Blockade of Calcium Influx through L-Type Calcium Channels Attenuates Mitochondrial Injury and Apoptosis in Hypoxic Renal Tubular Cells

TETSUHIRO TANAKA,* MASAOMI NANGAKU,* TOSHIO MIYATA,† REIKO INAGI, † TAKAMOTO OHSE,* JULIE R. INGELFINGER,‡ and TOSHIRO FUJITA*

*Division of Nephrology and Endocrinology, University of Tokyo School of Medicine, Tokyo, Japan; †Molecular and Cellular Nephrology, Institute of Medical Sciences and Department of Internal Medicine, Tokai University School of Medicine, Kanagawa, Japan; and ‡Division of Pediatric Nephrology, Massachusetts General Hospital, Boston, Massachusetts

Abstract. In hypoxia, ATP depletion causes cellular Ca\(^{2+}\) increase, mitochondrial injury, and apoptosis in renal tubular cells. However, the molecular basis of these observations is incompletely delineated. IRPTC, a rat renal proximal tubular cell line, was treated with antimycin A, and disturbances in cytoplasmic calcium ([Ca\(^{2+}\)]c) and mitochondrial calcium ion concentration ([Ca\(^{2+}\)]m), dissipation of mitochondrial membrane potential (\(\Delta\Psi_m\)), cytochrome c release, and resultant apoptosis were examined. Pharmacologic targeting of L-type Ca\(^{2+}\) channels in vitro and in vivo was used to clarify the involvement of voltage-dependent Ca\(^{2+}\) channels during this process. In vitro studies indicated that ATP depletion–induced apoptosis was preceded by increased [Ca\(^{2+}\)]c and [Ca\(^{2+}\)]m before activation of mitochondrial signaling. Antagonizing L-type Ca\(^{2+}\) channels offset these findings, suggesting [Ca\(^{2+}\)]c and [Ca\(^{2+}\)]m involvement. Azelnidipine administration ameliorated cellular and mitochondrial Ca\(^{2+}\) accumulation, mitochondrial permeability transition, cytochrome c release, caspase-9 activation, and resultant apoptosis (15.8 ± 0.8% versus 8.9 ± 0.7%; \(P < 0.01\)). Similar effects of azelnidipine were substantiated in an in vivo ischemia/reperfusion injury model. There were fewer terminal-deoxynucleotidyl transferase mediated dUTP nick-end labeling–positive cells in the azelnidipine-treated group (0.322 ± 0.038/tubule) as compared with the vehicle-treated group (0.450 ± 0.041; \(P < 0.05\)), although the antiapoptotic effect was smaller in vivo than in vitro, partly as a result of distinct levels of Bax expression. It is proposed that voltage-dependent Ca\(^{2+}\) channels are involved in cellular and mitochondrial accumulation of Ca\(^{2+}\) subsequent to ATP depletion and play an important role in regulating mitochondrial permeability transition, cytochrome c release, caspase activation, and apoptosis.

Hypoxia is a complex stress marked by interrelated cellular changes such as ATP depletion, generation of reactive oxygen species (ROS), and elevation of intracellular Ca\(^{2+}\). In ischemia-reperfusion (I/R) injury, an initial decrease in cellular ATP causes an increase in extracellular potassium and intracellular sodium (1). The resultant depolarization of plasma membranes leads to Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels. A steep rise in intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]c) is buffered to some degree by mitochondrial Ca\(^{2+}\) uptake. However, once a continuous increase in [Ca\(^{2+}\)]c exceeds the buffering capacity, these organelles become dysfunctional via opening of a nonspecific pore in the mitochondrial membrane, the permeability transition (PT) pore. Mitochondrial Ca\(^{2+}\) overload seems to be a consequence of the rise in the cytosolic Ca\(^{2+}\) concentration promoted by Ca\(^{2+}\) entry through plasma membrane receptor-operated and voltage-dependent Ca\(^{2+}\) channels. The mitochondrial dysfunction contributes to apoptotic cell death, and increasing evidence suggests the relative importance of apoptotic cell death in the pathogenesis of ischemic acute renal failure (2–4). However, the molecular mechanism by which this occurs has remained elusive. It has been shown that agents that suppress calcium influx or buffer [Ca\(^{2+}\)]c can prevent apoptosis in several conditions (5–10), making this possibility an attractive explanation for the observations of apoptotic cell death in I/R injury.

The mitochondrial PT is a crucial checkpoint in determining cell fate. The PT pore is putatively composed of the adenine nucleotide translocator in the inner mitochondrial membrane, cyclophilin-D in the matrix, and the voltage-dependent anion channel in the outer membrane (11–13). Once activated by stress such as Ca\(^{2+}\) overload, ROS generation, and high pH, the PT pore transiently opens and releases cytochrome c into the cytosol, which, together with apoptotic protease activating factor 1 and dATP, cleaves procaspase-9 into the
active form of caspase-9, thus conferring the apoptotic death signal (14).

