Apoptosis Antagonizing Transcription Factor Protects Renal Tubule Cells against Oxidative Damage and Apoptosis Induced by Ischemia-Reperfusion

Jun Xie and Qing Guo
Department of Physiology, the University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma

Apgptosis is characterized by distinct morphologic and biochemical alterations of the cell, such as mitochondrial dysfunction, activation of caspases, chromatin condensation, and DNA fragmentation and has been implicated in ischemia/reperfusion-induced cell injury in many different organ systems, including the kidney (1–3). Ischemia typically damages renal tubular epithelial cells and also glomerular cells and is characterized by several hallmark features at the cellular level: Profound intracellular ATP depletion and a fall in tissue oxygen and glucose content with a concomitant rise in intracellular calcium (4–7). Although ischemic events alone may lead to necrosis and apoptosis in the kidney, reperfusion occurs upon restoration of blood flow and is associated with production of reactive oxygen species (ROS) and oxidative stress and apoptotic cell death. Ischemia/reperfusion-induced renal injury (renal IRI) is the most common cause of acute renal failure. It now is reported that AATF is expressed in human kidney proximal tubule (HK-2) cells and in mouse primary renal tubule epithelial cells. Levels of AATF expression were altered significantly in these cells in a well-established in vitro model of renal IRI. In transfected HK-2 cells, RNA interference–mediated silencing of AATF exacerbated whereas overexpression of the full-length AATF ameliorated mitochondrial dysfunction, accumulation of superoxide and peroxynitrite, lipid peroxidation, caspase-3 activation, and apoptotic death that were induced by IRI. In primary renal tubule epithelial cells, overexpression of AATF mediated by recombinant adeno-associated virus (AAV) vectors resulted in significant antiapoptotic activity, whereas knockdown of AATF by small interference RNA led to exacerbated cell death after IRI. These results identify AATF as a novel cytoprotective factor against oxidative and apoptotic damage in renal tubular cells. AATF may represent a potential candidate for therapeutic application in IRI.

to a complete blockade of the proapoptotic signaling that is mediated by Par-4 (19–21). We now report that AATF protects against oxidative and apoptotic damage in renal tubular cells and may represent a novel candidate for therapeutic application in renal IRI.

Materials and Methods

Culture and Transfection of Human Kidney Proximal Tubular Cells

These methods were similar to those described in our previous studies (19–21). In brief, the human kidney proximal tubular cell line HK-2 cells (American Type Culture Collection, Manassas, VA) were maintained at 37°C in an atmosphere of 95% air and 5% CO₂ in keratinocyte-serum free medium (Life Technologies/BRL, Grand Island, NY) with 5 ng/ml recombinant EGF and 0.05 mg/ml bovine pituitary extract (complete growth medium). A full-length rat AATF cDNA (17) was subcloned into the expression vector pREP4 (Invitrogen, Carlsbad, CA), yielding a recombinant construct pREP4-AATF that encodes the full-length AATF protein of approximately 70 kD. Human HK-2 cell lines that stably express AATF were established by transfection using Lipofectamine 2000 Reagent (Invitrogen) with pREP4-AATF. Transfected cells were selected with hygromycin (400 μg/ml) for 4 wk, and surviving clones were selected. For control purposes, parallel cultures of HK-2 cells were stably transfected with pREP4 vector alone.

Primary Cultures of Mouse Renal Tubule Epithelial Cells

Cultures of mouse primary renal tubule epithelial (PRTE) cells were established from the kidney cortex of 3- to 4-wk-old mice in a hormone-supplemented serum-free medium with negligible contamination of fibroblasts and mesangial and endothelial cells, as described previously (22,23). In brief, after being minced into 1-mm-diameter pieces, renal cortex was digested with 1 mg/ml type IV collagenase in the presence of 1 mg/ml soybean trypsin inhibitor in serum-free culture medium. After incubation at 37°C for 15 min, the renal fragments were washed by centrifugation. For obtaining single-cell suspensions, the renal fragments were treated with 1% trypsin-0.3% EDTA in PBS. Trypsin treatment was terminated with soybean trypsin inhibitor. Primary cells were expanded in DMEM (low glucose)/Ham’s F-12 (Invitrogen) medium, supplemented with 50 ng/ml insulin, 200 ng/ml hydrocortisone, 5 μg/ml apotransferrin, 1% penicillin, and 1% streptomycin.

