Phosphorylation dynamics of radixin in hypoxia-induced hepatocyte injury

Jo Suda,1 Don C. Rockey,2 and Serhan Karvar2
1Division of Gastrointestinal and Liver Diseases, Keck School of Medicine, University of Southern California, Los Angeles, California; 2Division of Gastroenterology and Hepatology, Department of Medicine, Medical University of South Carolina, Charleston, South Carolina

Submitted 8 October 2014; accepted in final form 8 December 2014

Phosphorylation dynamics of radixin in hypoxia-induced hepatocyte injury. Am J Physiol Gastrointest Liver Physiol 308: G313–G324, 2015. First published December 11, 2014; doi:10.1152/ajpgi.00369.2014.—The most prominent ezrin-radixin-moesin protein in hepatocytes is radixin, which is localized primarily at the canalicular microvilli and appears to be important in regulation of cell polarity and in localizing the multidrug resistance-associated protein 2 (Mrp-2) function. Our aim was to investigate how hypoxia affects radixin distribution and Mrp-2 function. We created wild-type and mutant constructs (in adenoviral vectors), which were expressed in WIF-B cells. The cellular distribution of Mrp-2 and radixin was visualized by fluorescence microscopy, and a 5-chloromethylfluorescein diacetate (CMFDA) assay was used to measure Mrp-2 function. Under usual conditions, cells infected with wild-type radixin, nonphosphorylatable radixin-T564A, and radixin-T564D (active phospho-mimicking mutant) were found to be heavily expressed in canalicular membrane compartment vacuoles, typically colocalizing with Mrp-2. In contrast, after hypoxia for 24 h, both endogenous and overexpressed wild-type radixin and the radixin-T564A mutant were found to be translocated to the cytoplasmic space. However, distribution of the radixin-T564D mutant, which mimics constant phosphorylation, was remarkably different, being associated with canalicular membranes even in hypoxic conditions. This dominant-active construct also prevented dissociation of radixin from the plasma membrane. Hypoxia also led to Mrp-2 mislocalization and caused Mrp-2 to be dissociated from radixin; the radixin phospho-mimicking mutant (T564D) abrogated this effect of hypoxia. Finally, hypoxia diminished the secretory response (measured using the CMFDA assay) in WIF-B cells, and the dominant-active construct (radixin-T567D) rescued this phenotype. Taken collectively, these findings suggest that radixin regulates Mrp-2 localization and function in hepatocytes and is important in hypoxic liver injury.

REGULATION OF BLOOD FLOW is essential for liver function. Hypoxic liver injury is a condition of decreased oxygen supply to the liver, resulting in injury to liver cells, especially hepatocytes. The most common cause of hypoxic liver injury appears to be insufficient hepatic perfusion in the setting of passive liver congestion or chronic liver disease. However, other factors can contribute to hypoxic liver injury, including poor oxygen delivery by the blood, inadequate oxygen extraction by hepatocytes, and/or increased metabolic demands. Additional disorders, such as those that affect arterial blood flow, including sickle cell crisis and thrombosis of the hepatic artery, heart failure, sepsis, and hypovolemic shock, are also important in liver ischemia (6, 27, 42).

Ischemia also appears to lead to oxidative damage and inflammation. A major consequence of ischemic injury is tissue anoxia and hypoxia that interrupt intracellular energy metabolism and enzymatic function (6, 15). It has been shown that ischemia may play a major role in the impairment of liver transport systems including bile flow (13, 16, 28). After ischemia, cholestasis may develop. Morphological studies reveal changes of bile canicular structure including increase of canalicular space and loss of microvilli (1). Hepatocytes express different transporters in its sinusoidal (basolateral) and canalicular (apical) membrane. Apically localized transporters play a key role in the transfer of solutes and water into bile canaliculi (38). Among the hepatic apical transporters are members of the ATP-binding cassette (ABC) superfamily of export pumps. These include the bile salt export pump, the multidrug-resistance protein, and the ABC subfamily C, member 2 protein/multidrug resistance-associated protein 2 (ABCC2/ MRp-2). Disorders including ischemia that impair Mrp-2 transport result in cholestatic liver injury (20–22).