BclII and Bax are two distinct members of the BclII family that regulates apoptosis. BclII is an antiapoptotic protein that stabilizes the mitochondrial membranes and inhibits cytochrome c release (15), whereas Bax is proapoptotic and translocates from the cytosol to mitochondria, homodimerizes, and forms a large pore with the voltage-dependent anion channel that allows the passage of certain proteins, including cytochrome c (16,17).

The present study aimed to address the role of calcium disturbance in hypoxia-mediated tubular cell apoptosis. We first focused on molecular mechanisms underlying an increase in \([Ca^{2+}]_{c}\), mitochondrial injury, and resultant apoptosis in vitro. We then attempted to intervene in the pathologic process by administering an L-type \(Ca^{2+}\) channel antagonist, azelnidipine. We last substantiated the role of an L-type \(Ca^{2+}\) channel antagonist in an in vivo model, I/R injury in the rat kidney, comparing the differential signaling of Bax in vitro and in vivo experiments.

### Materials and Methods

#### Chemicals and Reagents

Chemicals used in this study—antimycin A (5 mM stock in PBS), a specific complex III mitochondrial inhibitor; FPL64176 (2 mM stock in DMSO), an L-type \(Ca^{2+}\) channel agonist; valinomycin (2.5 mM stock in DMSO), the \(K^{+}\) ionophore; and carbonyl cyanide m-chlorophenylhydrazone (CCCP, 2 mM stock in DMSO), the protonophore—were purchased from Sigma (St. Louis, MO). Cyclosporine A (1 mM stock in ethanol) and ruthenium-red (1 mM stock in PBS), a blocker of the mitochondrial \(Ca^{2+}\) uniporter, was obtained from Wako chemicals (Osaka, Japan). Azelnidipine, an L-type \(Ca^{2+}\) channel antagonist, was provided by Sankyo Pharmaceutical Co. (Tokyo, Japan). In each experiment, the final concentration of the solvent (DMSO and/or ethanol) was <0.2%, below levels that affect experimental results.

#### Cell Culture

IRPTC (immortalized rat proximal tubular cells) is a cultured cell line established from proximal tubular cells of 4-wk-old male Wistar rats, immortalized by transformation with origin-defective SV40 DNA (18). Cells were grown in DMEM buffered with 25 mM HEPES, supplemented with 5% FBS (JRH Biosciences, Lenexa, KS).

Figure 1. Antimycin A treatment induces disturbance of intracellular \(Ca^{2+}\) and cell death in IRPTC. A temporal increase in \([Ca^{2+}]_{c}\) (A) and cell death expressed as LDH release (B) is shown. Antimycin A treatment (10 \(\mu\)M) for up to 4 h resulted in an increase of both parameters, reaching 320.6 ± 44.3 nM of \([Ca^{2+}]_{c}\) (versus 127.7 ± 18.1 nM in control; \(P < 0.01\)) and 34.2 ± 1.9% of dead cells (versus 3.6 ± 2.3% in control; \(P < 0.01\)), respectively. There was a linear correlation between \([Ca^{2+}]_{c}\) levels and the percentage of dead cells (\(R^2 = 0.90\); C). Cellular \(Ca^{2+}\) levels were measured using a fluorescence probe, Fura 2, as described in Materials and Methods (n = 4; *\*\(P < 0.01\) versus control). The LDH release assay was done in quadruplicate each, and the representative data of three independent experiments are shown.
Figure 2. Molecular evidence of apoptosis and mitochondrial signaling. To obtain molecular evidence of apoptotic cell death during antimycin A treatment, we checked the chronological death profile by annexin V binding assay. As the treatment period became longer, the more proportion of cells moved from lower left to lower right, then to upper right, suggesting a temporal transition of the mechanisms of apoptotic
and maintained at 37°C under humidified 5% CO₂/95% air. For ATP depletion, medium was changed to PBS that contained 1.5 mM CaCl₂, 2 mM MgCl₂, and 10 mM antimycin A, and cells were stimulated for up to 4 h at 70% confluence, as described previously (19). In some specific experiments, BclII-overexpressing clones were used. Stable transfectants carrying the rat BclII-expressing vector have been established and characterized previously in our laboratory (20) and were maintained at 200 μg/ml G418 (Sigma).

Detection of Dead/Apoptotic Cells

The proportion of dead cells was quantified chronologically by LDH assay (Wako). Cells in 24-well culture dishes were exposed to antimycin A for 0, 0.5, 1, 2, and 4 h. After this exposure, the supernatants were set aside and the adherent cells were lysed with 1% Triton X-100. By measuring the optic absorbance of supernatants and cell lysates at 560 nm, the percentage of LDH-release was calculated. For another measure of cell viability, we used 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reduction to formazan (Promega, Madison, WI). We followed the manufacturer’s instructions. The presence of apoptotic cells was confirmed both by an annexin V binding assay (Medical and Biological Laboratories, Nagoya, Japan) and by Hoechst 33258 staining. By double staining cells with annexin V-FITC and propidium iodide (PI) and analyzing them with flow cytometry (FACScan and LYSIS II software; Becton Dickinson, Franklin Lakes, NJ), the subsets of cells that were annexin V-positive, PI-negative (apoptotic) and annexin V-positive, PI-positive (necrotic and/or cells in advanced apoptosis) were determined. In Hoechst staining, cells that showed nuclear/cytoplasmic shrinkage and chromatin condensation were categorized as apoptotic.

