

Role of Caspases in Hypoxia-Induced Necrosis of Rat Renal Proximal Tubules

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Abstract. The role of the caspases, a newly discovered group of cysteine proteases, was investigated in a model of hypoxia-induced necrotic injury of rat renal proximal tubules. An assay for caspases in freshly isolated rat proximal tubules was developed. There was a 40% increase in tubular caspase activity after 15 min of hypoxia in association with increased cell membrane damage as indicated by a threefold increase in lactate dehydrogenase release. The specific caspase inhibitor Z-Asp-2,6-dichlorobenzoyloxymethylketone (Z-D-DCB) attenuated the increase in caspase activity during 15 min of hypoxia and markedly decreased lactate dehydrogenase release in a dose-dependent manner. In the proximal tubules, Z-D-DCB also inhibited the hypoxia-induced increase in calpain activity, another cysteine protease. In contrast, when Z-D-DCB was added to purified calpain *in vitro*, there was no inhibition of calpain activity. The calpain inhibitor (2)-3-(4-iodophenyl)-

2-mercapto-2-propenoic acid (PD150606) also inhibited the hypoxia-induced increase in caspase activity in proximal tubules, but did not inhibit the activity of purified caspase 1 *in vitro*. In these experiments, caspase activity was detected with the fluorescence substrate Ac-Tyr-Val-Ala-Asp-7-amido-4-methyl coumarin (Ac-YVAD-AMC), which is preferentially cleaved by caspase 1. However, minimal caspase activity was detected with the fluorescence substrate Ac-Asp-Glu-Val-Asp-7-amido-4-methyl coumarin (Ac-DEVD-AMC), which is cleaved by caspases 2, 3, and 7. The present study in proximal tubules demonstrates that (1) caspase inhibition protects against necrotic injury by inhibition of hypoxia-induced caspase activity; and (2) caspase 1 may be the caspase involved. Thus, although the role of caspases in apoptotic cell death is well established, this study provides new evidence that caspases contribute to necrotic cell death as well.

The cysteine proteases consist of three major groups: cathepsins, calpains, and the newly discovered caspases. The cathepsins are lysosomal proteases that do not appear to play a role in lethal cell injury. Calpains, however, have been shown to be a mediator of hypoxic/ischemic injury to brain, liver, heart, and kidneys (1–6). Caspases play an important role in apoptosis (7,8), but may also be involved in necrotic cell death (9). Caspase inhibition has been demonstrated to reduce ischemic and excitotoxic neuronal damage (10–12). Moreover, mice deficient in interleukin-1 β -converting enzyme (ICE/caspase 1) (13,14) demonstrate reduced ischemic brain injury produced by occlusion of the middle cerebral artery (12). Inhibition of caspases also protects against necrotic cell death induced by the mitochondrial inhibitor antimycin A in PC12 cells, Hep G2 cells, and LLC-PK₁ cells (15,16). Rat kidneys subjected to ischemia demonstrate an increase in both caspase 1 and caspase 3 mRNA and protein expression (17). The role of caspases in hypoxia-induced injury in the rat renal proximal tubule, the major nephron site of injury with acute renal failure, has not been studied.

The caspases are a family of intracellular proteases that share a predilection for cleavage of their substrates after an aspartate residue at P1 (7,18). There are 11 members of the caspase family, caspases 1 to 11. Once activated, caspases can process other caspases in a cascade sequence (19). Caspases participate in two distinct signaling pathways: processing of proinflammatory cytokines and promotion of apoptotic cell death (19,20).

The signaling pathway of caspases during renal proximal tubular injury is not known. Because calpain is a mediator of hypoxic injury in freshly isolated rat proximal tubules (5,6,21), it could be postulated that both calpain and caspases participate in a cascade mechanism of activation during proximal tubular injury. Another reason to suspect that both calpain and caspases may interact during proximal tubular injury is the following: As expected, monocytes from caspase 1 knockout mice do not process interleukin-1 β (IL-1 β), since this process is caspase-dependent (13,14). However, it was unexpectedly found that these monocytes from caspase 1 knockout mice also do not process interleukin-1 α (IL-1 α), (13), a calpain-dependent process (22,23). It has been suggested that the reason the caspase 1 knockout mice do not process IL-1 α is that there is an absence of ICE interaction with or activation of calpain in these mice (13). Thus, there may be an interaction between calpain and caspases during hypoxic injury in proximal tubules.

With this background, the aims of the present study were to determine: (1) whether hypoxia increases caspase activity in rat proximal tubules; (2) the effect of selective caspase inhibition

Received October 6, 1998. Accepted March 24, 1999.

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1046-6673/1009-1940

Journal of the American Society of Nephrology

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on proximal tubular injury; and (3) the relationship between caspases and the increase in calpain activity during hypoxia.

Materials and Methods

Reagents

Hepes, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), digitonin, hyaluronidase type III from sheep testes, *N*-succinyl-Leu-Tyr-7-amido-4-methyl coumarin (*N*-succinyl-Leu-Tyr-AMC), and DMSO were purchased from Sigma (St. Louis, MO). *N*-succinyl-Leu-Leu-Val-Tyr-AMC, and AMC were made by Peptide Institute (Osaka, Japan) and obtained from Peptides International (Louisville, KY). Ac-Tyr-Val-Ala-Asp-7-amido-4-methyl coumarin (Ac-YVAD-AMC), Ac-Asp-Glu-Val-Asp-7-amido-4-methyl coumarin (Ac-DEVD-AMC), Z-Asp-2,6-dichloro benzoyl oxymethylketone (Z-D-DCB), and recombinant IL-1 β (murine) were obtained from Bachem (King of Prussia, PA). Purified μ -calpain (from porcine erythrocytes), PD150606 [(2)-3-(4-iodophenyl)-2-mercapto-2-prope-noic acid], and acetyl-Leu-Leu-norleucinol (Calpain inhibitor 1) were purchased from Calbiochem (San Diego, CA). Collagenase type B (lot no. 0FAA133) was from Boehringer Mannheim (Indianapolis, IN). Fatty acid-free bovine albumin was from ICN Biochemical (Cleveland, OH), and interleukin-1 receptor antagonist (IL-1Ra) was provided by Dr. Vann Parker (Amgen, Boulder, CO). Recombinant human ICE (caspase 1) purified to homogeneity from *Escherichia coli* was obtained from Dr. Nancy Thornberry (Merck Research Laboratories, Rahway, NJ) (24).

