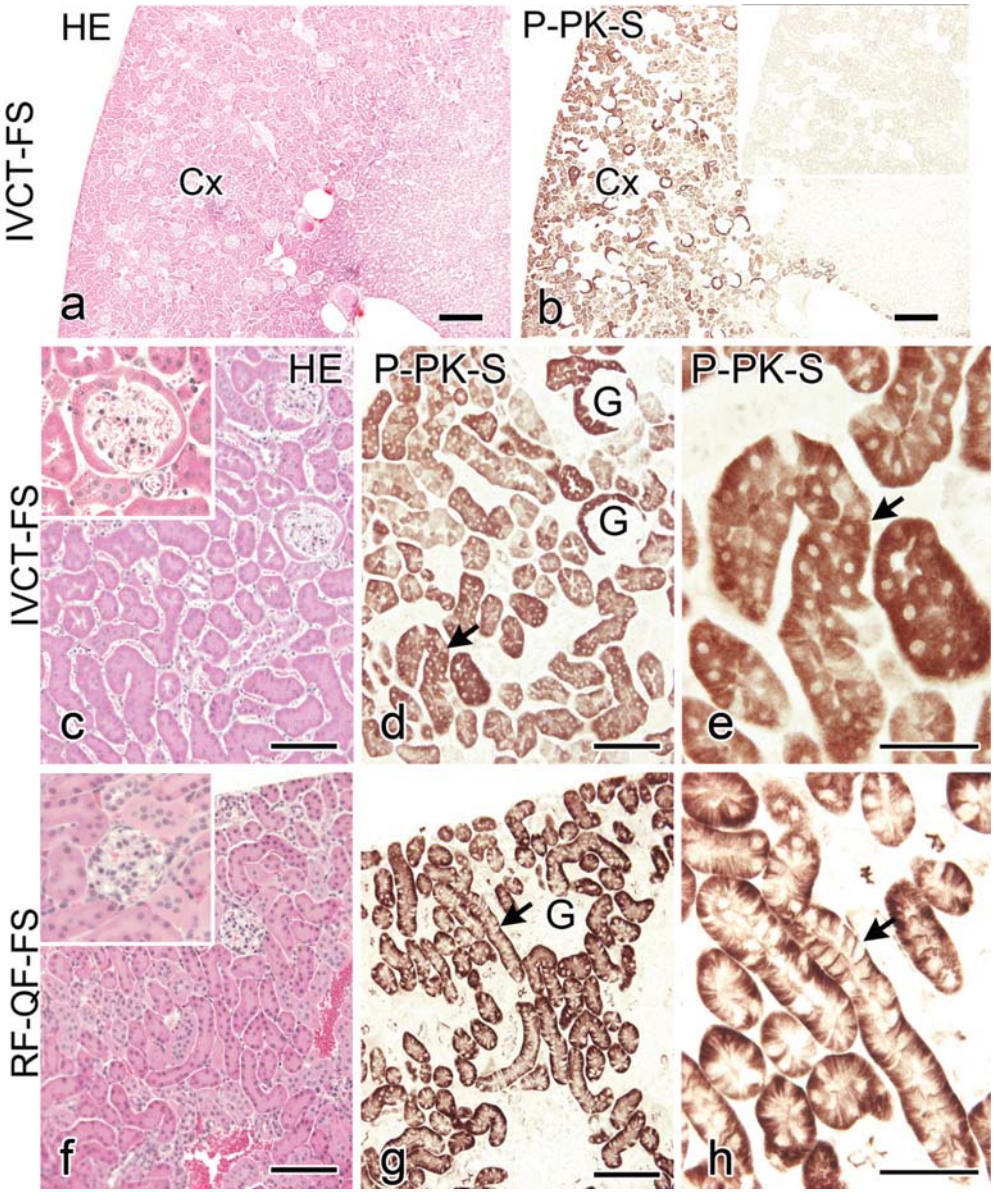

Fig. 1. Light micrographs of mouse kidneys prepared with the "in vivo cryotechnique" (*IVCT*) or resection of fresh tissues and quick-freezing (*RF-QF*), followed by freeze-substitution fixation (*FS*). **a**, **b** Serial sections are stained with hematoxylin and eosin (H&E) (**a**; *HE*) and immunostained for phospho-(Ser/Thr) protein kinase A/C substrate (**b**; *P-PK-S*). The P-PK-S-immunopositive areas are mostly observed in the cortex (*Cx*). *Inset* of **b** is the immunocontrol for P-PK-S in the renal cortex at the same magnification as **b**. **c–h** Normal kidneys on serial sections stained with H&E and immunostained for P-PK-S, by two different procedures with IVCT-FS and RF-QF-FS. **c–e** With IVCT-FS, immunolocalization for P-PK-S is detected in the cytoplasm of the proximal tubules (**d** and **e**; *arrows*) and each tubular cell shows different intensities of immunostaining. **e** Another higher-magnification view shows heterogeneous immunolocalization patterns of P-PK-S in the cytoplasm. **f–h** With RF-QF-FS, the immunolocalization of P-PK-S is detected along the basal striations of the renal proximal tubules (**g** and **h**; *arrows*). *Cx*, cortex; *G*, glomerulus. *Bars* **a**, **b** 300 μm; **c**, **d**, **f**, **g** 100 μm; **e**, **h** 50 μm