Intracellular and Mitochondrial Ca²⁺ Measurement

Intracellular Ca²⁺ concentration was measured in IRPTC in suspension with the Ca²⁺-sensitive dye Fura 2 (21). Cells were collected and loaded with 2.5 μM Fura 2-AM (Dojin Chemical, Kumamoto, Japan) for 30 min. After loading, cells were washed and resuspended in the experimental solution, and [Ca²⁺]c was calculated according to Grynkiewicz et al. (22). The ratio of 500 nm fluorescence emission obtained with alternate excitation by 340- and 380-nm wavelengths (R) was monitored with a CAM-110 fluorophotometer (Nihon Koden, Tokyo, Japan). After each measurement, the cell suspension was lysed with Triton X-100 (final concentration, 0.1%), and the maximum value of R and the fluorescence intensity were obtained with alternate excitation by 340- and 380-nm wavelengths (representative data, annexin V assay; n = 2). Intracellular localization of cytochrome c is shown in B. Cytochrome c (green) translocated from mitochondria to the cytosol upon stimulation, which was confirmed by double staining with MitoTracker (red), a specific probe for mitochondria. In cells that were treated with antimycin A, cytochrome c extended from the perinuclear area to its periphery at 1 h, which was more prominent with longer hypoxic stimulation. The yellow color is a computer-designated co-localization of red (MitoTracker) and green (cytochrome c), which signifies that cytochrome c is indeed localized in mitochondria. Furthermore, Western blotting (C) showed that Bax translocated from the cytosol to mitochondria (top), whereas the total amount of Bax remained unchanged (bottom). Immunoblotting with anti-cytochrome oxidase (COX) antibody served as quality controls of purification of the mitochondrial fraction, and CBB staining confirmed equal loading and transfer (lanes 1 and 2, control; lanes 3 and 4, antimycin A treated [10 μM, 4 h] groups; samples in lanes 1 and 2 and lanes 3 and 4 were prepared separately; lane 5, cytosolic fraction of sample 1 [served as negative control for COX]). Magnification, ×600 in B.
streptavidin (Molecular Probes). Slides were observed with a microscope equipped with fluorescein filters (Olympus, Tokyo, Japan). Negative controls were served by omitting the incubation with the primary antibody.

**In Vivo Study**

The role of L-type Ca$^{2+}$ antagonist was substantiated further in rats that were subjected to I/R injury. Six-week-old male Wistar rats were divided into (1) sham, (2) I/R (vehicle-treated), and (3) I/R plus azelnidipine groups (n = 10 each). Azelnidipine was administered at 3 mg/kg per d by gastric gavage from day −3 until the end of the study. Rats were anesthetized with intraperitoneal ketamine (50 mg/kg) and maintained at 37°C during the operation. Laparotomy was performed for carrying out right nephrectomy and cross-clamping of the hilum of the left kidney for 45 min, after which the left kidney was reperfused. Twenty-four hours after I/R injury, rats again were anesthetized; blood samples were obtained; and the left kidney was removed, fixed in buffered formalin, and processed for histologic evaluation. Paraffin sections (3 μm) were stained with periodic acid-Schiff, and tubulointerstitial injury was assessed on the basis of semiquantitative morphologic changes such as tubular dilation, cast formation, sloughing of tubular epithelial cells, and thickening of the tubular basement membrane as follows: grade 1, <10% of tubules involved;
grade 2, <25%; grade 3, <50%; grade 4, <75%; and grade 5, ≥75%.

Twenty consecutive fields in the cortex were examined at ×400 magnification and averaged per specimen. Tubular cell apoptosis was quantified by counting the number of terminal-deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL)-positive proximal tubular cells in the outer stripe of the corticomedullary junction, the part of the kidney most susceptible to hypoxia. At least 80 to 100 tubules were counted per ×200 field and averaged for five consecutive fields. A commercially available kit (Trevigen, Gaithersburg, MD) was used. All quantification was done in a blinded manner. Immunohistochemistry for cytochrome c and Bax was performed using an indirect peroxidase method. Antigen retrieval was done at 121°C for 10 min, and the primary antibodies were used at 1:125 (cytochrome c) and 1:50 (Bax). In another set of experiments, release of cytochrome c from mitochondria to the cytosol was visualized by fluorescence double staining, with modifications of the previously described method (23).