Preparation of Tubules

Proximal tubules were isolated from kidney cortex of male Sprague Dawley rats (200 to 300 g body wt), using collagenase digestion and Percoll centrifugation (25). Six-milliliter aliquots of tubule suspension (approximately 1 to 2 mg/ml) were placed in siliconized 25-ml Erlenmeyer flasks for a "recovery" period, which included gassing with 95% O₂/5% CO₂ for 5 min on ice. Flasks were then capped with rubber stoppers and kept at room temperature for 5 min, then placed in a shaking water bath at 37°C for 10 min. To create hypoxia, the tubule suspension was regassed for 5 min with 95% N₂/5% CO₂ at a rate of 3 L/min (26). After regassing, the flasks were closed and kept in the shaking water bath for the period of hypoxia studied. At the end of the hypoxic period, 1 ml of tubule suspension was sampled for measurement of lactate dehydrogenase (LDH) and 2 ml for caspase activity and calpain activity.

Caspase Inhibition Studies

A stock solution of 50 mM of the inhibitor Z-D-DCB in 100% ethanol was freshly prepared before each experiment. After the recovery period, the tubules were preincubated with the inhibitor or vehicle (ethanol) for 10 min at 37°C in the shaking water bath before induction of hypoxia.

Measurement of LDH Release and Protein

LDH release was measured to evaluate cell damage, as described previously (25,27). The percentage of LDH released from tubules was calculated by determining the ratio of LDH in the supernatant compared to that in the lysed tubule pellet plus the supernatant. Tubule protein was measured by the Lowry method, using bovine serum albumin as standard (28).

In Vitro Calpain Assay

The calpain assay used in this study is based on that described by Sasaki *et al.* for purified porcine kidney calpain (29). *N*-succinyl-Leu-Leu-Val-Tyr-AMC was used as a susceptible substrate for calpain (29). A stock solution of 10 mM was prepared in DMSO and stored at -20°C between use. The preparation of cytosolic extracts from proximal tubules and the calpain assay in freshly isolated proximal tubules has recently been described in detail by us (4–6).

Briefly, the calpain assay was performed as follows. After exposure to normoxia or hypoxia, the tubule pellet was separated from 2 ml of tubule suspension by centrifugation. The imidazole-HCl buffer used contained (in mM): 63.2 imidazole, 10 mercaptoethanol (pH 7.3). The tubule pellet was immediately resuspended in a calcium-free imidazole-HCl buffer containing in addition 1 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM ethyleneglycol-bis(β -aminoethyl ether)-*N,N'*-tetra-acetic acid (pH 7.3). The suspension was then incubated with digitonin (10 μ M) at 37°C in a shaking water bath for 5 min. Digitonin (10 μ M) selectively permeabilizes the plasma membrane but does not destroy lysosomal or mitochondrial membranes of hepatocytes (30) or mitochondrial membranes of rat proximal tubules (31). After incubation with digitonin, the tubule pellet and supernatant containing released calpain were again separated by centrifugation. Calpain activity in the tubule pellet after digitonin incubation was zero, confirming that all of the calpain had been released into the supernatant. The calpain assay was then performed on this supernatant as follows: 0.5 ml supernatant (0.5 to 1 mg tubule protein) and 10 μ l of the substrate *N*-succinyl-Leu-Leu-Val-Tyr-AMC (final concentration, 50 μ M) was added to the imidazole buffer with or without 5 mM CaCl₂. The total volume of the assay solution was made up to 2 ml with the imidazole buffer. The solution was incubated for 30 min at 37°C in a shaking water bath. In the assay performed without CaCl₂, the imidazole-HCl buffer containing 1 mM EDTA and 10 mM ethyleneglycol-bis(β -aminoethyl ether)-*N,N'*-tetra-acetic acid was used. After the 30 min incubation, fluorescence at 380 nm excitation and 460 nm emission was determined using a Hitachi F2000 spectrophotometer. Calpain activity was determined as the difference between the calcium-dependent fluorescence and the non-calcium-dependent fluorescence. An AMC standard curve was determined for each experiment. Calpain activity was expressed in pmol AMC released per minute of incubation time per mg of tubule protein.

Rat Lung Extract

Partially purified *m*-calpain controls were obtained from normal nonperfused rat lung tissue by a technique modified from Waxman and Krebs (32).

Caspase Assay

The caspase assay developed in rat proximal tubules is based on the biochemical characteristics of caspases (33) and the caspase assay in apoptotic cerebellar granule neurons described by Nath *et al.* (34). Ac-YVAD-AMC and Ac-DEVD-AMC were used as susceptible substrates for caspases (34). A stock solution of 10 mM was prepared in DMSO and stored at -20°C between use.

Proximal tubule extracts for the caspase assay were prepared as follows. After exposure to normoxia or to hypoxia, the tubule pellet was separated from 2 ml of tubule suspension by centrifugation. The tubule pellet was mixed with 1 ml of lysis buffer on ice for 10 min. The lysis buffer used contained 20 mM Hepes, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, and 1% Triton X-100, pH 7.2. The lysate was then centrifuged at 5000 \times g for 10 min. The supernatant was mixed with an equal quantity of 50%

glycerol. The caspase assay was then performed on this supernatant as follows: 200 μ l of supernatant (200 to 400 μ g protein) and 10 μ l of the substrate (final concentration, 50 μ M) were added to the caspase assay buffer with and without 100 μ M of the caspase inhibitor Z-D-DCB. The total volume of the assay solution was adjusted to 2 ml using the assay buffer. The assay buffer contained 100 mM Hepes, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 10% sucrose, 0.1% CHAPS, and 10% glycerol, pH 7.4. The solution was incubated for 30 min at 37°C in a shaking water bath. After the 30-min incubation, fluorescence at 380 nm excitation and 460 nm emission was determined using a Hitachi F2000 spectrophotometer. Both total fluorescence and Z-D-DCB-inhibitable fluorescence were determined. Caspase activity was determined as the Z-D-DCB-inhibitable fluorescence. An AMC standard curve was determined for each experiment. Caspase activity was expressed in pmol AMC released per minute of incubation time per mg of lysate protein. Lysate protein was measured by the Bradford method as described in the Bio-Rad protein assay kit with bovine serum albumin as standards.

