Reduced tolerance of immature renal tubules to anoxia by HSF-1 decoy

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Sreedharan, Rajasree, Michael Riordan, Shirley Wang, Gunilla Thulin, Michael Kashgarian, and Norman J. Siegel. Reduced tolerance of immature renal tubules to anoxia by HSF-1 decoy. Am J Physiol Renal Physiol 288: F322–F326, 2005. First published October 5, 2004; doi:10.1152/ajprenal.00307.2004.—Immature animals demonstrate an amplified heat shock response following a variety of insults compared with that seen in mature animals (M). The potential role of the heat shock response in modulating immature tolerance to injury was compared between rat pups, 10 postnatal days of age (P10), and M. Baseline levels of the heat shock transcription factor (HSF-1) were substantially elevated in P10 compared with M animals. In uninjured P10 pups, HSF-1 level was comparable to that of M animals subjected to 45 min of ischemia. As anticipated, the integrity of suspensions of tubules exposed to anoxia was preserved in P10 animals (23% LDH release) compared with M (40%), P < 0.01. The effect of targeted inhibition of HSF-1 on tubular integrity was studied using a cyclic oligonucleotide decoy. The HSF-1 decoy increased the severity of anoxic injury in P10 pups to a level comparable with M animals. LDH release was 33% in decoy-treated P10 tubules compared with 40% in M. When P10 tubules were treated with scrambled decoy, resistance to anoxia remained intact (24%). The increased vulnerability of the tubular suspension to injury was specific to the HSF-1 decoy and proportional to the dose of decoy applied. This study demonstrates maturation in the abundance of HSF-1 in the immature rat kidney. The loss of resistance of immature tubules to anoxia with specific inhibition of HSF-1 may be due to its effect on the heat shock response or other signaling pathways of critical pathobiological importance in renal cell injury.

newborn; renal proximal tubule; heat shock proteins; 70-kDa heat shock protein; ischemia

IMMATURE ANIMALS ARE WELL recognized to be afforded greater tolerance to anoxia than their mature counterparts (2, 15). Resistance to oxygen deprivation has been demonstrated in the kidney (10, 21, 22), myocardium (18), and central nervous system of immature animals (9, 13). Protection does not appear to be related to differences in glycolytic capacity, as inhibition of glycolysis does not worsen anoxic injury (12), and a role for the heat shock response has been postulated. Heat shock proteins (HSP) induce tolerance to hypoxic-ischemic injury in the mature kidney (8), myocardium (5, 7), and brain (3, 14). Levels of HSP expression vary with maturation (6, 21) and a disparity in the heat shock response between immature and mature animals has been noted (11, 18). In the kidney, immature rat tubules demonstrate increased interaction between the heat shock transcription factor (HSF-1) and the heat shock transcriptional element (HSE) following anoxic injury compared with tubules from mature animals (11). In addition, mRNA and protein levels of the HSF-1-regulated 70-kDa heat shock protein (HSP70) are increased in immature tubules following anoxia (11, 21).

To establish the role of HSF-1 in the response of the immature tubule, developmental and injury-induced changes in nuclear protein expression were examined. The effect of specific inhibition of HSF-1 on tolerance of the immature nephron was explored using circular, ethylene glycol bridged, decoy oligodeoxynucleotides.

METHODS

Animal model. Pups from two litters of Sprague-Dawley rats were killed at postnatal days 1, 3, and 10 (P1, P3, and P10). Animals were anesthetized with 50 mg/kg of intraperitoneal pentobarbital sodium (Nembutal sodium solution, Abbott Laboratories, Chicago, IL); the kidneys were rapidly removed, decapsulated, and homogenized in a Potter-Elvehjem homogenizer into 1 ml of phosphate-buffered saline containing 1 mM benzamidine, 2 mM sodium vanadate, and Complete Protease Inhibitor Cocktail (Roche).

