Neutrophil peptidyl arginine deiminase-4 has a pivotal role in ischemia/reperfusion-induced acute kidney injury

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Ischemia/reperfusion is a common cause of acute kidney injury (AKI). However, mechanisms underlying the sudden loss in kidney function and tissue injury remain to be fully elucidated. Here, we investigated the role of peptidyl arginine deiminase-4 (PAD4), which converts arginine to citrulline and plays a role in epigenetic regulation and inflammation, in renal ischemia/reperfusion injury. PAD4 expression was highly induced in infiltrating leukocytes 24 hours following renal ischemia and reperfusion. This induction was accompanied by citrullination of histone H3 and formation of neutrophil extracellular traps in kidneys of wild-type mice. By contrast, PAD4-deficient mice did not form neutrophil extracellular traps, expressed lower levels of pro-inflammatory cytokines and were partially protected from renal ischemia/reperfusion-induced AKI. Furthermore, PAD4-deficient mice recovered kidney function 48 hours after ischemia/reperfusion, whereas kidney function in the wild-type mice progressively worsened. Administration of DNase I, which degrades neutrophil extracellular traps or the PAD-specific inhibitor YW3-56 before ischemia, partially prevented renal ischemia/reperfusion-induced AKI. Notably, transfer of neutrophils from wild-type, but not from PAD4-deficient mice, was sufficient to restore renal neutrophil extracellular trap formation and impair kidney function following renal ischemia/reperfusion. Thus, neutrophil PAD4 plays a pivotal role in renal ischemia/reperfusion-induced AKI.

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Acute kidney injury (AKI) results in high morbidity and mortality but has no effective treatment beyond supportive care.[1,2] AKI induced by ischemia/reperfusion (I/R), the most common cause of AKI,[3] is characterized by injury to tubular epithelial cells[4,5] and vascular endothelial cells,[6,7] and robust inflammatory responses, including leukocyte infiltration and upregulation of chemokines and cytokines in the kidney.[8–10] Although inflammatory responses to viral and bacterial infection are vital to host defense, tissue, and wound repair, I/R-triggered "sterile" inflammation may lead to tissue injury.[11,12] A relatively recently described pathway of neutrophil-induced injury involves the formation of neutrophil extracellular traps (NETs), which involves the decondensation of chromatin and subsequent extravasation of DNA into the extracellular space.[13] Formation of NETs may aid clearance of bacteria during infection but also has been implicated in a growing list of autoimmune and inflammatory conditions,[13] including ischemic injury to the heart,[14] liver,[15] muscle,[16] brain,[17,18] and kidney.[19,20]

The peptidyl arginine deiminase (PAD) family is comprised of 5 closely related proteins (PADs 1–4 and PAD6) that posttranslationally convert arginine to citrulline in proteins, thereby mediating signal transduction for diverse stimuli and affecting many biological processes.[21] Although PADs 1–3 and PAD6 are cytoplasmic enzymes,[21] PAD4 occurs in both the cytoplasm and the nucleus[22,23] and is primarily expressed in leukocytes, particularly in granulocytes.[21] The conversion of histone arginines to citrullines by PAD4 reduces the overall positive charges of histones and weakens histone-DNA binding, thereby disrupting the nuleosomes and triggering nuclear DNA release and formation of NETs.[21] Thus, PAD4 may be involved in signal transduction, leading to the inflammatory responses after I/R injury. Indeed, Rabadi *et al.*[24] reported that renal tubular cell PAD4 is highly induced by I/R injury and that PAD4-deficient mice are protected against I/R-induced kidney injury. Although neutrophil-PAD4 is implicated in renal I/R injury,[20,24] nothing is known regarding its specific role in this pathologic context. Neutrophil PAD4 is especially relevant because it is essential to the formation of NETs, which evokes most aspects of inflammatory responses during autoimmunity and thrombosis.[13,25]

Here, we tested the hypothesis that neutrophil PAD4 and NET formation are mechanistically linked to renal I/R-induced AKI. We found, in wild-type (WT) mice, the induction of PAD4 after renal I/R. PAD4 promoted inflammation after renal I/R injury and was essential for NET formation. DNase I treatment before renal I/R suppressed NET formation and partially protected mice from I/R-induced renal injury. A PAD-specific inhibitor, YW3-56, potently suppressed I/R-induced kidney injury in mice. In PAD4-deficient mice, transfer of neutrophils derived from WT mice was sufficient to promote NET formation in the kidney and loss of kidney function after I/R. By contrast, PAD4-knockout mice receiving neutrophils from PAD4-deficient mice showed no detectable NETs in the kidney and were protected from I/R-induced renal injury.

RESULTS

PAD4 is upregulated in mouse kidneys after I/R injury

To establish the biological role for PAD4 in renal I/R injury, we first assessed the expression and distribution of PAD4 and one of its products, citrullinated histone H3 (Cit-H3), in the kidney after ischemia. Relative to sham controls, PAD4 mRNA levels measured by quantitative reverse transcription–polymerase chain reaction (RT-PCR) were 3-fold higher 24 hours after I/R injury ($n = 4$; $P < 0.01$; Figure 1a). Citrullination of histone-H3 was not detectable in mice that underwent sham surgery and was markedly increased in mice 24 hours after I/R (Figure 1b). Thus, renal I/R induces PAD4 expression and activity.