Generation of Replication-Deficient, Recombinant AAV Particles and Transduction of PRTE Cells

AAV vectors have a high affinity for renal tubule epithelial cells and have been used extensively for high-efficiency gene transfer and expression in the kidney (24–27). Replication-deficient recombinant AAV particles that express full-length AATF cDNA (rAAV-AATF) were produced using the AAV Helper-Free System (Stratagene, La Jolla, CA), following the manufacturer’s instructions. Briefly, a cDNA encoding full-length rat AATF was subcloned into the pAAV-MCS vector using standard molecular cloning protocols. The AAV-MCS vector contains AAV-2 inverted terminal repeats, which direct viral replication and packaging. The recombinant expression plasmid was co-transfected into AAV-293 cells with pHelper and pAAV-RC (Stratagene). rAAV stocks in transfected cells were subjected to four rounds of freezing and thawing. After cell debris was removed by centrifugation, the stocks were filtered using a low-protein-binding 5-μm syringe filter (Millipore, Bedford, MA), followed by a 0.8-μm syringe filter and subsequently by heparin agarose column (Sigma, St. Louis, MO) purification. The viruses finally were concentrated with a 100K MWCO Amicon filter (Millipore) and titrated as described (28). For overexpression of AATF in primary cultures of renal tubular epithelial cells, cells were plated in 35-mm culture dishes at 2 × 10⁵ cells/dish. Subconfluent cultures were incubated with recombinant AAV-AATF particles (1 × 10⁴ viral particles/cell) for 48 h. Parallel cultures were transduced with empty AAV vector alone and used as controls.

Induction of IRI in Cell Culture

These methods combine a widely used and extensively characterized cell culture model of ischemia injury with an in vitro reperfusion...
protocol described previously for renal tubule cells (29,30). In brief, for induction of IRI in cell culture, HK-2 cells (approximately 80% confluent) were washed in glucose-free buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl₂, 1.0 mM MgCl₂, 3.6 mM NaHCO₃, and 5 mM HEPES [pH 7.2]) and then incubated with 10 mM antimycin A plus 10 mM 2-deoxyglucose plus 1 μM calcium ionophore (A23187) for 60 min (to induce ischemic injury in vitro). The in vitro reperfusion was achieved by incubating cells in glucose-replete complete growth medium.

Western Blot Analysis
Levels of expression of AATF were determined by Western blot analysis as described previously (19–21). The polyclonal antibody that specifically recognizes AATF was described previously, and it reacts with AATF of mouse, rat, and human origins and reveals a strong band at approximately 70 kD in whole-cell protein extracts (20). For examination of caspase-3 activation, the protein samples were probed with a rabbit polyclonal antibody that is specific for cleaved caspase-3 (9661; Abcam, Cambridge, MA) and visualized by chemiluminescence (ECL kit, GE Healthcare, Pittsburgh, PA). The uncropped gel is shown as Figure 2e.
Figure 3. Specific knockdown of AATF by RNA interference (RNAi) significantly increases vulnerability of human kidney proximal tubule cells to IRI: Effects on caspase-3 activation and apoptosis. (a) Representative Western blot analysis showing specific knockdown of AATF expression by small interference RNA (siRNA) cocktail targeted against AATF in HK-2 cells. (Top) Cultures of HK-2 cells were either mock transfected (control) or transfected with siRNA against AATF. A validated siRNA against green fluorescence protein (GFP) was used as a negative control. Forty-eight hours after siRNA transfection, cells were subjected to IRI for 8 h. The cells then were processed for AATF immunoreactivity by Western blotting. Note that the induction of AATF
Cell Signaling Technology, Beverly, MA), and Western blotting was performed by following the manufacturer’s instructions. This antibody detects endogenous levels of the large fragment (17/19 kD) of activated caspase-3 that results from cleavage adjacent to Asp175 but does not recognize full-length caspase-3 or other cleaved caspases. Equal loading was verified by probing the blots with the monoclonal anti-β-actin or anti-β-tubulin antibody (Sigma Aldrich). Western blot images were acquired and quantified using Kodak Image Station 2000R and Kodak Digital Science 1D 3.6. software (Eastman Kodak, Rochester, NY).