Figure 4. Dissipation of mitochondrial membrane potentials ($\Psi_m$). Antimycin A–induced dissipation of $\Psi_m$ was measured by rhodamine123 uptake and analyzed with flow cytometry. Antimycin A induced a significant reduction in the uptake of rhodamine123, seen as the left-side shift of the fluorescence histogram. In the presence of ruthenium red (RuRed; 2 μM), a decrease in rhodamine123 uptake was attenuated, indicating that $\Delta\Psi_m$ is mediated by mitochondrial Ca$^{2+}$ influx. Similar amelioration of $\Psi_m$ fall was observed by pretreatment of azelnidipine (1 μM; 1 to 4). Dissipation of $\Psi_m$ was also attenuated by cyclosporine A (5), suggesting that the dissipation of $\Psi_m$ is closely linked to the permeability transition (PT). Last, zVAD-fmk, a pan-caspase inhibitor, had no impact on $\Psi_m$ dissipation subsequent to ATP depletion (6; representative data).
MitoTracker (assumed final concentration, 0.1 μM) was injected via tail veins 30 min before rats were killed, and the kidneys were snap-frozen in Cryomolds and subsequently sectioned (4 μm).

Blood urea nitrogen (BUN) levels were measured by the urease-indophenol method with Urea N B (Wako), serum creatinine levels were measured by Jaffe method (Wako), and the BP was monitored using an occlusive tail-cuff plethysmograph. BUN and BP were obtained at day 0 and day 1.

Statistical Analyses

Data are expressed as means ± SEM. All analyses were carried out using a StatView software (Ver. 5.0; SAS Institute, Cary, NC). The difference among groups was compared using unpaired t tests with the correction of Bonferroni/Dunn method. Nonparametric data were analyzed with the Kruskal-Wallis test, when appropriate. P < 0.05 was considered statistically significant.

Results

Antimycin A Treatment Induces Intracellular Ca$^{2+}$ Accumulation and Apoptosis in IRPTC through Mitochondrial Pathways

We first substantiated that ATP depletion resulted in disturbance of intracellular Ca$^{2+}$ and death in IRPTC in vitro. Figure 1, A and B, shows the time course of changes in [Ca$^{2+}$]c and the proportion of nonviable cells. Antimycin A treatment (10 μM) for up to 4 h induced a temporal increase in both [Ca$^{2+}$]c and cell death, reaching 320.6 ± 44.3 nM [Ca$^{2+}$]c (versus 127.7 ± 18.1 nM in control; P < 0.01) and 34.2 ± 1.9% of dead cells (versus 3.6 ± 2.3% in control; P < 0.01), respectively. There was a linear correlation between [Ca$^{2+}$]c levels and the percentage of dead cells, measured as LDH release ($R^2 = 0.90$; Figure 1C). Similar correlations were found between [Ca$^{2+}$]c levels and cell viability measured by MTS conversion to formazan ($R^2 = 0.85$).

To delineate whether cell death induced by antimycin A was from apoptosis or necrosis, we analyzed the time course with an annexin V assay (Figure 2). The longer the treatment with antimycin A, the more cells moved from lower left to lower right (signifying apoptosis), after which they moved to the upper right (necrosis and/or advanced stages of apoptosis). This pattern was blunted significantly in BclII-overexpressing clones (Figure 2A). Intracellular movement of cytochrome c is shown in Figure 2B. Cytochrome c changed in localization from perinuclear to diffuse, cytosolic areas by the treatment.

Figure 5. Alterations in mitochondrial Ca$^{2+}$ accumulation and oxidative stress. Pharmacologic intervention of azelnidipine to antimycin A–induced [Ca$^{2+}$]m rise and reactive oxygen species (ROS) generation is shown. Accumulation of mitochondrial Ca$^{2+}$ was measured with the fluorescence probe Rhod 2, and the mean fluorescence intensity was analyzed by flow cytometry (A). The relative fluorescence intensity was elevated to 138 ± 10% (P < 0.01) by antimycin A treatment, whereas pretreatment with azelnidipine (1 μM) reduced it to 115 ± 6% (P < 0.05 versus antimycin A group; A; n = 4; (***)P < 0.01 versus control; (##)P < 0.05 versus antimycin A group). Generation of ROS was estimated by 2',7'-dichlorodihydrofluorescein diacetate staining and flow cytometry (B). In contrast to [Ca$^{2+}$]m, ROS generated by antimycin A was essentially not altered by azelnidipine (1 μM). Pretreatment with ruthenium red (2 μM) also failed to attenuate the ROS levels, indicating that ROS produced in these conditions is largely independent of mitochondrial Ca$^{2+}$ accumulation (representative data; histogram 2 is displayed in a merged view with that of control, histograms 3 and 4 with that of the antimycin A group).
cells that were treated with antimycin A, cytochrome c started to extend from the perinuclear area to its periphery at as early as 1 h, which was more prominent with longer hypoxic stimulation. Double staining with MitoTracker confirmed the translocation of cytochrome c from mitochondria to the cytosol.