The dominant ezrin/radixin/moesin (ERM) protein in hepatocytes is radixin, which is localized primarily at the canalicular microvilli and virtually absent in other liver cells (22, 32, 39). Radixin plays an important role in mediating the binding of F-actin to the plasma membrane and thus is involved in many cellular functions, such as membrane trafficking, cell polarization, and motility (3, 7, 8). All ERM proteins contain two ERM association domains; the NH2 terminus is homologous to the membrane-binding domain and at the COOH terminus has an F-actin-binding domain (29, 33). The NH2-terminal domain of ERM proteins binds to membrane indirectly via adaptor molecules such as Na+/H+ exchanger regulatory factor (EBP-50/NHERF1) or E3-KARP/NHERF2. The primary cellular function of NHERF1 is to act as a scaffold protein linking transmembrane proteins, such as transporters to cytoskeletal proteins. NHERF1 has also been found to associate with Mrp-2 (19, 23).

In the present study, we have hypothesized that hypoxia-induced inhibition of radixin-T564 phosphorylation will lead to disruption of Mrp-2 trafficking and function. We have used the well validated WIF-B culture model [WIF-B cells maintain polarization and a distinct canalicular membrane in culture, and, in addition, protein trafficking has been extensively studied (5, 14, 30–32)] to study the role of radixin in hypoxia. We found that the radixin intracellular localization, radixin phosphorylation, and Mrp-2 localization were markedly abnormal in hypoxic WIF-B cells. We also found that hypoxia-induced radixin dephosphorylation led to an abnormal secretory phenotype.

MATERIALS AND METHODS

Cell culture. WIF-B cells were grown at 37°C in 5% CO2 in modified Coon’s F12 medium supplemented with 5% fetal calf serum and 10 μmol/l hypoxanthine, 0.04 μmol/l aminopterin, and 1.6 μmol/l
thymidine, as described (18). Cultures were passaged using standard trypsinization procedures. Cells were seeded on 3.5-cm glass-bottom and 6-cm plastic cell culture dishes at a density of \(3.8 \times 10^4\) cells/cm\(^2\), and experiments were undertaken with cells at confluence and after they were polarized, typically after 7–10 days of culture. At this time, 70–80% of cells form intercellular phase-lucent, spherical structures (apical vacuoles) defined to be bile canalicular-like spaces as described (2, 18, 31, 32).

**Hypoxia.** Conditions for exposure of cells to hypoxic incubations have been described in detail (17, 41). Briefly, cells were incubated in a temperature- and humidity-controlled environmental chamber in an atmosphere containing 0.5% O\(_2\)-5% CO\(_2\) and balanced with N\(_2\). Oxygen tension inside the chamber was monitored continuously with an oxygen-sensitive electrode.

**Generation of recombinant adenovirus.** A commercial recombinant adenoviral expression system was used to generate radixin-cyan fluorescent protein (CFP) wild-type (WT) and mutants (T564A and T564D) (32). After construct generation, recombinant viruses were grown by transfection of HEK T293 cells. Adenovirus was purified by ultracentrifugation on a CsCl gradient and stored at \(-80^\circ\)C until use. Cells were infected with recombinant adenovirus at the multiplicity of infection of 10 infectious units (IU)/cell for 24 h.

**Subcellular fractionation and immunoblotting.** WIF-B cells were infected with recombinant adenoviruses expressing radixin-CFP WT, radixin-CFP T564A, and radixin-CFP T564D. Uninfected cells were used as a control. Cells were treated with 1% Triton X-100, and cell lysates were centrifuged at 4°C (15,000 g) for 15 min. Radixin-CFP-infected and control supernatant and pellet fractions were separated by SDS-PAGE and subjected to immunoblotting. In brief, after SDS-PAGE and transfer of separated proteins to nitrocellulose, blots were incubated with primary antibody, washed, and exposed to secondary antibody and chemiluminescence kit (Thermo Scientific, Rockford, IL). Signals corresponding to specific bands were quantified and presented graphically.