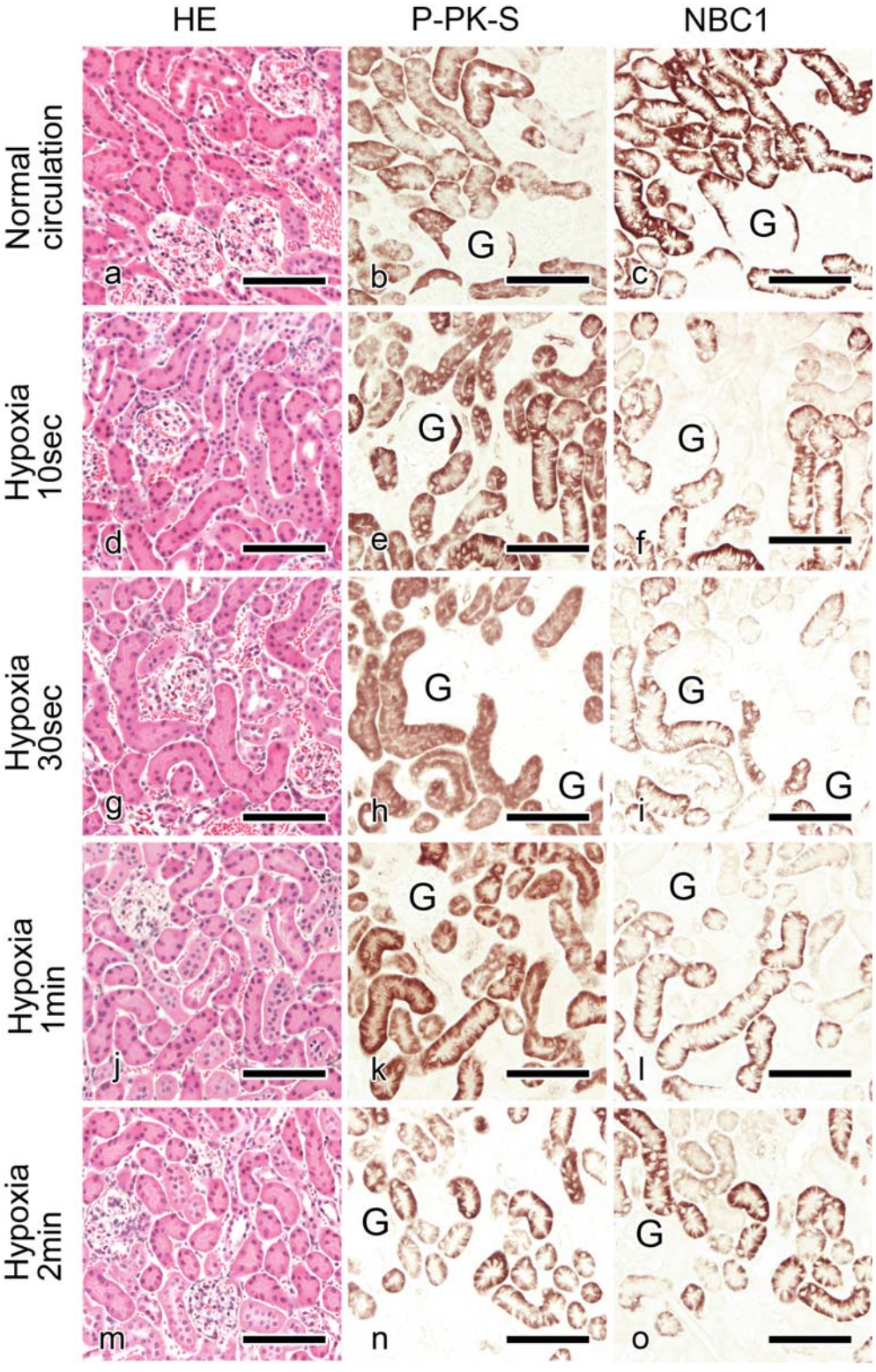

Fig. 2. Light micrographs of mouse kidney tissue specimens prepared with IVCT-FS under normal blood circulation (**a–c**) and hypoxia of 10 s (**d–f**), 30 s (**g–i**), 1 min (**j–l**), or 2 min (**m–o**) before cryofixation. Serial sections are stained with H&E (**a**, **d**, **g**, **j**, **m**) and immunostained for P-PK-S (**b**, **e**, **h**, **k**, **n**) and Na^+/HCO_3^- cotransporter 1 (*NBC1*) (**c**, **f**, **i**, **l**, **o**). The tissue areas of P-PK-S-immunopositive proximal tubules are more widely distributed than those of NBC1. *G*, glomerulus. *Bars* 100 μm

of ischemia caused by tissue resection (Fig. 1f), as previously described.[19] In serial sections of the same samples prepared with IVCT-FS, the immunoreactivity of P-PK-S was heterogeneously detected throughout the cytoplasm of the proximal tubules in mosaic-like patterns (Fig. 1d,e). In contrast, it was detected mainly in the basal striations of the proximal tubules in the tissue samples prepared with RF-QF-FS (Fig. 1g,h). These findings indicate that the immunolocalization of P-PK-S was quickly altered by the tissue resection procedure.