Furthermore, Western blotting (Figure 2C) showed that Bax increased in the mitochondrial fraction (mit. fr.), indicating that it had translocated from the cytosol to mitochondria (top), whereas the total amount of Bax (whole fr.) was unchanged (bottom). These lines of evidence confirmed that IRPTC death

**Table 1. General characteristics of each treatment group (in vivo I/R injury)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Before I/R</th>
<th>After Injury (Day 1)</th>
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<tbody>
<tr>
<td></td>
<td>Systolic BP (mmHg)</td>
<td>BUN (mg/dl)</td>
</tr>
<tr>
<td>Sham</td>
<td>120 ± 10</td>
<td>15.1 ± 5.2</td>
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<tr>
<td>I/R 45min (vehicle)</td>
<td>119 ± 9</td>
<td>79.7 ± 16.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>I/R + azelnidipine (3mg/kg)</td>
<td>104 ± 12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61.0 ± 17.5&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<sup>a</sup> I/R, ischemia/reperfusion; BUN, blood urea nitrogen; PTC, proximal tubular cells.

<sup>b</sup> <i>P < 0.01 versus</i> sham.

<sup>c</sup> <i>P < 0.05 versus</i> vehicle.
Figure 7. Azelnidipine treatment ameliorates tubular cell apoptosis in the ischemia/reperfusion (I/R) injury of the rat kidney. (A through C) Representative figures of terminal-deoxynucleotidyl transferase meditated dUTP nick-end labeling (TUNEL) staining. At day 1 after I/R injury, a significant number of TUNEL-positive tubular cells were seen in the outer layer of the corticomedullary junction and the outer cortex (B). In the azelnidipine-treated group, TUNEL-positive cells were sparse and restricted in the corticomedullary area (C). There was no positive
induced by antimycin A was mediated, in large part, by apoptosis via mitochondrial pathways.

**Disturbance of Intracellular Ca\(^{2+}\) and the Involvement of L-Type Ca\(^{2+}\) Channels**

Next we determined the relative contribution of L-type Ca\(^{2+}\) channels to the alteration of [Ca\(^{2+}\)]c during ATP depletion. Figure 3A shows the presence of the αc chain of L-type Ca\(^{2+}\) channels in IRPTC and the functional operation of this channel. By adding 50 mM potassium to the experimental solution to cause depolarization of plasma membranes and monitoring changes in Fura 2 fluorescence, a substantial rise in fluorescence intensity was observed, which was suppressed significantly by pretreatment with 1 μM azelnidipine. A second set of [Ca\(^{2+}\)]c tracing was obtained in the presence of the protonophore, 2 μM CCCP, to prevent Ca\(^{2+}\) uptake by mitochondria. In these conditions, addition of external potassium allowed us to observe a more rapid increase in [Ca\(^{2+}\)]c, which was facilitated in the presence of FPL 64176 (agonist) and blunted by the administration of azelnidipine (antagonist). Collectively, these results indicate that the voltage-dependent L-type Ca\(^{2+}\) channels operate in cultured proximal tubular cells and that mitochondria might be working to buffer Ca\(^{2+}\) influx from the external space.

We then measured changes in [Ca\(^{2+}\)]c in the presence and/or absence of azelnidipine and FPL64176 (Figure 3B). Pretreatment with azelnidipine significantly abrogated the increase in [Ca\(^{2+}\)]c subsequent to antimycin A treatment (144.6 ± 11.8 nM versus 320.6 ± 44.3 nM in control; P < 0.01), whereas preincubation with FPL64176 further accelerated the rise (440.9 ± 44.9 nM; P < 0.05 versus control). Importantly, virtually no changes in [Ca\(^{2+}\)]c were observed unless antimycin A was added. These results suggest that the Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels is induced by ATP depletion.

**Dissipation of ΔΨ\(_{m}\) and the Effect of Intervention on Ca\(^{2+}\) Flux**

ΔΨ\(_{m}\) were measured by flow cytometry (Figure 4). Antimycin A induced dissipation of Ψ\(_{m}\) as measured by rhodamine123 uptake (Figure 4, 1 and 2). In the presence of ruthenium red, decrease in rhodamine123 uptake was attenuated (Figure 4, 3), indicating that ΔΨ\(_{m}\) is partly mediated by mitochondrial Ca\(^{2+}\) influx and that Ca\(^{2+}\)-induced depolarizations are distal to mitochondrial Ca\(^{2+}\) uptake. Similar amelioration of Ψ\(_{m}\) disturbance was observed by preadministration of azelnidipine (Figure 4, 4). Importantly, Ψ\(_{m}\) fall was also attenuated by cyclosporine A (Figure 4, 5), a drug that targets cyclophilin-D in the mitochondrial matrix (5). Given that activation of the PT pore by [Ca\(^{2+}\)]m can be inhibited by cyclosporine A, we speculate that the calcium signal–induced loss of Ψ\(_{m}\) is due to the opening of the PT pore. Furthermore, zVAD-fmk, a pancaspase inhibitor (Figure 4, 6), failed to inhibit Ψ\(_{m}\) dissipation (6), suggesting that caspase activation is distal to the mitochondrial PT and is not required to facilitate the PT pore opening.