The effect of bilateral renal ischemia on HSF-1 expression was studied in adult male Sprague-Dawley rats, as previously described (20). After 45-min ischemia, the kidneys were rapidly removed, decapsulated, and harvested as above. Kidneys harvested from sham-operated animals served as controls.

Nuclear protein extraction. Nuclear protein extraction was performed using hypotonic lysis followed by high-salt extraction of nuclei (1). Homogenate was centrifuged for 10 s at 18,000 g using a Microfuge (Beckman Coulter) and the supernatant was discarded. The pellet was resuspended in 400 μl of a buffer containing 10 mM HEPES-KOH (pH 7.9) at 4°C, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 1 mM sodium vanadate, and Complete Protease Inhibitor Cocktail (Roche), incubated on ice for 10 min, and centrifuged at 18,000 g for 10 s. The supernatant was discarded and the remaining pellet was suspended in a volume of buffer equal to the volume of the pellet and containing 20 mM HEPES-KOH (pH 7.9) at 4°C, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 1 mM sodium vanadate, and Complete Protease Inhibitor Cocktail (Roche). Samples were incubated on ice for 20 min, centrifuged at 18,000 g for 2 min at 4°C, and the supernatant was removed and stored at −80°C.

Preparation of suspensions enriched in tubule segments. For each experiment, tubule segments were obtained from the kidneys of 12 immature (P10) rat pups and 1 mature (8–10 wk) male Sprague-Dawley rat. Animals were anesthetized with pentobarbital sodium; the kidneys were removed and bisected longitudinally. Kidneys were incubated for 45 min at 37°C with 21 FALGPA units of collagenase (Sigma, St. Louis, MO) in 7.5 ml of freshly prepared buffer (buffer C) containing 4 mM sodium lactate, 2 mM alanine, 3 mM butyrate, 2 mM glutamine, 5 mM malate, 5.5 mM glucose, 112 mM NaCl, 3 mM KCl, 1.2 mM MgSO4, 1 mM KH2PO4, 1.5 mM CaCl2, and 25 mM...
NaHCO₃, equilibrated with 95% O₂-5% CO₂, and having a final pH of 7.4 (10). After digestion, tubules were separated by gentle scraping with a razor blade and resuspended in buffer C. To eliminate gomerauli, the tubules were filtered through a fabric mesh (Tetko, Briarcliff Manor, NY), 115 µm for the mature suspension, 50 µm for the immature suspension. The suspension was washed in buffer C by spinning at 500 g three times for 2 min. The final pellet was resuspended in buffer C and kept on ice until studied.

Induction of anoxia and subsequent recovery. Tubules from both the immature and mature rats were suspended in 6 ml of buffer C and studied in 500-µl aliquots. Before the induction of anoxia, tubule suspensions were bubbled with 95% O₂-5% CO₂ at 37°C for 10 min to allow for steady-state equilibration. Samples of tubular suspension obtained following equilibration were used to provide baseline measurements (mature and immature controls). Anoxia was produced by bubbling each aliquot for 2 min with 95% N₂-5% CO₂. Anoxia was confirmed by measurement of PO₂ using a Clarke oxygen electrode. Each Eppendorf tube was capped and incubated at 37°C in a shaking water bath for 20 min, rebubbled with 95% N₂-5% CO₂, and incubated for a further 25 min, giving a total anoxic period of 45 min. Tubes were opened at the end of the anoxic period, bubbled with 95% O₂-5% CO₂, and incubated at 37°C in a shaking water bath for 1 h. Aliquots of tubular suspension for Western blotting (150 µl) were removed, sonicated, and stored at −80°C. Aliquots for additional studies were obtained and stored as described below.