**AATF Knockdown by RNA Interference and Detection of Apoptosis**

The methods for silencing gene expression by RNA interference (RNAi) were described in our previous studies (21). In brief, the small interference RNA (siRNA) that were targeted against human AATF were generated by *in vitro* transcription using the Silencer siRNA Cocktail Kit (Ambion, Austin, TX), following the manufacturer’s instructions. 17 promoter sequences were added to DNA template (a cDNA fragment that contains nucleotides 1161 to 1639 of the human AATF coding sequence) by PCR using the following primers: Forward 5′-taatacgactcactatagggtactctttgaacgctcaat-3′ and reverse 5′-taatacgactcactatagggtactctttgaacgctcaat-3′. The transcription reaction was assembled, and the resulting complementary RNA was annealed for maximum duplex yield. Double-strand RNA were purified, and siRNA cocktail was obtained by RNase III digestion. siRNA that were targeted against mouse AATF were generated using similar strategies, except that a cDNA fragment that contained nucleotides 1058 to 1537 of the mouse AATF coding sequence was used as DNA template (31). Cells were transfected with siRNA cocktails at a concentration of 100 nM using TransMessenger Transfection Reagent (Qiagen, Valencia, CA). Using this method, an average of approximately 70 to 80% siRNA transfection efficiency was achieved in a variety of cells studied (21). A nonsilencing siRNA and a validated siRNA against green fluorescence protein (GFP, Qiagen) were used as a negative control. Quantification of apoptosis using fluorescence microscopy was described previously (19) and involves the staining of cells with the fluorescence DNA-binding dye Hoechst 33342. Cells that showed nuclear chromatin condensation and fragmentation are counted as apoptotic cells.

Assays of Mitochondrial Transmembrane Potential, Oxidative Stress, and Caspase Activation by Confocal Laser Scanning Microscopy