**Effect of L-Type Ca\(^{2+}\) Channels on Mitochondrial Calcium Accumulation and Oxidative Stress**

To investigate further the relationship between blockade of voltage-dependent Ca\(^{2+}\) channels and the inhibition of Ψ\(_{m}\) dissipation, we measured mitochondrial Ca\(^{2+}\) accumulation ([Ca\(^{2+}\)]\(_{m}\)) and cellular oxidative stress levels, two major factors that trigger mitochondrial PT and ΔΨ\(_{m}\), in the presence or absence of azelnidipine (Figure 5). The relative [Ca\(^{2+}\)]\(_{m}\) was elevated to 138 ± 10% (P < 0.01) by antimycin A treatment, whereas pretreatment with azelnidipine reduced it to 115 ± 6% (P < 0.05 versus antimycin A group; Figure 5A). It is deduced that blockade of L-type Ca\(^{2+}\) channels in the plasma membrane not only inhibits an increase in [Ca\(^{2+}\)]c but also stabilizes mitochondrial Ca\(^{2+}\) homeostasis. In contrast, ROS generated by antimycin A was not altered by azelnidipine (Figure 5B). Pretreatment with ruthenium red also failed to lessen the ROS levels, indicating that ROS produced here is largely independent of mitochondrial Ca\(^{2+}\) accumulation. Taken together, these results suggest that the positive effect of azelnidipine in stabilizing mitochondrial membranes is due to its ability in stabilizing [Ca\(^{2+}\)]\(_{m}\) rather than ROS generated during the process.

**Blockade of Ca\(^{2+}\) Influx through L-Type Ca\(^{2+}\) Channels Inhibits Cytochrome c Release, Caspase-9 Activation, and Apoptotic Cell Death.**

On the basis of the positive effect of azelnidipine in stabilizing Ψ\(_{m}\) through the suppression of [Ca\(^{2+}\)]\(_{m}\) rise, we examined the downstream event of mitochondrial signaling and subsequent apoptosis. Cytochrome c release was analyzed by Western blotting (Figure 6A). The amount of cytochrome c released upon stimulation seemed less in the azelnidipine-
treated group. Caspase-9 activity was measured and summarized in Figure 6C. Antimycin A increased the relative activity of caspase-9 by 293 ± 42%, whereas the addition of azelnidipine reduced it to 153 ± 10% (P < 0.01). The resultant apoptosis is summarized in Figure 6C. Hoechst 33258 staining revealed that antimycin A induced 15.8 ± 0.8% of apoptosis (versus 2.3 ± 0.3% in control; P < 0.01). This was ameliorated by azelnidipine treatment to 8.9 ± 0.7% (P < 0.01). Overall cell death measured as LDH-release was 20.0 ± 5.4%, reduced by 41.5% by pretreatment with azelnidipine (see Figure 1B). Thus, blocking Ca2+ influx through L-type Ca2+ channels seems to inhibit cytochrome c release, caspase-9 activation, and subsequent cell death, presumably through stabilization of Ψm and suppression of mitochondrial PT.

**In Vivo Study**

In view of the aforementioned evidence that azelnidipine inhibits hypoxia-mediated apoptotic cell death via stabilization of Ψm in vitro, we sought to determine whether the Ca2+ antagonist could suppress tubular cell apoptosis in an in vivo model of acute ischemic injury. General characteristics of each treatment group are shown in Table 1. Systolic BP was reduced significantly in the azelnidipine-treated group by ~15 mmHg (P < 0.01 versus sham and vehicle-treated groups). Twenty-four hours after I/R injury, the BUN level rose to 79.7 ± 16.8 mg/dl in the vehicle group, which was attenuated in the azelnidipine group to 61.0 ± 17.5 mg/dl (P < 0.05). Similar results were obtained by measuring serum creatinine levels (2.25 ± 0.56 mg/dl in the vehicle group versus 1.59 ± 0.32 mg/dl in the azelnidipine group; P < 0.05). Semiquantitative analysis of tubulointerstitial injury confirmed amelioration in damage by the administration of azelnidipine (2.3 ± 0.1 versus 2.7 ± 0.1 in the vehicle-treated group; P < 0.05).

Representative TUNEL staining is shown in Figure 7. A through C. At day 1, a significant number of TUNEL-positive tubular cells were seen mainly in the outer layer of the corticomedullary junction and, to a lesser extent, in the outer cortex. In the azelnidipine-treated group, however, the distribution of TUNEL-positive cells was restricted in the corticomedullary area. To provide supportive evidence that apoptosis was mediated in part via mitochondrial signaling in vivo, we stained serial sections for TUNEL (Figure 7D) and cytochrome c (Figure 7E). In contrast to TUNEL-negative viable cells characterized by a perinuclear, mitochondrial staining pattern for cytochrome c, TUNEL-positive apoptotic cells show a diffuse, cytosolic distribution of this molecule, reminiscent of the release of cytochrome c from mitochondria to the cytosol. Release of cytochrome c was substantiated further by double staining with MitoTracker (Figure 7F). In contrast to the colocalized distribution of cytochrome c with mitochondria in the sham-operated group, some dilated tubules in the I/R group showed the mostly irrelevant localization of cytochrome c and mitochondria. The quantitative data are shown in Figure 7G, which indicate that the number of TUNEL-positive proximal tubular cells in the outer corticomedullary junction was less in the azelnidipine group (0.322 ± 0.038) as compared with the vehicle-treated group (0.450 ± 0.041; P < 0.05).