The caspase selectivity of the assay was determined by the following:

1. Purified human recombinant ICE (Caspase 1) (14 to 228 ng) (24) was added to the caspase assay using the substrate Ac-YVAD-AMC. There was a linear dose-dependent increase in caspase activity from 2.3 pmol AMC/min for 14 ng ICE to 28 pmol AMC/min for 228 ng recombinant ICE. Z-D-DCB (100 μ M) added to the assay resulted in a 95% decrease in the caspase activity of the higher dose of recombinant ICE.
2. Purified μ -calpain (10 to 40 μ g) and rat lung extract (260 to 520 μ g), which is rich in *m*-calpain, were added to the caspase substrate Ac-YVAD-AMC in the presence of 5 mM CaCl₂ to activate the purified calpain (*n* = 3). There was no increase in fluorescence activity of the Ac-YVAD-AMC, indicating that calpain could not cleave the caspase substrate.
3. Caspase activity in the assay was defined as activity that was inhibited by the specific caspase inhibitor Z-D-DCB. In this regard, it has been demonstrated that Z-D-DCB has no effect on cathepsin activity *in vitro* (33).
4. In proximal tubules in culture, the activity of caspases measured with the substrate Ac-YVAD-AMC is not inhibited by inhibitors of serine proteases (phenylmethylsulfonylfluoride, aprotinin), aspartic proteases (pepstatin A), metalloprotease (EDTA, 1,10-phenanthroline), and cysteine proteases (E-64-d, leupeptin) (15).

Statistical Analyses

Multiple group comparisons were done using ANOVA with post-test according to Newman-Keuls. A *P* value <0.05 was considered statistically significant. Values are expressed as means \pm SEM.

Results

Mechanism of Cell Death

The presence of necrosis rather than apoptosis during 5 to 30 min of hypoxia in this model of freshly isolated rat proximal tubules has been demonstrated (35). In this study, there was endonuclease activation without the morphologic features of apoptosis.

The following study was performed to confirm that the mechanism of cell death is necrosis rather than apoptosis. Freshly isolated rat proximal tubules were exposed to 15 min of hypoxia. The proximal tubules were then stained with the DNA-specific dyes Hoechst 33342 and propidium iodide (PI).

Hoechst 33342 is cell membrane-permeable. PI is membrane-impermeable and stains nuclei only in cells with a damaged cell membrane (31). Individual proximal tubules were visualized with video imaging fluorescence microscopy as we have described previously (36). Normoxic control tubules showed a normal structure of nuclei stained mostly by Hoechst 33342 (Figure 1A). These normoxic tubules effectively excluded PI thereby demonstrating maintenance of membrane integrity. In contrast, the hypoxic tubules displayed extensive necrosis characterized by loss of membrane integrity and PI staining (Figure 1B). The nuclei of these tubules were still normal with light staining in the center. Proximal tubules treated with 0.1 mM hydrogen peroxide for 1 h were used as a positive control for apoptosis. These tubules excluded PI, but demonstrated apoptotic bodies (arrow) and highly condensed nuclei with Hoechst stain (Figure 1C). These studies demonstrate that proximal tubules exposed to 15 min of hypoxia undergo necrotic rather than apoptotic cell death.

Caspase Activity and LDH Release after 15 Min of Hypoxia

Caspase activity was 10.7 ± 1.5 pmol/min per mg in normoxic controls and increased significantly to 17.7 ± 1.5 pmol/min per mg after 15 min of hypoxia (*P* < 0.01 *versus* hypoxia, *n* = 5). There was a dose-dependent decrease in caspase activity with the caspase inhibitor Z-D-DCB. Caspase activity was 15.5 ± 2.1 with 50 μ M Z-D-DCB (NS *versus* hypoxia, *n* = 5), 12.7 ± 2.5 with 200 μ M Z-D-DCB (*P* < 0.01 *versus* hypoxia, *n* = 5), and 9.6 ± 0.8 with 500 μ M Z-D-DCB (*P* < 0.01 *versus* hypoxia, *n* = 5) (Figure 2). This dose-dependent decrease in hypoxia-induced caspase activity with Z-D-DCB was accompanied by a dose-dependent decrease in cell membrane damage as assessed by LDH release. LDH release was $12 \pm 1\%$ in normoxic controls, $38 \pm 2\%$ after 15 min of hypoxia (*P* < 0.001 *versus* hypoxia, *n* = 7), $36 \pm 2\%$ with 50 μ M Z-D-DCB (NS *versus* hypoxia, *n* = 7), $29 \pm 2\%$ with 200 μ M Z-D-DCB (*P* < 0.01 *versus* hypoxia, *n* = 7), and $21 \pm 2\%$ with 500 μ M Z-D-DCB (*P* < 0.001 *versus* hypoxia, *n* = 7) (Figure 2).

Caspase Activity after 25 Min of Hypoxia

Caspase activity was 12.6 ± 3.5 pmol/min per mg in normoxic controls and increased significantly to 22.9 ± 4.3 pmol/min per mg after 25 min of hypoxia (*P* < 0.01 *versus* hypoxia, *n* = 4). These data demonstrate that caspase activity remains elevated after a longer period of hypoxia.

Calpain Activity in Normoxic, Hypoxic, and Z-D-DCB-Treated Proximal Tubules

It is known that calpain activity is increased during hypoxic injury in rat proximal tubules (4–6,37,38). The caspase inhibitor Z-D-DCB inhibited the hypoxia-induced increase in calpain activity in a dose-dependent manner. Calpain activity in proximal tubules was 24 ± 12 pmol/min per mg in normoxic controls, 73 ± 16 pmol/min per mg after 15 min of hypoxia (*P* < 0.001 *versus* hypoxia, *n* = 4), 67 ± 16 pmol/min per mg