**Subcellular fractionation without detergent.** WIF-B cells were resuspended in cold buffer (1 mM MgCl\(_2\), 1 mM dithiothreitol, and 1 mM EDTA) and incubated 5 min on ice. Cells were pelleted by centrifugation and resuspended in 0.25 M sucrose and 3 mM imidazole, pH 7.4, with added protease inhibitors (leupeptin, antipain, PMSF, and benzamidine) and sonicated for 3 min at 4°C. The homogenate was centrifuged at 2,000 g for 60 min at 4°C to obtain the cytosolic fraction and the membrane-containing pellet. Before high-speed centrifugation, we aliquoted PNS (which contains 50 \(\mu\)g of protein). After high-speed centrifugation, supernatant fractions were collected, and the entire supernatant and pellet fractions were subjected to immunoblot analysis to detect HIF-1α, as in MATERIALS AND METHODS; representative immunoblots are shown (top). Bottom: data were scanned, quantitated, and presented graphically (n = 3; \(* P < 0.05, ** P < 0.01\) after 24 h of hypoxia compared with 0 h of hypoxia).

**Detection of radixin knockdown by immunoblotting.** Radixin siRNA (5’-UCAUUUGAGAUCCUGUG-3’) and scramble were purchased from Thermo Scientific. Cells were transfected with 50 nM siRNA using Lipofectamine 2000 (Life Technologies, Grand Island, NY). Medium was changed 12 h after transfection, and WIF-B cells were exposed to hypoxia for 24 h. Control cells were transfected with scramble siRNA. Untransfected cells were served as an additional control.

**Immunohistochemistry and live cell imaging.** Cells were fixed with 4% formaldehyde and permeabilized with 0.1% Triton X-100, washed, and probed with anti-radixin (Sigma), anti-phospho-ERM (Cell Signaling Technology), or anti-Mrp-2 (Sigma) antibodies. CFP was detected by using rabbit polyclonal anti-GFP antibody ( Immunology Consultants Laboratory, Newberg, OR). For live cell imaging, WIF-B cells were seeded on glass-bottom cell culture dishes and infected with recombinant adenoviruses. Images were obtained using a Zeiss fluorescence microscope (Carl Zeiss Microscopy, Thornwood, NY).

**GSFM secretion by hepatocytes.** Glutathione-methylfluorescein (GSFM) is a substrate of the apical Mrp-2 transporter and is efficiently transported and accumulated in canalicular lumina. For the assay, the accumulation of dye by cells in normal media is compared with those where tight junctions between cells are disrupted by Ca\(^{2+}\)-free media, thus liberating the GSFM. Cells were first equilibrated in a standard buffer consisting of 0.35 g/l KCl, 0.25 g/l MgSO\(_4\), 0.18 g/l CaCl\(_2\), 0.16 g/l KH\(_2\)PO\(_4\), 4.8 g/l HEPES, 7.9 g/l NaCl, and 0.9 g/l glucose, pH 7.4, at 37°C. Cultures were then preincubated either with standard buffer or in Ca\(^{2+}\)-free standard buffer containing 10 mM EGTA at 37°C for 10 min. To start the assay, 2 \(\mu\)M 5-chloromethylfluorescein diacetate (CMFDA) was added to the cells with and without Ca\(^{2+}\). After 30 min, cells were washed in PBS and lysed in 1% Triton X-100 in PBS, and fluorescence in cell lysates was measured using a FLUOstar Omega microplate reader (ex = 485 nm, em = 530 nm) (BMG Labtech, Cary, NC) (32, 40). Measurements of total and canalicular fluorescence were acquired using NIH ImageJ, and data are presented as percentages of canalicular excretion as previously described (19, 32, 40).

**Fluorescence imaging.** Cells were infected with recombinant adenoviruses expressing radixin-CFP WT, radixin-CFP T564A, and radixin-CFP T564D. Uninfected cells were used as a control. Cells were treated with 1% Triton X-100, and cell lysates were centrifuged at 4°C (15,000 g) for 5 min to prepare PNS, which is the postnuclear supernatant (PNS). The PNS was centrifuged at 100,000 g for 60 min at 4°C to prepare cytosolic and membrane fractions. The fractions were immunoblotted for 5-nucleotidase (5-NT) (kindly provided by Dr. Ann Hubbard), GADDH (Abcam, Cambridge, MA), radixin (Sigma, St. Louis, MO), phosphoradixin (Cell Signaling Technology, Danvers, MA), and Mrp-2 (Sigma), and their relative distributions were determined by densitometry. To obtain equal amounts of protein in each condition (i.e., in normal, hypoxia, and hypoxia/reoxygenation conditions), we have used 50 \(\mu\)g of protein from PNS (WIF-B cell homogenates were centrifuged at 2,000 g at 4°C for 5 min to prepare PNS, which is the starting material). PNS from WIF-B cells were used for high-speed centrifugation (100,000 g for 60 min) to obtain the cytosolic fraction and the membrane-containing pellet. Before high-speed centrifugation, we aliquoted PNS (which contains 50 \(\mu\)g of protein). After high-speed centrifugation, supernatant fractions were collected, and the entire supernatant and pellet fractions were subjected to immunoblot analysis to detect HIF-1α, as in MATERIALS AND METHODS; representative immunoblots are shown (top). Bottom: data were scanned, quantitated, and presented graphically (n = 3; \(* P < 0.05, ** P < 0.01\) after 24 h of hypoxia compared with 0 h of hypoxia).
control. Cells were harvested 24 h after transfection, and cell lysates were separated by SDS-PAGE and subjected to immunoblotting to detect radixin, phospho-radixin, and α-tubulin (Cell Signaling Technology). Furthermore, WIF-B cells from each treatment condition as well as from radixin knockdown experiments were used for Trypan blue and lactate dehydrogenase (LDH) assays (see below).