In addition, Bax staining was more pronounced in some proximal tubular cells in the corticomedullary area after I/R injury (Figure 8A) and was not ameliorated by azelnidipine (deleted). The relative changes in Bax expression (whole lysates) in each group is shown in Figure 8B. Bax expression was increased in the I/R (vehicle-treated) group as compared with the sham-operated group, which was not altered in essence by azelnidipine (discussed later).

**Discussion**

The present study demonstrates that ATP depletion induces a rise in [Ca2+]c and [Ca2+]m and dissipation of Ψm, cytochrome c release and results in apoptosis. We have also shown that azelnidipine, an L-type Ca2+ channel antagonist, protects IRPTC from apoptosis partly by blunting an accumulation of mitochondrial calcium and the mitochondrial PT. Similar cytoprotective effects were also substantiated in in vivo I/R injury.

It was essentially important to show the functional operation of voltage-dependent Ca2+ channels first, because we dealt with nonexcitable, renal tubular cells throughout the study. In our experiments, depolarization of plasma membranes led to a substantial increase in [Ca2+]c, which was augmented or blunted by use of L-type Ca2+ channel agonists/antagonists. Pretreatment of cells with CCCP further accelerated the increase in [Ca2+]c, which supports the notion that mitochondria serve as the buffering system for Ca2+ overload through L-type Ca2+ channels. The presence of L-type Ca2+ channels in renal proximal tubular cells has been described elsewhere (24–26), and our findings are in agreement with theirs. One limitation that we need to heed is that channels that have activities that are independent of voltage sensitivity yet are dihydropyridine sensitive exist. For example, a parathyroid hormone–responsive, dihydropyridine-sensitive Ca2+ channel in renal distal tubules failed to uncover any voltage sensitivity (27). Although there is no denying officially the possible involvement of such voltage-independent channels in hypoxic proximal tubular cells, we can at least conclude that they are, in large part, dihydropyridine sensitive and treatable with L-type Ca2+ channel blockers.

Targets of Ca2+-mediated apoptotic signal could be various, including Ca2+-activated cytosolic proteins such as calpains, protein kinase C, and calcineurin. However, mitochondria are obvious candidates, given that key events such as ATP generation and the opening of the PT pore occurring in the mitochondrial matrix are regulated by variations of [Ca2+]m (9,28). The mitochondrial PT seems to constitute a crucial checkpoint that determines the release of cytochrome c and apoptosis. Major factors involved in this process are mitochondrial Ca2+ influx, generation of ROS, and pH shift. We primarily addressed the first two of these in this study.

Calcium mobilization can be categorized into three main classes: (1) Ca2+ release from internal stores, such as the endoplasmic reticulum (ER); (2) influx through voltage-dependent Ca2+ channels; (3) and receptor-mediated processes that exploit Ca2+ entry from the external milieu, albeit far more
diverse and complex given the central importance of calcium homeostasis in cell signaling. In the present study, we aimed to clarify the involvement of the voltage-dependent Ca\(^{2+}\) channels in mitochondrial injury subsequent to hypoxia. Our results suggest that the administration of L-type Ca\(^{2+}\) channel blockers offsets an increase in [Ca\(^{2+}\)]\(_c\) and [Ca\(^{2+}\)]\(_m\) and inhibits activation of its downstream cascade. We therefore propose that voltage-dependent Ca\(^{2+}\) channels are important in cellular and mitochondrial accumulation of Ca\(^{2+}\) subsequent to ATP depletion and likely play an important role in regulating the mitochondrial PT and apoptosis. However, it is possible that the other two Ca\(^{2+}\) mobilization pathways could be involved (29). For example, it is worth mentioning that the potential involvement of the ER, given the close proximity to mitochondria, and that local Ca\(^{2+}\) concentration at these sites can reach very high levels may be important (30). It is well known that hypoxia induces ER stress and that Ca\(^{2+}\) released from the ER affects the buffering function of mitochondria in a variety of cells (31,32). Furthermore, Ca\(^{2+}\) release from the ER activates caspase-12 through calpain activation and induces apoptosis independent of mitochondrial signaling (33,34), although there is, to our knowledge, no present evidence that those mechanisms are involved in renal hypoxic injury.