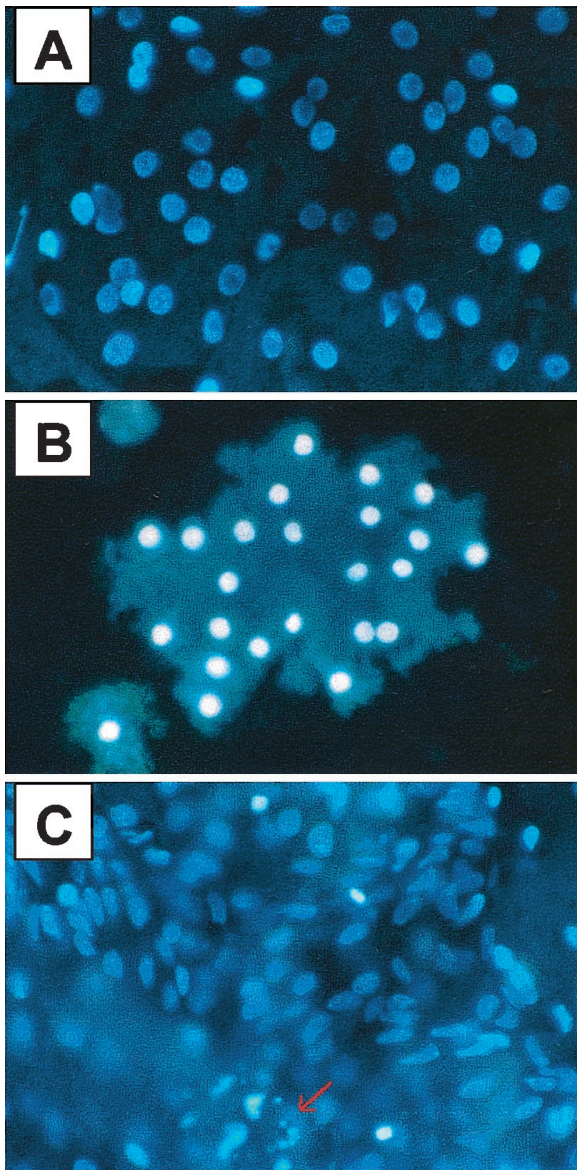


Figure 1. Freshly isolated rat proximal tubules exposed to 15 min of hypoxia undergo necrotic rather than apoptotic cell death. Proximal tubules were exposed to 15 min of hypoxia. The proximal tubules were then stained with the DNA-specific dyes Hoechst 33342 and propidium iodide (PI). Individual proximal tubules were visualized with video imaging fluorescence microscopy. Normoxic control tubules showed a normal structure of nuclei stained mostly by Hoechst 33342 (A). These normoxic tubules effectively excluded PI thereby demonstrating maintenance of membrane integrity. Hypoxic tubules displayed extensive necrosis characterized by loss of membrane integrity and PI staining (B). The nuclei of these tubules were still normal with light staining in the center. Proximal tubules treated with 0.1 mM hydrogen peroxide for 1 h were used as a positive control for apoptosis. These tubules excluded PI, but demonstrated apoptotic bodies (arrow) and highly condensed nuclei with Hoechst stain (C).

with 50 μM Z-D-DCB (NS versus hypoxia, $n = 4$), 14 ± 10 pmol/min per mg with 200 μM Z-D-DCB ($P < 0.001$ versus hypoxia, $n = 4$), and 4 ± 3 pmol/min per mg with 500 μM Z-D-DCB ($P < 0.001$ versus hypoxia, $n = 4$) (Figure 3).

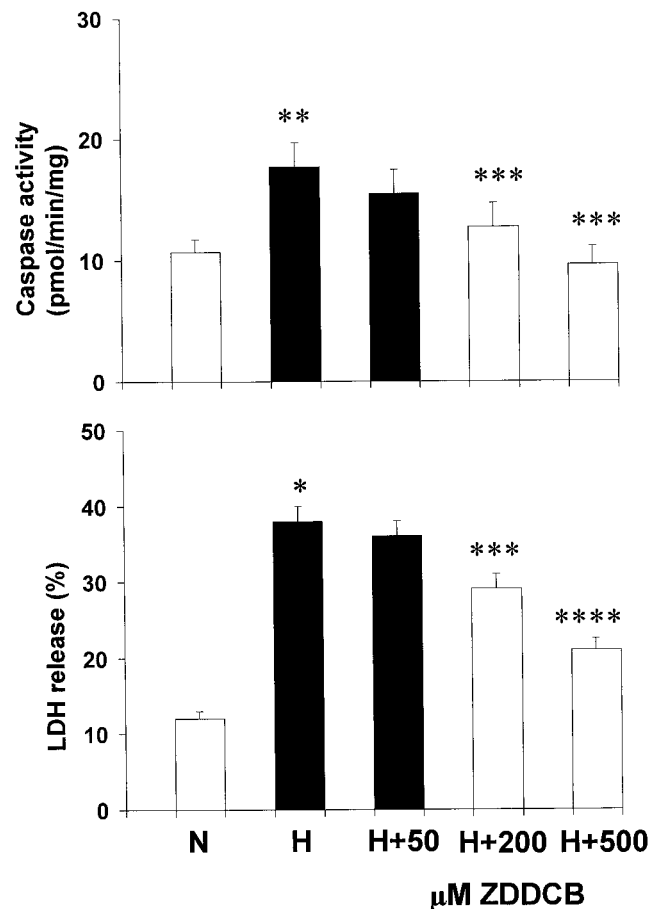


Figure 2. Effect of the specific caspase inhibitor Z-Asp-2,6-dichlorobenzoyloxymethylketone (Z-D-DCB) on caspase activity (top panel) and lactate dehydrogenase (LDH) release (bottom panel) after 15 min in normoxic (N) and hypoxic (H) proximal tubules. Proximal tubules were preincubated with the caspase inhibitor Z-D-DCB for 10 min before being exposed to hypoxia (95% N_2 /5% CO_2). After hypoxia, tubules were sampled for determination of both LDH release and caspase activity. Caspase activity was determined using the fluorescent substrate Ac-Tyr-Val-Ala-Asp-7-amido-4-methyl coumarin (Ac-YVAD-AMC), as described in Materials and Methods. * $P < 0.001$ versus normoxia; ** $P < 0.01$ versus normoxia; *** $P < 0.01$ versus hypoxia; **** $P < 0.001$ versus hypoxia ($n = 5$).

Effect of Caspase Inhibitor Z-D-DCB on Calpain Activity *in Vitro*

To ascertain whether Z-D-DCB directly inhibits calpain activity *in vitro*, we measured the *in vitro* activity of purified calpains in the absence and presence of Z-D-DCB and the calpain inhibitors PD150606 and calpain inhibitor 1. The substrate *N*-succinyl-Leu-Tyr-AMC, which we have found to be more sensitive than *N*-succinyl-Leu-Leu-Val-Tyr-AMC for detecting activity of purified calpains, was used. The calpain activity of purified calpains was not affected by 100 μM Z-D-DCB but was almost completely inhibited by the calpain inhibitors PD150606 and calpain inhibitor 1 (Figure 4). The calpain activity (pmol/min per mg) of purified μ -calpain (20 μg) was 329 ± 46 with no added Z-D-DCB and 283 ± 36 after addition of 100 μM of the caspase inhibitor Z-D-DCB (NS