**Cell viability assays**. Cell viability was determined utilizing both Trypan blue and LDH assays, as previously described (11, 24). For the Trypan blue method, cell viability is calculated as the number of viable cells divided by the total number of cells within the grids on the hemocytometer. If cells take up Trypan blue, they are considered nonviable. To calculate the number of viable cells per milliliter of

---

**Fig. 2.** Localization of radixin, phospho-radixin, and multidrug resistance-associated protein 2 (Mrp-2) in hypoxia. Cultured WIF-B cells were maintained in normal and hypoxic conditions for 24 h as in MATERIALS AND METHODS. Some cells were subsequently reoxygenated for 6 h after hypoxic conditions. Cells were fixed and labeled to detect radixin (green, A), phospho-radixin (green, B), and Mrp-2 (red, A and B) as in MATERIALS AND METHODS. Representative images are shown, including merged images (overlay). The bar marker represents 10 μm.
culture, use the formula below. Cell viability was calculated using the following formula: % viable cells = \( \frac{1.00 \times (\text{number of blue cells/number of total cells})}{100} \).

An LDH assay was performed by measuring LDH levels in conditioned medium and in cells. Culture medium was collected and centrifuged for 5 min at 300 g, and LDH levels were measured in the supernatant. To detect the LDH levels in whole-cell lysates, cells were harvested with phosphate buffer containing 1% Triton X-100. Samples were homogenized and centrifuged at 800 g for 10 min at 4°C. LDH levels were measured in supernatants and whole-cell lysates using the Spotchem EZ Chemistry Analyzer (Arkray, Edina, MN). Media-released LDH ratio was calculated using the following formula: media-released LDH ratio (\%) = \( \frac{\text{LDH}_{\text{media}}}{\text{LDH}_{\text{whole-cell lysate}}} \) \times 100.

Statistical analysis. The Student’s two-sample t-test was used to compare data sets. The criterion for significance was \( P < 0.05 \).

RESULTS

Hypoxia-induced activation of HIF-1α. To determine whether hypoxia affects expression of hypoxia-inducible factor (HIF)-1α in WIF-B cells, they were exposed to hypoxia for 6 and 24 h. This led to a greater than twofold increase in HIF-1α protein expression at 24 h (Fig. 1).

Cellular localization of radixin in hypoxia. We next explored the effect of hypoxia on radixin and phospho-radixin and Mrp-2 (Fig. 2) localization in WIF-B cells (which have similar radixin and Mrp-2 localization). Immunofluorescence localization of radixin and phospho-radixin demonstrated that it was normally present in the canaliculair plasma membrane (Fig. 2, A, top, and B, top). Mrp-2 was localized predominantly to the pericanalicular membranes (Fig. 2, A, top, and B, top). Hypoxia led to redistribution of radixin, phospho-radixin, and Mrp-2 to the cytoplasmic space (Fig. 2, A, middle, and B, middle). Of note, minimal amounts of radixin and phospho-radixin were still localized at the apical membranes in hypoxia (Fig. 2, A, middle, and B, middle). After reoxygenation, there was partial restoration of radixin, phospho-radixin, and Mrp-2 localization (Fig. 2, A, bottom, and B, bottom). Phospho-radixin and radixin were associated with the canalicular plasma membrane similar to Mrp-2.