Decoy treatment. Inhibition of HSF-1 was achieved using a transcription factor decoy as previously described (17). Briefly, multiple copies of a 23-bp oligodeoxynucleotide, of a sequence corresponding to HSE, were added to suspensions of immature tubules. After absorption, the decoy provides an alternative, and ineffective, target for binding of activated HSF-1. The decoy consisted of complementary strands of DNA joined at either end by ethylene glycol bridges to HSE, were added to suspensions of immature tubules. After absorption, multiple isoforms of HSF-1 between 70 and 85 kDa in molecular mass (Fig. 1).

Baseline levels of HSF-1 expression increased between days 1 and 10 of postnatal life (P₁, P₃, P₁₀) and in mature rats. Western blotting demonstrated multiple isoforms of HSF-1 between 70 and 85 kDa in molecular mass (Fig. 1).

To determine the developmental pattern of HSF-1 abundance, nuclear protein extracts of immature and mature kidney homogenates were studied at days 1, 3, and 10 of postnatal life (P₁, P₃, P₁₀) and in mature rats. Western blotting demonstrated multiple isoforms of HSF-1 between 70 and 85 kDa in molecular mass (Fig. 1).

The effect of HSF-1 inhibition on cellular viability after an anoxic insult was studied in immature and mature kidneys. After anoxia, HSF-1 antibody (17), Western blots of nuclear protein extracts were probed with the HSF-1 antibody (top). The relative intensity of HSF-1 expression averaged over all 3 animals at each developmental stage and condition was assessed by densitometry (bottom). Densitometry included each isoform (all bands) at each time point in each animal. C, control.
The effect of decoy treatment on the integrity of immature tubules was assessed. Cellular release of lactate dehydrogenase (LDH) was compared in tubule preparations from immature (P10) and mature animals. Immature tubules demonstrated a baseline release of 9% of total LDH compared with 13% in mature tubules (Fig. 3, control bars).

Following 45 min of anoxia and 1 h of reoxygenation, tubules from both groups demonstrated a significant increase in LDH release (injury bars). As previously reported, immature tubules were more resistant to anoxia compared with mature tubules (10). LDH release in immature tubules (23%) was significantly (P < 0.01) less than that in adult tubules (40%). Treatment of immature tubules with 80 μg of the HSF-1 decoy substantially diminished the tolerance of the immature tubules to anoxia. Decoy-treated P10 tubules demonstrated a severity of injury similar to that seen in mature tubules. LDH release in decoy-treated immature tubules was 33% compared with 40% in the mature tubules (P = not significant). When tubules were treated with scrambled decoy, resistance to anoxia remained intact (24%).

To determine whether the magnitude of injury could be modulated in proportion to the dose of decoy administered, immature tubules were treated with 40, 80, and 160 μg of decoy. The degree of anoxic injury was proportional to the dose of decoy administered (Fig. 4).

DISCUSSION

Immature animals exhibit an extraordinary tolerance to hypoxia/anoxia compared with mature animals of the same species. In 1670, Boyle (2) reported that kittens survived three times longer than mature cats when subjected to anoxia. Similarly, in 1813 Le Gallois (15) demonstrated that signs of life persisted for 15 min in newborn rabbits following asphyxia; in contrast, signs of life were extinguished in 2 min in mature rabbits. Subsequent modern work confirmed the results of early studies, although the mechanisms underlying this phenomenon remain incompletely understood (9).

Tolerance to injury has also been demonstrated within individual organ systems. Lambs exposed to hypoxemia demonstrate increasing preservation of glomerular filtration rate and sodium reabsorption with decreasing postnatal age (22). Proximal convoluted tubules, removed from immature rabbits, show greater preservation of active transport and cellular integrity following anoxia compared with tubules from mature animals (4).

Increased glycolytic capacity and preservation of high-energy phosphates have been proposed as potential mechanisms of resistance to injury in neonatal animals. However, although glycolysis is increased in immature proximal renal tubules exposed to hypoxia, inhibition of glycolysis does not alter immature tolerance (12).