Blockade of electron transfer can lead to the increased reduction of ubiquinone and increased levels of partially reduced ubisemiquinone, some of which seems to be a major source of oxygen radicals by reacting directly with oxygen (35,36). ROS, along with Ca\(^{2+}\), is a major inducer of the mitochondrial PT. In our experiments, however, an antioxidative effect of azelnidipine was minimal, suggesting that ROS generated by antimycin A is irrelevant of cellular and mitochondrial Ca\(^{2+}\) disturbance, although a caution is required that ROS production by antimycin A occurs mainly at the ubiquinone region of complex III, not at complex I, the major pathophysiologically relevant mitochondrial ROS-generating site (37,38).

The cytoprotective effect of voltage-dependent Ca\(^{2+}\) channel antagonists was also verified in the rat model of ischemic acute renal failure. Our results indicated that azelnidipine was able to ameliorate I/R injury, as observed 24 h after ischemia with relative blunting of increases in BUN levels, tubulointerstitial injury, and the number of apoptotic tubular cells. The relative importance of intracellular Ca\(^{2+}\) accumulation in the I/R model has been previously suggested; a recent microarray analysis indicated that most of the induced transcripts after I/R were associated with cell structure, extracellular matrix, intracellular calcium binding, and cell division/differentiation (39).

In addition, mice heterozygous for knockout of the Na\(^+/\)Ca\(^{2+}\) exchanger have a decrease in observed renal injury after I/R as compared with wild-type counterparts, mainly as a result of decreased Ca\(^{2+}\) influx via Na\(^+/\)Ca\(^{2+}\) exchanger (40). The key finding in the present study is that the pharmacologic blockade of L-type Ca\(^{2+}\) channels also permits [Ca\(^{2+}\)]\(_c\) stability. On the basis of the general properties of Ca\(^{2+}\) channel blockers that

![Figure 8](image)

**Figure 8.** Upregulated Bax expression in *in vivo* tubular cells. Bax staining was performed in kidney sections (A). Contrary to *in vitro* results, the signal was obviously stronger in proximal tubular cells in the corticomedullary area, after I/R injury, which was not altered in essence by azelnidipine treatment (deleted). Arrows indicate the translocated Bax from the cytosol to mitochondria, upon stimulation (inset). The relative changes in Bax expression are also shown in Western blotting (B). Bax expression was stronger in the I/R (vehicle-treated) and azelnidipine-treard groups than in the sham-operated group, making an obvious contrast to the *in vitro* results (cf. Figure 2C, bottom). (A total of 100 µg of protein in the cortex was loaded in each lane. Lane 1, sham; lane 2, I/R 45 min (vehicle); lane 3, I/R + azelnidipine [3 mg/kg]). Magnification, ×400 in A.
the pharmacologic effects can be seen early and are overall well tolerated, the administration of Ca\(^{2+}\) channel blockers after renal ischemia might be expected to prove beneficial.

Unfortunately, however, the antiapoptotic effect of azelnidipine in vivo seemed partial as compared with in vitro experiments. To explain this disparity, we chose to perform immunostaining with Bax. In dying cells in the corticomedullary area of ischemic kidneys, not only did the Bax signal translocated to the perinuclear area (reminiscent of mitochondrial translocation), but also its intensity was apparently stronger than that in sham-operated controls. Azelnidipine did not improve this situation. Because the amount of Bax did not change after in vitro antimycin A treatment, the quantitative increase of Bax may partly explain the lesser antiapoptotic effect of azelnidipine in vivo as compared with in vitro. Transcriptional upregulation of Bax in a hypoxic ambiance has been observed previously both in vitro (20,41,42) and in vivo (43). Furthermore, Bax has been suggested to induce cytochrome c release through Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent mechanisms (41) or in a mitochondrial PT-dependent and PT-independent manner (35).

Findings in the present study may be potentially pertinent to the chronic tubulointerstitial injury observed in various forms of progressive renal diseases, because they are presumably linked to chronic hypoxia in the tubulointerstitium (44,45) and apoptosis is a major contributor to the pathologic progression (46). Indeed, the accumulation of mitochondrial Ca\(^{2+}\) has been observed in the rat remnant kidney model (47), and it can be assumed that the accumulation of mitochondrial Ca\(^{2+}\) contributes to the development of mitochondrial respiratory uncoupling, but the underlying mechanisms and their pathogenetic roles are still unclear and await further study.

In conclusion, we have shown evidence that administration of azelnidipine, a calcium antagonist, protects tubular cells from apoptosis subsequent to hypoxic injury, by stabilizing cellular and mitochondrial Ca\(^{2+}\) homeostasis and inhibiting the mitochondrial permeability transition, cytochrome c release, and the downstream cascade. Similar antiapoptotic effects of Ca\(^{2+}\) antagonists were also seen in in vivo acute ischemic injury, albeit smaller than in in vitro antimycin A treatment. The apparent disparity between them was partly due to the distinct expression levels of Bax. Further studies that focus on Ca\(^{2+}\) movement in a hypoxic milieu will lead us to new insight into the pathogenesis of ischemic renal diseases and, ultimately, allow us to develop novel antidotes against them, with future therapeutic perspectives.

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