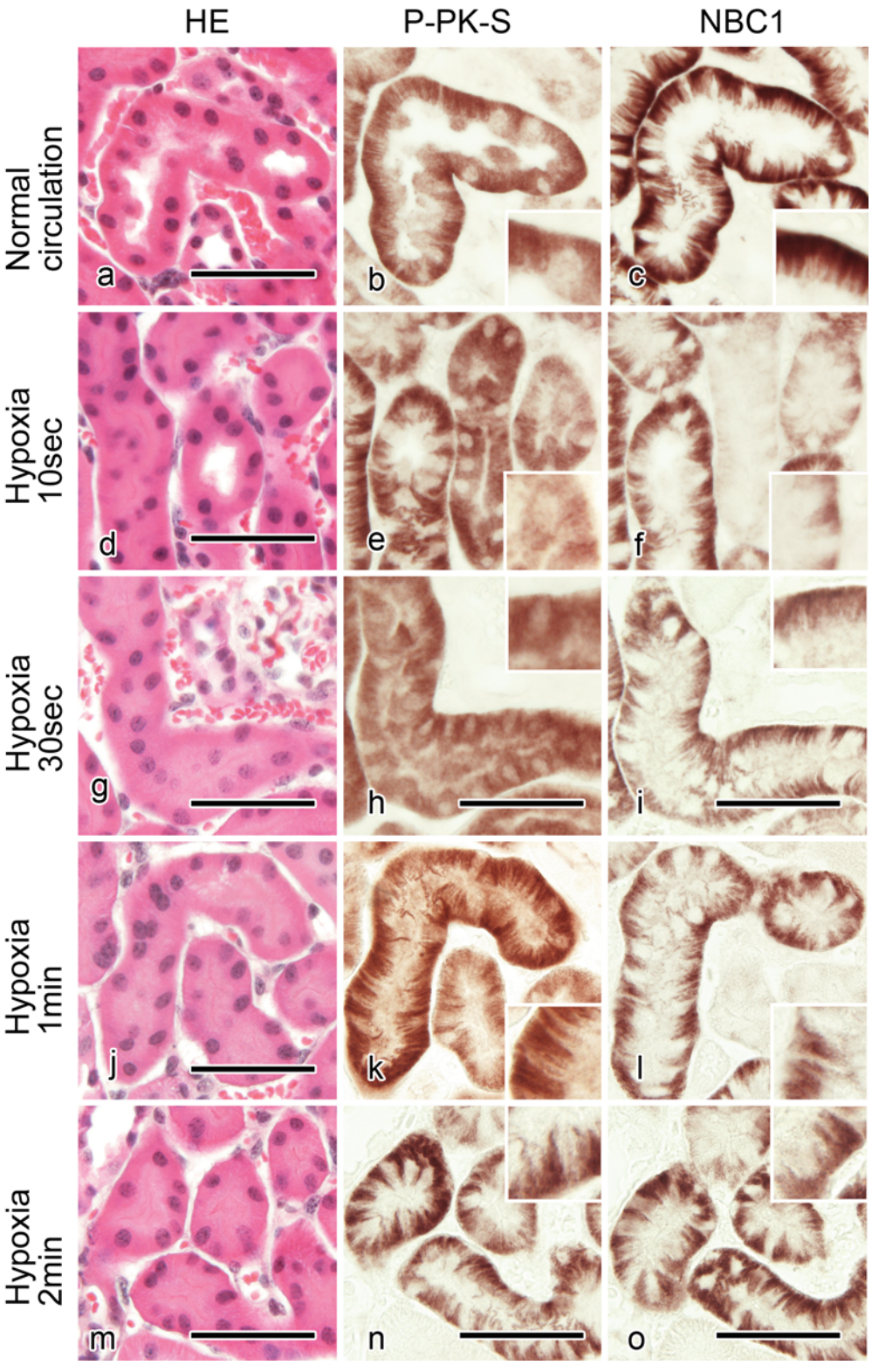

Fig. 3. Higher-magnification micrographs of Fig. 2, in the serial sections stained with H&E and also immunostained for P-PK-S and NBC1. *Insets* show the basal cytoplasm of the epithelial cells, thus indicating the immunolocalization of the P-PK-S in the whole cytoplasm (**b**, **e**, **h**) under normal blood circulation or 10 s and 30 s hypoxia and along the basal striations (**k**, **n**) under 1 min and 2 min hypoxia. The NBC1 is almost always immunolocalized along the basal striations (**c**, **f**, **i**, **l**, **o**). *Bars* 50 µm

Immunolocalization of P-PK-S in hypoxic mouse kidneys prepared with IVCT-FS

To examine the transient effects of hypoxia on the immunolocalization of P-PK-S, both the morphology of H&E-stained tissue and the immunolocalization of P-PK-S, NBC1 (an ion cotransporter), and 4.1B (a membrane skeletal protein) (data not shown) were compared in serial sections from renal proximal tubules under various hypoxic conditions (Figs. 2, 3). Under normal blood circulation, P-PK-S was immunolocalized diffusely throughout the cytoplasm of most epithelial cells in the proximal tubules and occasion-