Effect of hypoxia on radixin distribution. We next examined the distribution of radixin and phospho-radixin and putative association with the actin cytoskeleton biochemically during hypoxia and reoxygenation (Fig. 3). We have used two cell fractionation methods (with and without Triton X-100).

Fig. 3. Membrane localization of radixin and phospho-radixin in hypoxia. A sequential centrifugation protocol (with and without Triton X-100) was used to isolate cytosolic (supernatant, S) and membrane (pellet, P) from WIF-B cells. The fractions were immunoblotted for 5-nucleotidase (5-NT) as a membrane marker and GAPDH as a cytosolic marker (A). Cells were harvested without (B) and with (C) detergent (Triton X-100) to obtain cytosolic (S) and membrane (P) fractions. In B and C, equal fractions of each extraction from all samples were separated by SDS-PAGE and analyzed by immunoblot analysis with anti-radixin and anti-phospho-radixin antibody. In D and E, data from Triton X-100 extraction (C) were scanned, quantitated, and presented graphically. Data from membrane and cytosolic fractions were normalized individually for each experiment (membrane plus cytosolic = 100%, n = 3 independent experiments, * \( P < 0.05 \)).
WIF-B cells, as described in MATERIALS AND METHODS. 5-NIT and GAPDH were used as markers to validate the cytosolic (GAPDH) and membrane (5-NIT) localization of protein fractions (Fig. 3A). 5-NIT is a well-characterized apical membrane marker in WIF-B cells (5, 30, 32). Soluble (cytosolic) and nonsoluble (membrane bound) radixin was extracted first with 1% Triton X-100 buffer. Triton X-100, which is a nonionic detergent, may break protein–lipid associations but not protein–protein interactions. The Triton X-100-based extraction method is widely used for the isolation of membrane proteins, especially ezrin (43). However, to avoid the possible strong unspecific protein extraction effect of Triton X-100, we have also used a nondetergent (without Triton X-100)-based extraction method to evaluate membrane-bound and cytosolic radixin. Figure 3, B and C, shows that both methods have similar results and demonstrates that hypoxia increases cytosolic radixin. Under normal conditions, radixin was recovered in the detergent-insoluble [membrane-bound (P)] fraction as well as in the detergent-soluble [cytosolic (S)] fraction, whereas phospho-radixin was localized largely in the membrane-bound fraction (Fig. 3, B and C). After 24 h of hypoxia, radixin and phospho-radixin in the detergent-insoluble fraction decreased, suggesting that radixin had dissociated from the cytoskeleton (Fig. 3, B and C). Both of these trends were reversed after reoxygenation (Fig. 3, B and C). There was no phospho-radixin immunoreactivity in detergent-soluble fractions.

Expression of Mrp-2 in hypoxia. To further investigate Mrp-2 protein expression in the context of hypoxia, cell lysates from WIF-B cells were examined. Mrp-2 expression was reduced after hypoxia (27% of control, \( P < 0.05 \)) but recovered after reoxygenation (49% of control, \( P < 0.05 \)) (Fig. 4).

**Distribution dynamics of radixin in live cells after hypoxia.** Under normal conditions, radixin heavily localized on the apical membrane vacuoles, with relatively little or no radixin on the cytoplasmic space. Hypoxia led to redistribution of radixin to the cytoplasm (Fig. 5, top), and reoxygenation caused radixin to relocate to its normal location. The dominant-negative construct (radixin-CFP T564A) was unable to rescue this phenotype (Fig. 5, middle). However, the dominant-active construct (radixin-CFP T564D) led to persistent radixin localization in apical membrane vacuoles despite hypoxic conditions (Fig. 5, bottom). Of note, however, was that some cytoplasmic accumulation of radixin remained after reoxygenation.

**Cellular distribution of exogenously expressed radixin constructs in hypoxia.** After we infected WIF-B cells with radixin-CFP WT and mutant viruses (containing each T564A and T564D), we measured protein expression levels in normal and hypoxic conditions and found them to be relatively equal (Fig. 6, A and B). Furthermore, we analyzed the membrane and cytosolic distribution of exogenously expressed radixin in WIF-B cells (Fig. 6C). WIF-B cells were infected with recombinant adenoviral constructs incorporating radixin-CFP WT, the T564A mutant, and the T564D mutant for 24 h. Western blots of CFP overexpressed WIF-B cells in normal, hypoxia, and hypoxia/reoxygenation conditions (Fig. 6C) in the normal state, consistent with the live cell images demonstrating that radixin was about equally localized at the membrane and cytosolic fractions (Fig. 6C, top). Whereas the radixin-T564A construct was similarly distributed in membrane and cytosolic fractions, the radixin-T564D was found predominantly in the membrane fraction (Fig. 6C, top). Notably, hypoxia shifted localization of radixin to the cytoplasmic fraction, but the radixin-T564D construct caused radixin to retain its normal localization, even after hypoxia (Fig. 6C, middle). Reoxygenation led to reversal of hypoxia-induced radixin mislocalization (Fig. 6C, bottom).