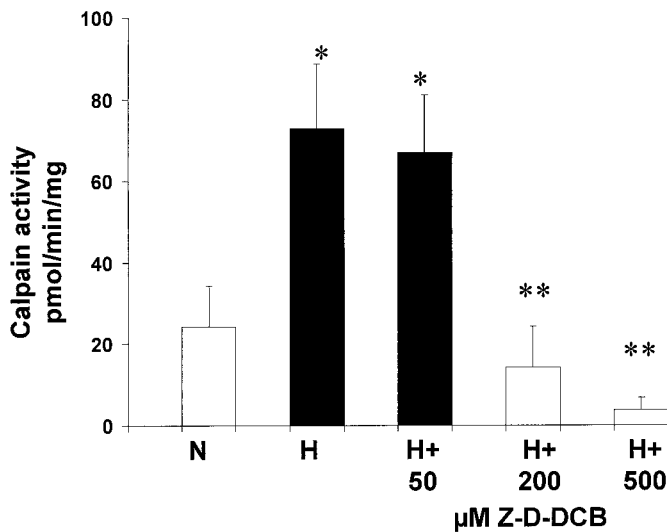


Figure 3. Effect of the specific caspase inhibitor Z-D-DCB on calpain activity after 15 min in normoxic (N) and hypoxic (H) proximal tubules. Proximal tubules were preincubated with the caspase inhibitor Z-D-DCB for 10 min before being exposed to hypoxia (95% N₂/5% CO₂). After hypoxia, tubules were sampled for determination of calpain activity using the fluorescent substrate *N*-succinyl-Leu-Leu-Val-Tyr-AMC, as described in Materials and Methods. **P* < 0.001 versus normoxia; ***P* < 0.001 versus hypoxia (*n* = 4).

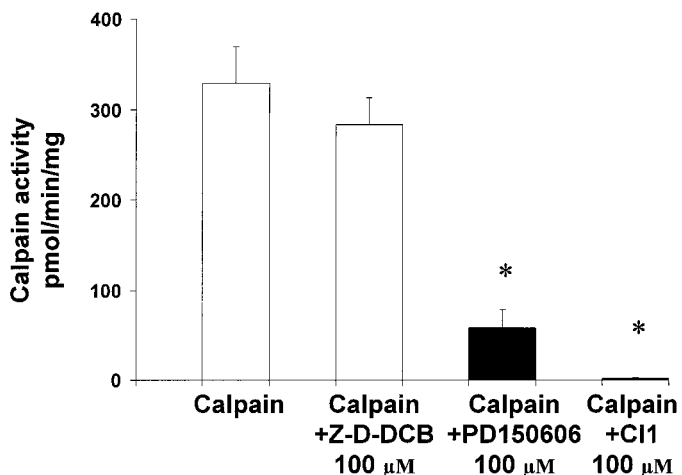


Figure 4. Effect of the specific caspase inhibitor Z-D-DCB and the specific calpain inhibitors PD150606 and calpain inhibitor 1 (CI1) on the activity of purified calpain *in vitro*. Purified μ -calpain (20 μ g) was preincubated with the inhibitors or the vehicle in the calpain assay buffer in the presence of 5 mM CaCl₂ for 10 min at 37°C. The fluorescent substrate *N*-succinyl-Leu-Tyr-AMC (final concentration, 50 μ M), was then added. After an additional 30-min incubation, calpain activity was determined as described in Materials and Methods. **P* < 0.001 versus purified calpain alone (*n* = 4).

versus purified calpain alone, *n* = 4). However, after addition of 100 μ M of the calpain inhibitor PD150606, calpain activity decreased to 58 \pm 29 (*P* < 0.001 versus purified calpain alone, *n* = 4) and was 0 after addition of 100 μ M of calpain inhibitor 1 (*P* < 0.001 versus purified calpain alone, *n* = 4) (Figure 4).

Effect of Calpain Inhibitor PD150606 on Caspase Activity in Proximal Tubules

The effect of calpain inhibition on hypoxia-induced caspase activity was determined. The calpain inhibitor PD150606 was used (39). We have demonstrated previously that PD150606 inhibits calpain activity and protects against hypoxic injury in proximal tubules (5). Caspase activity was 6 \pm 0.5 pmol/min per mg in normoxic controls and increased to 13 \pm 1.4 pmol/min per mg after 15 min of hypoxia (*P* < 0.01 versus normoxia, *n* = 5). There was a decrease in caspase activity with the calpain inhibitor PD150606. Caspase activity was 6.4 \pm 0.5 with 100 μ M of PD150606 (*P* < 0.01 versus hypoxia, *n* = 5) (Figure 5).

Effect of Calpain Inhibition on Caspase Activity in Vitro

To ascertain whether PD150606 directly inhibits caspase activity *in vitro*, we measured the *in vitro* activity of purified caspase 1 with Z-D-DCB and in the absence and presence of the calpain inhibitors PD150606 and calpain inhibitor 1. The caspase activity of purified caspase 1 was not affected by the calpain inhibitors PD150606 and calpain inhibitor 1, but was almost completely inhibited by the caspase inhibitor Z-D-DCB. The caspase activity (pmol/min per μ g) of purified caspase 1 (57 ng) was 390 \pm 10 with no added inhibitors, 415 \pm 55 after addition of 100 μ M of the calpain inhibitor PD150606 (NS versus purified caspase alone, *n* = 3), and 410 \pm 10 after addition of 100 μ M of calpain inhibitor 1 (NS versus purified caspase alone, *n* = 3). After addition of both 10 and 100 μ M of the caspase inhibitor Z-D-DCB, caspase activity was reduced to zero (*P* < 0.001 versus purified caspase alone, *n* = 3) (Figure 6).

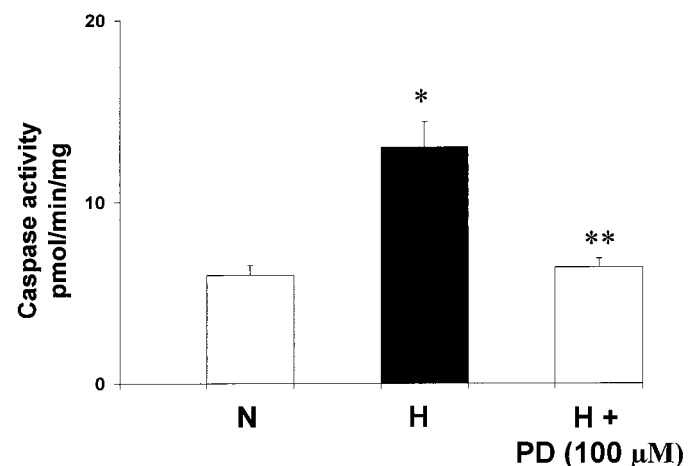


Figure 5. Effect of the specific calpain inhibitor PD150606 (PD) on caspase activity after 15 min in normoxic (N) and hypoxic (H) proximal tubules. Proximal tubules were preincubated with the calpain inhibitor PD150606 for 10 min before being exposed to hypoxia (95% N₂/5% CO₂). After hypoxia, tubules were sampled for determination of caspase activity using the fluorescent substrate Ac-YVAD-AMC, as described in Materials and Methods. **P* < 0.01 versus normoxia; ***P* < 0.01 versus hypoxia (*n* = 5).