ally along the basal striations (Figs. 2b, 3b). The diffuse immunolocalization of P-PK-S in the proximal tubules as shown in Figs. 2b and 3b was very similar to that shown in Fig. 1e. After 10 or 30 s hypoxia, their signals were also detected in the proximal tubules (Fig. 2e,h), some of which were immunolabeled with anti-NBC1 (Fig. 2f,i) or 4.1B (data not shown) antibody, showing the proximal S1–S2 segments. Higher magnification demonstrated that immunolocalization of P-PK-S in the cytoplasm (Fig. 3e,h) was almost identical to that under normal blood circulation (Fig. 3b). After 1 or 2 min hypoxia, it was also detected in the proximal tubules at low magnification (Fig. 2k,n). Higher magnification showed that it had changed to become localized, mainly in the basal striations (Fig. 3k,n). In contrast, the immunolocalization of both NBC1 and 4.1B was always detected in the basal striations of the S1–S2 proximal tubules under all conditions (NBC1: Figs. 2c,f,i,l,o; Fig. 3c,f,i,l,o; 4.1B, data not shown). These findings indicate that immunolocalization of P-PK-S was almost completely changed from the whole cytoplasm to the basal striations in the proximal tubules after 1 min hypoxia.

Immunolocalization of P-Akt-S in normal and hypoxic mouse kidneys

To examine the immunostaining patterns of another antibody against a similar phospho-specific motif, anti-P-Akt-S antibody was used for the IVCT-FS-prepared kidneys of living mice under normal blood circulation and hypoxic conditions. The P-Akt-S was immunolocalized in the cytoplasm of the same P-PK-S-immunopositive proximal tubules in the normal mouse kidneys (Fig. 4b,c). After 1 min hypoxia, the P-Akt-S was also immunolocalized in the basal striations, similar to the P-PK-S-immunopositive proximal tubules (Fig. 4e,f). These findings indicate that the substrates phosphorylated by Arg-directed protein kinases also changed from a cytoplasmic pattern to the basal striations in the renal proximal tubules after 1 min hypoxia.

Immunoblotting analyses of P-PK-S in hypoxic mouse kidneys prepared with IVCT

Under normal blood circulation or after 2 min hypoxia, a single band of 40 kDa was clearly observed in the kidney tissues prepared with IVCT for immunoblotting. The immunoblotting of the secondary antibody alone did not show any significant band as an immunocontrol (Fig. 5). These findings indicate that the main P-PK-S was not changed under normal circulation or 2 min hypoxia.

Discussion

In this study, IVCT-FS demonstrated that immunolocalization of P-PK-S and P-Akt-S changed from the whole cytoplasm to the basal striations in the mouse proximal tubules