**Effect of radixin activation on Mrp-2 in hypoxia.** Experiments to evaluate the effect of hypoxia and hypoxia/reoxygenation on the cellular distribution of radixin-CFP WT (Fig. 7, top), radixin-CFP T564A (Fig. 7, middle), and radixin-CFP T564D (Fig. 7, bottom) are shown. WIF-B cells were infected with radixin-CFP WT and mutant constructs (T564A and T564D). Cells were fixed and stained for radixin-CFP and for Mrp-2 at 24 h of hypoxia or 24 h of hypoxia/6 h of reoxygenation. Radixin-CFP WT, radixin-CFP T564A, and radixin-CFP T564D appeared to be localized to the apical vacuoles along with Mrp-2, with relatively little CFP signal located on the cytoplasmic area in control cells. However, a significant amount of radixin-CFP T564A localized on the cytoplasmic space compared with radixin WT and T564D mutant. Cells infected with radixin-CFP WT and radixin-CFP T564A apical vacular localization of CFP tended to disappear, and radixin-CFP as well as endogenous Mrp-2 was distributed throughout the cytoplasmic space after 24 h of hypoxia. In contrast, WIF-B cells expressed radixin-CFP T564D prominently on the apical membranes similar to live cells. Mrp-2 was also associated with the apical membrane. Nonetheless, some radixin-CFP T564D localized on the cytoplasmic area along with Mrp-2. Reoxygenation caused radixin-CFP WT to relocalize to its normal location. The dominant-negative construct (radixin-CFP T564A) was unable to rescue this phenotype. However,
the dominant-active construct (radixin-CFP T564D) led to persistent radixin localization in apical membrane vacuoles. Some cytoplasmic accumulation of radixin remained after reoxygenation.

Canalicular excretion of fluorescence-tagged Mrp-2 substrate.

The canalicular membrane localization of radixin implies that it may play a role in canalicular membrane structure and polarity as well as Mrp-2 function. Therefore, we analyzed canalicular secretion with the well validated GSMF assay (32). GSMF was efficiently transported and accumulated into canalicular lumina for many cells; quantitative evaluation of CMFDA uptake of uninfected control WIF-B cells, and cells infected with adenovirus alone (CFP) or adenovirus expressing WT radixin revealed a robust secretory response (Fig. 8, left). The dominant-negative construct inhibited the secretory response, whereas the dominant-active stimulated it (Fig. 8, left). The secretory response of all cells was diminished by hypoxia. However, the secretory response was partially restored by the radixin-T564D mutant (Fig. 8, middle). The secretory response of all cells was increased after reoxygenation, and the radixin-T564D mutant restored the secretory response to above normal levels, suggesting that radixin phosphorylation is important in canalicular excretory function in hypoxia.

Effect of radixin knockdown on cell viability in hypoxia.

To determine the effect of radixin knockdown on cell viability in hypoxic conditions, we knocked down radixin in WIF-B cells using siRNA in cells under hypoxia (Fig. 9A). Radixin siRNA markedly decreased radixin and phospho-radixin expression.

Fig. 5. Radixin localization in hypoxia. WIF-B cells as in Fig. 2 were infected with adenovirus-containing recombinant cyan fluorescent protein (CFP)-labeled wild-type radixin (radixin-CFP WT), CFP-labeled dominant-negative radixin (radixin-CFP T564A), and CFP labeled dominant-active radixin (radixin-CFP T564D). After 24 h in hypoxic conditions or after hypoxia for 24 h followed by 6 h of reoxygenation (24 h/6 h hypoxia/reoxygenation), radixin localization (blue CFP fluorescence) was evaluated. Representative images are shown. The asterisk shows the canalicular membrane, and the N indicates the cell nucleus. The bar marker represents 10 μm.