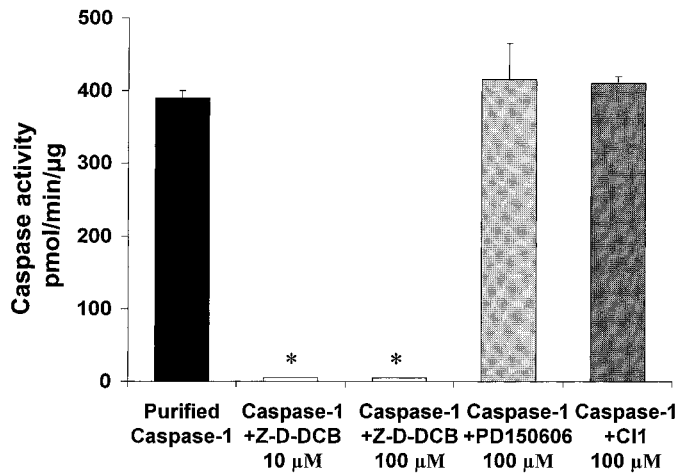


Figure 6. Effect of the calpain inhibitors PD150606 and calpain inhibitor 1 (C11) on the activity of purified caspase 1 *in vitro*. Purified caspase 1 (57 ng) was preincubated with the inhibitors or the vehicle for the inhibitors. The fluorescent substrate Ac-YVAD-AMC was then added. After an additional 30-min incubation, caspase activity was determined as described in Materials and Methods. The caspase inhibitor Z-D-DCB was used as a positive control. **P* < 0.001 versus purified caspase alone (*n* = 3).

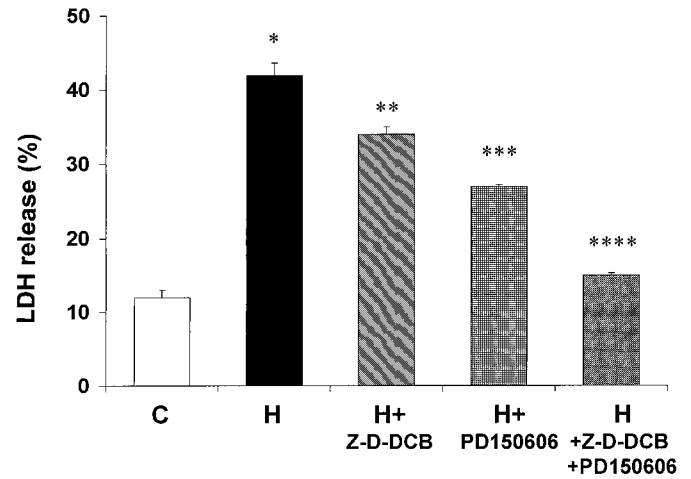


Figure 7. Effect of the combined caspase and calpain inhibition on LDH release after 15 min in normoxic (N) and hypoxic (H) proximal tubules. Proximal tubules were preincubated with either the caspase inhibitor Z-D-DCB (100 μM) or the calpain inhibitor PD150606 (100 μM), or a combination of both Z-D-DCB (100 μM) and PD150606 (100 μM) for 10 min before being exposed to hypoxia (95% N₂/5% CO₂). After hypoxia, tubules were sampled for determination of LDH release as described in Materials and Methods. **P* < 0.001 versus normoxia; ***P* < 0.05 versus hypoxia; ****P* < 0.01 versus hypoxia; *****P* < 0.001 versus hypoxia (*n* = 5).

Effect of Combined Caspase and Calpain Inhibition on Proximal Tubule Cell Membrane Damage

The effect of a combination of the calpain inhibitor PD150606 (100 μM) and the caspase inhibitor Z-D-DCB (100 μM) on cell membrane damage as assessed by LDH release was determined. A total of 100 μM of each inhibitor was used. In this regard, 200 μM of either inhibitor alone does not give complete protection against LDH release (Figure 2) (5). LDH release was 12.3 ± 1% in normoxic controls, 42 ± 4% after 15 min of hypoxia (*P* < 0.01 versus hypoxia, *n* = 4), 34 ± 3% with 100 μM Z-D-DCB (*P* < 0.05 versus hypoxia, *n* = 4), 27 ± 1% with 100 μM PD150606 (*P* < 0.01 versus hypoxia, *n* = 4), and 15 ± 2% with a combination of 100 μM Z-D-DCB and 100 μM PD150606 (*P* < 0.001 versus hypoxia, NS versus controls, *n* = 4) (Figure 7). Thus, the combination of caspase and calpain inhibition gave additive and complete protection against hypoxic proximal tubular injury.

Caspase Activity using a Different Substrate

The caspase assay was developed using the fluorescence substrate Ac-YVAD-AMC, which is preferentially cleaved by caspases 1, 4, and 5. The fluorescence substrate Ac-DEVD-AMC, which is cleaved by caspases 2, 3, and 7, was also used. Both total fluorescence and Z-D-DCB-inhibitable fluorescence (caspase activity) were measured in normoxic and hypoxic tubules with both substrates. Significant fluorescence activity was detected with Ac-YVAD-AMC but not with Ac-DEVD-AMC (Figure 8), suggesting that caspase 1 is involved in hypoxic injury.