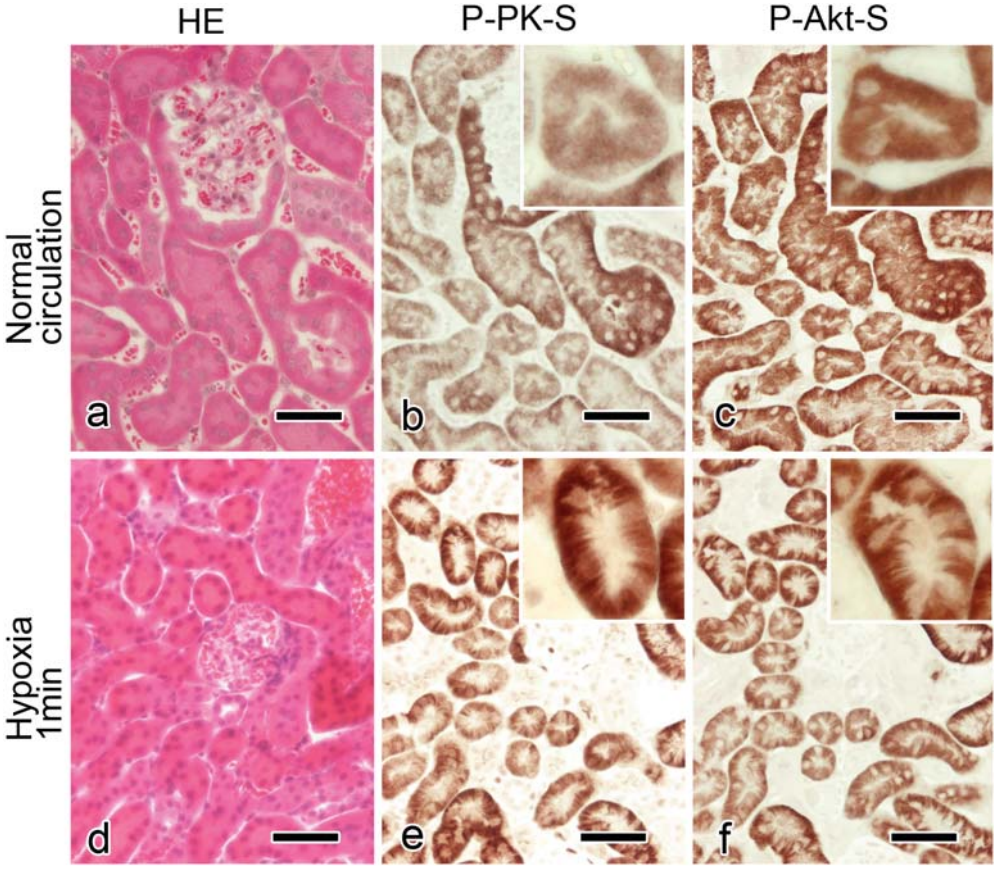

Fig. 4. Light micrographs of mouse kidney tissue specimens under normal blood circulation (**a–c**) and 1 min hypoxia (**d–f**), prepared with IVCT-FS. The serial sections are stained with H&E (**a**, **d**) and immunostained for P-PK-S (**b**, **e**) and phospho-(Ser/Thr) Akt substrate (*P-Akt-S*) (**c**, **f**). The immunolocalization of both P-PK-S and P-Akt-S has changed from the whole cytoplasm (**b**, **c**) to the basal striations in the proximal tubules after 1 min hypoxia (**e**, **f**). *Bars* 100 μm

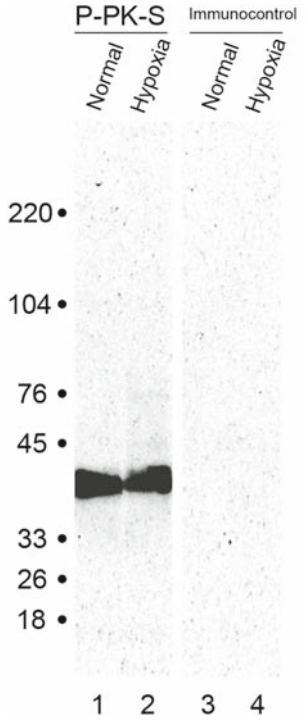

Fig. 5. Immunoblotting analyses of P-PK-S in the mouse kidneys under normal blood circulation and 2 min hypoxia, as prepared with IVCT. The immunoblotting for P-PK-S indicates a single 40-kDa band in the kidney under normal blood circulation (*lane 1*) and 2 min hypoxia (*lane 2*). Immunocontrols show no significant bands in mice under normal blood circulation (*lane 3*) and 2 min hypoxia (*lane 4*)