Although PAD4 is expressed mainly in hematopoietic cells,[21] it also has been described in renal epithelial cells.[24] We performed immunolocalization of PAD4 and Cit-H3 in kidneys after ischemia to determine the sites of PAD4 expression and activity. Immunofluorescence microscopy showed few detectable PAD4 (Figure 1c) or Cit-H3 (Figure 1d) signals co-localizing with nuclear DNA in kidneys from WT and PAD4 knock-out (PAD4KO) mice after sham surgery. By contrast, 24 hours after I/R injury, PAD4 (Figure 1c) and Cit-H3 (Figure 1d) were dramatically increased in the kidneys of WT mice, but they were barely detectable in the PAD4KO mice. Double labeling of PAD4 or Cit-H3 with a neutrophil marker, Ly6G, revealed that most of the PAD4- and Cit-H3-expressing cells in the ischemic kidneys were neutrophils (Figure 1, c and d). Although faint PAD4 signals were observed in renal epithelial cells from sham WT kidneys, they did not increase after I/R. Thus, renal I/R injury induces PAD4 expression and triggers Cit-H3 production mainly in infiltrating neutrophils.

PAD4 mediates kidney I/R injury

To determine whether PAD4 is involved in renal I/R injury, we assessed kidney function 24 hours and 48 hours after I/R in WT and PAD4KO mice. As expected, WT mice developed severe kidney dysfunction, as reflected by elevated plasma creatinine (Figure 2a) and blood urea nitrogen (BUN; Figure 2b) concentrations. In contrast, BUN ($P < 0.001$) and creatinine ($P < 0.001$) levels were significantly lower in PAD4KO mice, compared with WT mice 24 hours after I/R. Indeed, 48 hours after I/R injury, levels of both creatinine and BUN returned to the normal ranges in the PAD4KO mice, but they continued to climb in the WT mice (Figure 2, a and b). Likewise, mRNA levels of kidney injury molecule-1 (KIM-1), an established biomarker for renal proximal tubule injury,[26] were barely detectable in the kidneys of both WT and PAD4KO mice that underwent sham surgery but increased dramatically 24 hours after renal I/R injury in WT mice (Figure 2c). The induction of KIM-1 expression after I/R was lower in PAD4KO mice ($P < 0.001$; Figure 2c). Finally, histologic evidence of injury was assessed in kidneys from WT and PAD4KO mice subjected to I/R (Figure 2d). Kidneys from sham-operated mice of either genotype displayed normal morphology, with well-preserved brush border membranes and no loss of tubular epithelial cells. Kidneys from WT mice subjected to I/R showed marked tubular injury as reflected by loss of brush border, cast formation, and extensive loss of tubular epithelial cells, tubular dilation, and intra-tubular debris (Figure 2d). Kidneys from PAD4KO mice subjected to I/R also showed evidence of injury, but less than that seen in WT mice (Figure 2d). Semiquantitative assessment of kidney tissue injury yielded tubular necrosis scores of 0.07 ± 0.01 and 0.09 ± 0.04, respectively, in WT and PADK4KO mice that received sham surgery, and 3.4 ± 0.06 and 2.1 ± 0.4 ($P < 0.05$) in WT and PAD4KO mice 24 hours after I/R injury (Figure 2e). These data support the conclusion that PAD4 mediates renal I/R-induced injury.

PAD4 promotes inflammation after renal I/R injury

Infiltration of inflammatory cells into the renal parenchyma occurs early in the course of renal I/R injury.[10,27] Neutrophils are key effectors of the inflammatory cascade in a variety of kidney injury models, including I/R.[10,27] We therefore assessed the role of PAD4 in renal I/R-triggered inflammatory responses and neutrophil infiltration. As shown in Figure 3a, neutrophil elastase (NE)–positive neutrophils were hardly detectable in the kidneys of WT and PAD4KO mice subjected to sham surgery. I/R induced significant neutrophil infiltration in kidneys of WT mice, and to a lesser extent, in PAD4KO mice 24 hours after I/R ($P < 0.01$; Figure 3a). To test whether PAD4 deficiency affects neutrophil mobility, we performed a Boyden trans-well migration assay using 10% fetal bovine serum as a chemo-attractant. Supplementary Figure S1 shows that neutrophil migration rates were similar in WT and PAD4KO mice. In addition, no difference in total number of white blood cells in the peripheral blood was observed (Supplementary Figure S2A). Next, we tested whether PAD4 deficiency reduced the circulating platelet number, which could impair neutrophil infiltration after I/R injury.[19] Supplementary Figure S2B shows that total platelet count was similar in WT and PAD4KO mice.

To identify the role of PAD4 in pro- and anti-inflammatory cytokine production, we performed quantitative RT-PCR analyses 24 hours after renal I/R injury. Basal