A positive control for the substrate Ac-DEVD-AMC was performed. Thymus, which is rich in thymocytes that undergo caspase 3-mediated apoptosis (40), was prepared for the

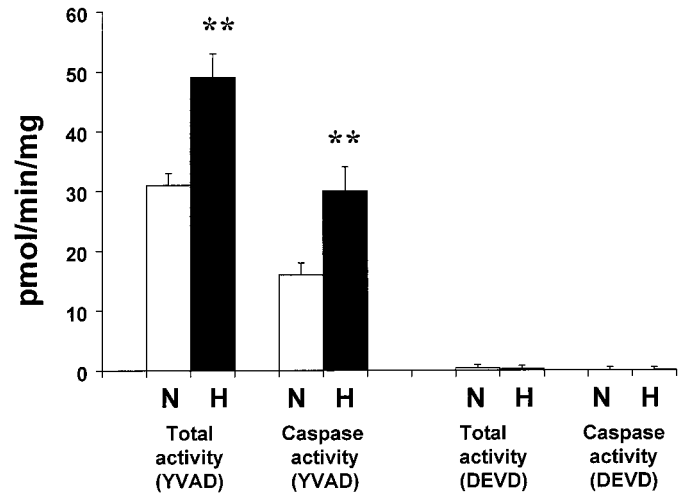


Figure 8. Caspase activity was measured in proximal tubules using the fluorescent substrate Ac-DEVD-AMC (DEVD) in addition to Ac-YVAD-AMC (YVAD). The caspase assay was performed as described in Materials and Methods. Both total fluorescence and Z-D-DCB-inhibitable fluorescence (caspase activity) was measured in normoxic (N) and hypoxic (H) tubules. The fluorescent substrate Ac-YVAD-AMC (YVAD) is preferentially cleaved by caspases 1, 4, and 5, and Ac-DEVD-AMC (DEVD) is cleaved by caspases 2, 3, and 7 and CED-3. ***P* < 0.01 versus normoxia (*n* = 4).

caspase assay in the same manner as the proximal tubules. The thymus extract was assayed using the substrates Ac-YVAD-AMC and Ac-DEVD-AMC. Significant caspase activity was only detected with Ac-DEVD-AMC, and minimal activity was detected with Ac-YVAD-AMC (Figure 9).

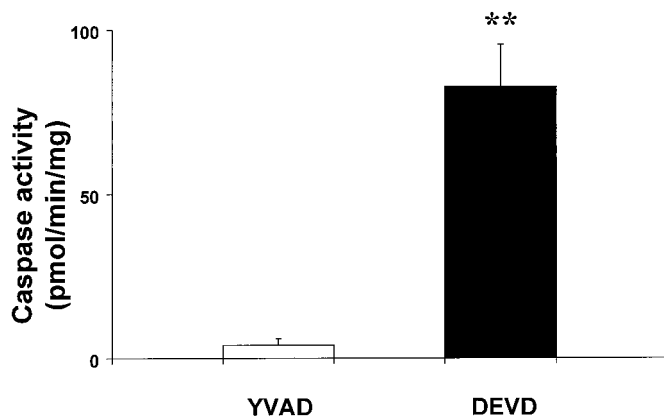


Figure 9. Caspase activity was measured in the rat thymus using the fluorescent substrates Ac-DEVD-AMC (DEVD) and Ac-YVAD-AMC (YVAD). Thymus, which is rich in thymocytes that undergo caspase 3-mediated apoptosis (40), was prepared for the caspase assay as described in Materials and Methods. Significant caspase activity was only detected with Ac-DEVD-AMC (which is cleaved by caspases 2, 3, and 7). Minimal caspase activity was detected with Ac-YVAD-AMC (which is cleaved by caspases 1, 4, and 5). * $P < 0.001$ versus YVAD ($n = 4$).

Effect of Interleukin-1 β on Hypoxic Injury

Pro-interleukin-1 β is cleaved at the aspartic acid-alanine position (amino acids 116 to 117) to mature IL-1 β by caspase 1 (ICE) (41). It is possible that caspase 1 (ICE) activation during hypoxic injury produces IL-1 β , which mediates the cell membrane damage. Thus, the effect of IL-1Ra on hypoxic injury was determined. The IL-1Ra had no protective effect on hypoxic injury. LDH release (%) was 14.5 ± 1 in normoxic controls, 36 ± 3 in hypoxia ($P < 0.01$ versus normoxia, $n = 3$), 34 ± 4 in hypoxic tubules preincubated with 20 $\mu\text{g/ml}$ IL-1Ra (NS versus hypoxia alone, $n = 3$), and 33 ± 1 in hypoxic tubules preincubated with 40 $\mu\text{g/ml}$ IL-1Ra (NS versus hypoxia alone, $n = 3$). To further determine whether IL-1 β mediates cell injury, the additive effect of recombinant IL-1 β on hypoxic injury was determined. A shorter time of hypoxia with less cell membrane damage was used so that the additive effect of recombinant IL-1 β on a mild degree of cell membrane damage could be determined. LDH release (%) was 10 ± 1 in normoxic controls, 19 ± 4 after 10 min of hypoxia ($P < 0.05$ versus normoxia, $n = 4$), and 18 ± 3 in hypoxic tubules preincubated for 10 min with 20 ng/ml recombinant IL-1 β (NS versus hypoxia alone, $n = 4$).

Discussion

There is considerable evidence for the role of caspases in apoptotic forms of cell death. The role of caspases in necrotic forms of cell death is less clear. In the present study, freshly isolated rat proximal tubules were used to investigate the role of caspases in renal hypoxic injury. When these tubules are exposed to short periods of hypoxia (up to 30 min), there is evidence of necrotic rather than apoptotic cell death. Using immunofluorescence microscopy with the DNA specific dyes Hoechst 33342 and PI, we confirmed that there are no mor-

phologic features of apoptosis (highly condensed and fragmented nuclei) during 15 min of hypoxia (Figure 1). The presence of necrosis rather than apoptosis during hypoxia in this model was confirmed in another study that demonstrated that there is endonuclease activation without morphologic features of apoptosis (35). The presence of necrosis rather than apoptosis may be related to the level of ATP depletion. In cultured mouse proximal tubules subjected to ATP depletion below 15% of control values, the cells died of necrosis, whereas in ATP depletion to 25 to 70% of control values all the cells died of apoptosis (42). In freshly isolated rat proximal tubules, we have demonstrated that ATP levels decrease to approximately 20% of normal during 15 min of hypoxic injury (25). Thus, these low ATP levels would also be expected to induce necrosis rather than apoptosis. It is not known whether caspases participate in this hypoxia-induced necrotic injury.