under hypoxia. However, with the conventional cryofixation method (RF-QF-FS), their immunoreactivity was detected only in the basal striations in the proximal tubules. The immunoreactivity of an intranuclear protein, phosphorylated cyclic adenosine monophosphate (cAMP)-responsive element-binding protein (pCREB), is clearly detected in paraffin sections prepared by IVCT without antigen retrieval by microwave treatment, which is often used for specimens prepared by perfusion-fixation followed by alcohol dehydration.[4] In addition, the current results demonstrated that IVCT-FS was useful to prevent diffusion artifacts during the tissue preparation procedures, which always induce the translocation of soluble proteins, such as immunoglobulins and albumin.[5] The present immunohistochemical findings showed that functioning molecules, such as P-PK-S and P-Akt-S, were quickly redistributed from one cytoplasmic compartment into another compartment under ischemic and hypoxic conditions, as revealed by the different preparation methods, IVCT-FS and RF-QF-FS. These results indicate that living mouse kidney tissues prepared with IVCT-FS probably reflect the physiological states of P-PK-S and P-Akt-S without serious ischemia or hypoxia that is inherent with the other preparation methods.

The basal striations in the proximal tubules that are observed by light microscopy contain many mitochondria and basal infolding of cell membranes, which are easily observed at an ultrastructural level. The translocation of the signal transduction molecules, including the Arg-directed kinases, occurs in response to cell death, such as apoptosis and necrosis, under ischemic and anoxic conditions.[20,21] The cellular polarity of the renal tubules with the specific localization of Arg-directed kinases has been thought to be a key point to integrate signal transduction pathways under hypoxia.[22] The time-dependent translocation of their molecules may be related to less mitochondrial energy consumption, a first step to apoptosis and cell membrane influx of sodium or calcium ion and water, resulting in cytoplasmic swelling and nuclear pyknosis for necrosis. We are now investigating whether the immunolocalization of P-PK-S and P-Akt-S could be reversed after reoxygenation using a ischemia-reperfusion model[23] by IVCT. Although the membrane skeletal protein 4.1B, which forms a complex with NBC1,[18] has a similar P-PK-S motif,[24] its immunolocalization was always detected at the basal striations under normal blood circulation, showing no translocation of the membrane skeletal protein under hypoxia. The Western blotting analysis showed a 40-kDa band in frozen tissues treated with IVCT under both normal and hypoxic conditions (see Fig. 5). The 40-kDa protein has an important role associated with the cell membranes of basal infolding in the proximal tubules under ischemic and hypoxic conditions. Another experiment to examine a shift of P-PK-S from cytosolic fraction to membrane fraction and also identify the translocated protein is now under investigation. However, it is necessary to carefully treat the frozen samples without inducing similar anoxic artifacts during the tissue preparation step of dissolving the frozen tissues into solutions. An Arg-directed protein kinase, PKC, has been reported to actively function during the time-dependent ischemic injury of the renal proximal tubules.[23] The translocation of PKC from the cytosolic fraction to the membrane fraction is evidence of this process under ischemic conditions.[25,26] The PKC has at least 12 different isoforms, which contain the conserved four regions, termed C1–C4. The PKC isoforms and the receptor for activated C kinase (RACK1) are assumed to be phosphorylated by postischemic injury in various tissues, including the kidneys.[27] It will also be necessary to determine if such phosphorylation of PKC isoforms is related to the P-PK-S immunolocalization.[28]

For the past decade, direct visualization of the γ-subspecies of PKC has been demonstrated in various cultured cells by use of green fluorescent protein, showing its movement in real time, which clarified the dynamic translocation in response to various extracellular signals.[29,30] Moreover, intravital microscopy with fluorescence resonance energy transfer (FRET) has also been developed to directly observe the movement of proteins, which practically showed Ca^{2+} modulation in HeLa cells[31] and translocation of α-PKC in Cos-7 cells.[32] Therefore, the immunohistochemical images obtained from the conventionally prepared samples with chemical fixation hardly integrate the intact biological phenomenon of cells and tissues in vivo and protein analyses in hypoxic conditions.[6,8] The newly developed IVCT is a reliable method for the examination of time-dependent regulation of signaling pathways in vivo and does not have the technical limitations of the usual methods that interfere

with molecular translocation, including soluble Arg-directed kinases. The immunohistochemical findings must therefore be compared with the live images obtained from intravital microscopy, such as FRET.

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