Heat shock response has been proposed as a potential mediator of immature tolerance to hypoxia-anoxia because: 1) overexpression of HSP induces tolerance to hypoxia-ischemia in mature animals (8, 24); 2) constitutive levels of inducible...
HSP increase during postnatal development (21); 3) immature animals demonstrate a robust and amplified heat shock response following a variety of insults compared with that seen in mature animals of the same species (11, 18, 21); and 4) immature renal tubules exposed to anoxia demonstrated increased activation and binding of trimerized HSF-1 to the HSE (11).

The current study demonstrates a maturation of abundance of HSF-1 in immature rat renal tubules. Increased expression of HSF-1 during development parallels that of HSP70, one of the many heat shock proteins under the control of this promoter (21). The relative total abundance of this potent transcriptional activator in P10 rat pups is considerably greater than that seen in uninjured mature rats. Renal ischemia increases the abundance of HSF-1 in mature animals. However, the level of HSF-1 after 45 min of ischemic injury in mature rats remains lower than that seen in uninjured P10 pups. The process of development appears analogous to injury-induced preconditioning in the mature animal, which is associated with tolerance to a subsequent insult (16).

Previous studies demonstrated that the activation of HSF-1 in immature renal tubules is markedly more robust than that seen in tubules from mature rats following a variety of injuries including anoxia, hyperoxia, and hyperthermia (11). Results of the current study suggest that constitutive upregulation of the heat shock response in the immature kidney is primed by high levels of the primary transcriptional regulator of this stress-induced system, HSF-1.

When immature tubules were incubated with the HSF-1 decoy, expression of the principle synthesis product of the heat shock response, HSP70, was substantially reduced. This observation is in keeping with the predicted molecular mechanism of action of the oligonucleotide decoy to dampen the molecular sequence of events following the activation of HSF-1.

In the present study, vulnerability to anoxia was increased in immature tubules treated with the HSF-1 decoy. The severity of injury following targeted inhibition of HSF-1 was similar to that seen in mature tubules. The loss of tolerance to anoxia was specific to decoy-sharing sequence homology with the HSE and occurred in a dose-dependent manner. Increased injury following decoy treatment of immature tubules may be a reflection of direct inhibition of heat shock protein synthesis. Previous work in our laboratory indicated that treatment of cultured proximal tubule cells with an HSF-1 decoy produces a significant downregulation of both HSP70 and HSP25 following cellular ATP depletion. Decay-induced dampening of the heat shock response was associated with a substantial loss of cellular polarity and more severe injury following energy depletion (17). The HSF-1 decoy increases both the severity of injury in cultured renal cells and the vulnerability of immature tubules to anoxia, suggesting the possibility of a common mechanism that modulates renal cell injury.

Although it is tempting to assume that the increased abundance of HSF-1 and HSP70 in P10 tubules is responsible for the tolerance of the immature kidney to anoxia, it is important to recognize that HSF-1 governs transcription and regulation of a myriad of genes that affect essential cellular functions. Homozygous HSF-1-deficient mice exhibit multiple phenotypes including prenatal lethality and abnormalities during extraembryonic development. Of note, basal heat shock protein expression is not altered appreciably in HSF-1 knockout mice, suggesting that HSF-1 might be involved in regulating other genes or signaling pathways important to critical pathobiological processes including cellular resistance to injury (23). In fact, HSF-1, although traditionally regarded as a transcription promoter, can act to repress transcription, which might suppress induction of pathways that propagate cell death (19).

The present series of studies indicates that there is a progressive increase in expression of HSF-1 in renal tubules during early postnatal development. In essence, the immature nephron responds to stress as if the process of development is analogous to a preconditioning insult in the mature kidney. Treatment with an HSF-1 decoy is associated with a loss of tolerance to anoxia in immature renal tubules. The precise cellular mechanism of this increased vulnerability to injury may be related to the modulation of the heat shock response or other critical cellular functions influenced by HSF-1. The immature nephron may serve as a paradigm of augmented heat shock response in the absence of prior injury.

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