Interleukin-1 (IL-1) is an important mediator of the inflammation that occurs during reperfusion injury (41). In support of this, IL-1 is a chemoattractant that can recruit leukocytes to areas of inflammation during ischemic acute renal failure (43). IL-1 levels are increased in ischemic acute renal failure in the mouse (44). Pro-interleukin-1 β is cleaved at the aspartic acid-alanine position (amino acids 116 to 117) to mature IL-1 β by caspase 1 (ICE) (41). Thus, it is possible that the IL-1 β directly mediates the cell membrane damage. In this situation, the role of caspase 1 activation would be to generate increased IL-1. To exclude such an effect, the effect of IL-1Ra on hypoxic injury was determined. The IL-1Ra had no protective effect on hypoxic injury. To further determine whether IL-1 β mediates cell injury, the additive effect of recombinant IL-1 β on hypoxic injury was determined. Recombinant IL-1 β did not worsen the hypoxic injury. Thus, IL-1 β does not directly mediate hypoxia-induced proximal tubule injury. These data confirm studies, using an IL-1Ra *in vivo* and IL-1 receptor knockout mice, that IL-1 is not involved in the recovery of renal function after ischemia (43).

To further study the role of caspases in proximal tubule injury, an assay for caspase activity was developed in freshly isolated rat proximal tubules. The specific caspase substrate Ac-YVAD-AMC was used. The specificity of this substrate was confirmed by the demonstration that the cysteine protease calpain did not increase the fluorescence of Ac-YVAD-AMC. The specific aspartate-based caspase inhibitor Z-D-DCB was added to the assay to measure the Z-D-DCB-inhibitable activity during hypoxia (Figures 1 and 4) (45). Z-D-DCB inhibits both ICE-like and CPP32-like caspases (34,46) and can completely block apoptotic cell death of human myeloid leukemia cells (47). It is specific for caspases and does not inhibit the other cysteine proteases such as cathepsin B (45). We confirmed that Z-D-DCB also does not inhibit the cysteine protease calpain *in vitro* (Figure 4). Thus, to ensure the caspase specificity of our assay, caspase activity was expressed as the Z-D-DCB-inhibitable activity rather than total activity.

The caspase inhibitor Z-D-DCB attenuated the hypoxia-induced increase in caspase activity in proximal tubules and markedly protected against hypoxia-induced cell membrane damage, as assessed by LDH release, in a dose-dependent

manner. These results suggest that caspases play a role in hypoxia-induced cell membrane damage in the proximal tubule. We have demonstrated previously that another cysteine protease, calpain, is also a mediator of hypoxic injury in the same model (4–6). Since an interaction between caspases and calpain during cell injury has been suggested (13,34,48,49), we studied the effect of the specific caspase inhibitor Z-D-DCB on the hypoxia-induced increase in calpain activity. Z-D-DCB attenuated the hypoxia-induced increase in calpain activity in a dose-dependent manner. However, Z-D-DCB did not inhibit the activity of purified calpain *in vitro*.

Next, the effect of calpain inhibition on caspase activity was determined. The specific calpain inhibitor PD150606 (39) inhibits calpain activity and protects against hypoxic injury in rat proximal tubules (5). PD150606 attenuated the hypoxia-induced increase in caspase activity in proximal tubules. However, PD150606 did not inhibit the activity of purified caspase 1 *in vitro*.

These findings demonstrate the following: (1) the caspase inhibitor Z-D-DCB inhibits the hypoxia-induced increase in both caspase and calpain activity in proximal tubules; and (2) the calpain inhibitor PD150606 inhibits the hypoxia-induced increase in both calpain and caspase activity in proximal tubules. The specificity of the inhibitors was confirmed in *in vitro* experiments using purified calpain and caspase 1. Thus, these data suggest that both caspase-mediated activation of calpain and calpain-mediated activation of caspases occurs during hypoxic proximal tubule injury. This is not surprising, because cell death is known to involve the activation of proteolytic cascades involving different caspases and calpains (7,50). These proteolytic cascades are not yet well defined but are initiated after caspase and calpain activation (50). In fact, the simultaneous activation of both calpain and caspases during nerve cell death has been found (34,48,49). Also, a recent study demonstrated that calpain activation is upstream of caspases in radiation-induced apoptosis (51). However, another study demonstrated caspase-mediated fragmentation of the calpain inhibitor protein calpastatin during apoptosis, suggesting that caspases are upstream of calpain (52). Thus, it is possible that during hypoxic proximal tubule injury there are different proteolytic pathways involving different caspases and calpains. To further test this hypothesis, it was determined whether a combination of caspase and calpain inhibition would give additive protection against hypoxic injury. Additive protection would suggest that the different inhibitors may be inhibiting separate pathways of cell membrane damage. Figure 7 demonstrates the additive and complete protective effect of combined calpain and caspase inhibition against hypoxia-induced proximal tubule injury.

The members of the caspase family have an absolute requirement for an aspartate residue at P1 (7,53). The 11 members of the caspase family can be divided into three subfamilies based on substrate specificity at P2, P3, and P4 positions (19). The peptide preferences within each group are remarkably similar (54). Caspases 1, 4, and 5 prefer the tetrapeptide sequences WEHD and YVAD. In contrast, the optimal peptide sequence motif for caspases 2, 3, and 7 is DEVD (54,55).

Caspases 6, 8, 9, and 10 prefer the sequence (L/V)EXD. With this background, we determined the substrate specificity of the caspase in proximal tubules. Two substrates were used in our study: Ac-YVAD-AMC and Ac-DEVD-AMC. Caspase activity in proximal tubules was only detected with Ac-YVAD-AMC and not with Ac-DEVD-AMC (Figure 5). Thymus was used as a positive control for Ac-DEVD-AMC (Figure 6). These results strongly suggest that caspase 1 is involved in hypoxic injury in rat proximal tubules.

In summary, the present study in rat proximal tubules demonstrates that: (1) hypoxia increases caspase activity; (2) the specific caspase inhibitor Z-D-DCB attenuates the increase in caspase activity during 15 min of hypoxia and markedly protects against hypoxic injury; (3) Z-D-DCB also inhibits the hypoxia-induced increase in calpain activity in proximal tubules, but not *in vitro*; (4) the calpain inhibitor PD150606 inhibits the hypoxia-induced increase in caspase activity in proximal tubules, but not *in vitro*; and (5) based on preferential cleavage of the substrate Ac-YVAD-AMC, caspase 1 may be the caspase involved in hypoxia-induced proximal tubule injury. In conclusion, although most of the literature focuses on the role of caspases in apoptosis, this study provides new evidence that caspases contribute to necrotic cell death as well.

Acknowledgments

This research was supported by Grants DK35098 and DK52599 from National Institutes of Health. We thank Dr. Hong Ling for helping to perform the video imaging fluorescence microscopy.

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