Fig. 6. Distribution of exogenously expressed radixin constructs in hypoxia. In A and B, immunoblots of radixin-CFP-overexpressed WIF-B cells in normal, hypoxia, and hypoxia/reoxygenation conditions are shown. Conditions for cell culture included control uninfected cells, control virus-infected cells (CFP), and cells infected with adenovirus alone (CFP) or adenovirus expressing WT radixin revealed a robust secretory response (Fig. 8, left). The dominant-negative construct inhibited the secretory response, whereas the dominant-active stimulated it (Fig. 8, left).

The secretory response of all cells was diminished by hypoxia. However, the secretory response was partially restored by the radixin-T564D mutant (Fig. 8, middle). The secretory response of all cells was increased after reoxygenation, and the radixin-T564D mutant restored the secretory response to above normal levels, suggesting that radixin phosphorylation is important in canalicular excretory function in hypoxia.
PHOSPHORYLATION OF RADIXIN IN HYPOXIA
Radixin siRNA significantly decreased cell survival after hypoxia for 24 h, determined by Trypan blue assay \( (P < 0.05) \) (Fig. 9b).

In addition, we examined cell viability using a Trypan blue and LDH assay (Fig. 9, C and D, respectively). WIF-B cells expressing the radixin-T564A mutant appeared to be significantly more injured than control cells \( (P < 0.05) \). However, the phospho-mimicking radixin-T564D mutant appeared to protect cells from hypoxic injury \( (P < 0.05 \text{ vs. the T564A mutant or WT cells}) \). These data suggest that...
radixin activation preserves cellular function in the setting of hypoxia.

**DISCUSSION**

In this study, we report that hypoxia caused radixin dissociation and its dephosphorylation. This was associated with Mrp-2 mislocalization (away from its typical apical plasma membrane location) and marked impairment in hepatocellular secretory function. We also found that hypoxia was associated with some cellular injury (Fig. 9). Reoxygenation restored radixin phosphorylation, Mrp-2 distribution dynamics, and secretory function. Importantly, overexpression of a phospho-mimicking radixin (radixin-T564D) partially rescued the abnormal phenotype induced by hypoxia (Figs. 5–7).

ERM proteins contain two ERM association domains (ERMADs); the N-ERMAD at the NH2 terminus is considered to be the membrane-binding domain, and the C-ERMAD at the COOH terminus has an F-actin-binding domain. ERM proteins bind directly to some membrane proteins and lipids and ERM proteins also bind to membrane indirectly via adaptor molecules such as NHERF-1 or NHERF-2 (4, 7, 8). Several studies have suggested that phosphorylation may be essential for dissociating intramolecular N- and C-ERMAD interaction, thus promoting open and active forms of ezrin, radixin, and moesin monomers (9, 25). Phosphorylation on threonine 564 is sufficient to disrupt the N-C binding of radixin, as predicted from in vitro studies on other ERM proteins and structural studies. Radixin, a protein linking actin filaments and plasma membrane, is a known component of the hepatocellular microvilli (32, 40). Radixin knockout mice show a selective loss of Mrp2 from the canalicular membrane and begin to develop conjugated hyperbilirubinemia (20). Furthermore, suppression of radixin expression in hepatocytes resulted in mislocalization of Mrp-2 as well as other apical transporters (40). Previously, we have characterized the intracellular localization of endogenous radixin and Mrp-2, as well as exogenously expressed radixin and forms with point mutations at the radixin-threonine 564 site (T564A and T564D) in WIF-B cells (32). Together, these findings suggest that radixin is essential for maintaining normal hepatic canalicular membrane morphology and the functional activity of bile salt transporters in hepatocytes and that radixin may be required for retaining/tethering of Mrp-2 in the apical canalicular domain.

Ischemia is a complex and multifaceted cellular problem. The normal architecture of the actin cytoskeleton becomes disrupted early after ischemia and ATP depletion, and this disruption directly results in impaired cell-cell and cell-substrate adhesion, loss of tight-junction barrier function, and mixing of apical and basolateral transporters, with resultant impairment in cell and organ function. Ischemia also leads to loss of actin from the apical microvilli and stress fibers, which in turn results in membrane blebbing and disruption of apical microvilli (10, 12, 26, 37).

Our work extends previous work by demonstrating that the ERM protein, radixin, an important cytoskeletal linker in hepatocytes, is involved in hypoxia-mediated secretory dys

---

**Fig. 8.** Canalicular excretion of fluorescence-tagged Mrp-2 substrate. WIF-B cells as in Fig. 1 were exposed to fluorescence-tagged 5-chloromethylfluorescein diacetate (CMFDA), and the cellular accumulation of CMFDA uptake and excretion was measured 24 h after infection with radixin-CFP WT, radixin-CFP T564A, and radixin-T564D constructs. The biliary excretion index was calculated as in MATERIALS AND METHODS. The data were collected from assays in 5 separate culture preparations and normalized within each experimental run by setting the CMFDA uptake for uninfected control cells (control) at 100%. There was no significant difference in rates of CMFDA excretion between the adenovirus-infected cells and uninfected control cells (in normal culture condition, *P < 0.05, radixin-CFP T564A infected cells compared with control, *P < 0.05, radixin-CFP T564D compared with control). CMFDA excretion was significantly decreased in hypoxic condition (*P < 0.05, radixin-CFP T564D compared with control). In reoxygenated cells (hypoxia/reoxygenation), CMFDA excretion was increased compared with hypoxic cells (*P < 0.05, radixin-CFP T564A-infected cells compared with control, *P < 0.05, radixin-CFP T564D compared with control) (n = 5 independent experiments).
function. It has been shown that ERM proteins are important for cell survival in ischemia/reperfusion injury (35, 36). Our work is consistent with previous research that demonstrated that hepatic ischemia-reperfusion injury reduced Mrp-2 mRNA and protein expression and caused it to be mislocalized (34), similar to the effect of hypoxia on WIF-B cells, as in this study. Our work emphasizes that the effect of hypoxia on Mrp-2 and hepatocellular dysfunction is tightly integrated with the ERM-cytoskeletal system. Furthermore, our results show that radixin is also critical for cell survival in hypoxia.

Although the maintenance of secretory polarity of the hepatocyte is critical for its normal function, little is known about how these cells establish and maintain this functionally distinct apical domain. Radixin is essential for maintaining the polarized targeting and retaining of canalicular membrane transporters and is a critical determinant of the overall structure and function of the apical membrane of hepatocytes.

Hypoxic liver injury is an important clinical problem. Hypoxia is critical in ischemic hepatitis as well as in ischemia-reperfusion injury. Unfortunately, effective clinical therapies to treat ischemic hepatitis are lacking, and approaches to prevent ischemia-reperfusion injury are unsatisfactory. Our data raise the possibility that manipulation of the ERM system could be beneficial clinically. Our data also suggest that further in vivo studies to determine whether dynamic support of radixin phosphorylation and activation are warranted.

We recognize limitations of our study. Because of the multifactorial and complex nature of hypoxia, there are currently no suitable in vivo animal models available for the study of hypoxia-induced liver injury. The liver ischemia and reperfusion animal model is well characterized; however, this in vivo model represents generally a research tool for study of ischemia and cold-storage preservation in liver transplantation. Thus, in the current study, we used WIF-B cells to examine
PHOSPHORYLATION OF RADIXIN IN HYPOXIA

G323

hypoxia. Although we considered studying primary hepatocytes, we chose to evaluate WIF-B cells because they are polarized and represent an ideal functional model for hepatocytes (2, 18, 31, 32). Furthermore, live cell imaging (Fig. 5) can be readily performed with WIF-B cells. Another advantage of the WIF-B system is that these cells can be readily infected under carefully controlled circumstances.

In summary, our data strongly suggest that radixin activation is essential for Mrp-2 distribution and function in hypoxia and also that, when radixin is active, it may overcome the effects of hypoxia. We propose that characterization of additional radixin/Mrp-2 regulatory proteins, including adapter proteins (NHERF1 and NHERF2), protein kinases, and other radixin-interacting proteins, will enhance the understanding of the cellular dynamics and function of radixin in hypoxia.

ACKNOWLEDGMENTS

We thank Drs. Voyno-Yasenetskaya and Liu (University of Illinois) for radixin cDNA and Dr. Ann Hubbard (Johns Hopkins University) for WIF-B cells and 5-NT antibody.

GRANTS

S. Karvar is been recipient of an AGA/Foundation for Digestive Health and Nutrition Research Scholar Award, which helped support the work reported in this study.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