The loss of mitochondrial membrane potential is a hallmark for apoptosis. The dyes JC-1 (5,5′,6,6′-tetrachloro-1,1′,3′,3′′ tetraethylbenzimidazolylcarbocyanine iodide) and rhodamine 123 (Rhd123; Molecular Probes, Eugene, OR) were used to measure mitochondrial transmembrane potential, as described previously (21,32,33). In healthy cells, the intact mitochondrial membrane potential allows JC-1 to enter the mitochondrial matrix and stains the mitochondria bright red (32). In apoptotic cells, the mitochondrial membrane potential collapses, and JC-1 remains in the cytoplasm in a green fluorescence monomeric form. For JC-1 staining, cells were plated into a 96-well plate. At designated times after IRI, cells were washed with PBS and incubated for 30 min at 37°C in the presence of 10 μM JC-1. Cells were then washed in PBS. Cellular red fluorescence (excitation 550 nm, emission 600 nm) and green fluorescence (excitation 485 nm, emission 535 nm) of JC-1 were measured using a Fluoroskan Ascent fluorescence plate reader (Thermo Electron Corp., Milford, MA). The levels of fluorescence at both emis-
Figure 4. AATF ameliorates mitochondrial dysfunction and oxidative damage that are induced by IRI. (a) Representative confocal laser scanning microscope images of Rh123 fluorescence, a measure of mitochondrial transmembrane potential, in HK-2 cells before and 12 h after IRI. Note that expression of AATF largely prevented the decrease in Rh123 fluorescence induced by IRI. (b) Statistical analysis of the average pixel intensity of Rh123 fluorescence/cell in untransfected HK-2 cells and cells transfected with vector alone, AATF, or AATF siRNA 12 h after IRI. ***p < 0.01 versus control Rh123 fluorescence levels within their respective groups; ****p < 0.001 versus corresponding values in untransfected or vector-transfected cell groups. (c) Effect of AATF on mitochondrial transmembrane potential as measured by JC-1 fluorescence. The ratio of red to green fluorescence of JC-1 was measured in untransfected HK-2 cells and cells transfected with vector alone, AATF, or AATF siRNA 12 h after IRI. ***p < 0.01 versus the control JC-1 fluorescence ratio within their respective groups; ****p < 0.001 versus corresponding values in untransfected.
sion wavelengths were quantified, and the ratio of red to green fluorescence was calculated. Levels of intracellular superoxide anion radical were measured with hydroethidine (HE), which is oxidized to fluorescence ethidium cation by superoxide, using methods similar to those described previously (34). The dye dihydrorhodamine (DHR) was used to quantify relative levels of mitochondrial peroxynitrite by using methods similar to those described previously (34). DHR localizes to mitochondria and fluoresces when oxidized to the positively charged rhodamine 123 derivative. A thiobarbituric acid reactive substances (TBARS) fluorescence-based method was used as a measure of membrane lipid peroxidation (34). Levels of caspase-3 activity also were assessed using a previously described protocol that uses DEVD, a pseudo-substrate and inhibitor of caspase-3 (19). Images of cellular fluorescence were acquired using a Zeiss LSM 510 confocal laser scanning microscope (488 nm excitation and 510 nm emission) with a 60× oil immersion objective. The average pixel intensity of fluorescence per cell was determined using the LSM 510 software (Carl Zeiss Microlmaging, Thornwood, NY).

**Results**

**Levels of AATF Expression Are Altered Significantly by IRI in Renal Tubule Epithelial Cells**

AATF expression in HK-2 cells was apparent on Western blotting (Figure 1A). For examination of whether levels of AATF are altered after IRI *in vitro*, HK-2 cells were incubated with 10 mM antimycin A plus 10 mM 2-deoxyglucose plus 1 μM calcium ionophore (A23187) for 60 min (to induce ischemic injury *in vitro*). The *in vitro* reperfusion was achieved by incubating cells in glucose-replete complete growth medium. As shown in Figure 1, A and B, a biphasic change in levels of AATF was observed in HK-2 cells after IRI. A significant increase in AATF expression was observed within 8 h after IRI, which was followed by a significant decrease in AATF levels. By 24 and 48 h after IRI, levels of AATF had dropped significantly, to below pre-IRI levels. Similar changes in AATF expression that were induced by IRI also were observed in mouse PRTE cells (Figure 1B). These drastic changes in AATF expression suggest that AATF plays a significant role in renal IRI.

**Overexpression of AATF Inhibits IRI-Induced Apoptosis of Human Proximal Tubule Cells**

To examine whether the early increase in AATF expression that is induced by IRI contributes to cell death or represents a cytoprotective mechanism in response to IRI (20,35), we examined the effect of overexpression of AATF on apoptotic cell death of HK-2 cells after IRI. Cultures of HK-2 cells were transfected with either the full-length AATF or the vector alone and then were subjected to chemical ischemia followed by reperfusion for 48 h. As shown in Figure 2A, stable transfection with AATF led to significant increase in AATF expression in HK-2 cells. Exposure of HK-2 cells to IRI resulted in apoptotic cell death in approximately 80% of the HK-2 cells 48 h after reperfusion, which largely was prevented by overexpression of AATF (Figure 2B). Time course analysis of IRI-induced apoptosis in HK-2 cells showed that a significant amount of cell death was detected 24 h after IRI, which continued to increase within 48 h after IRI. Transfection of AATF largely prevented the apoptosis of HK-2 cells, whereas transfection of vector alone was ineffective (Figure 2C). These results indicate that induction of AATF expression plays a significant cytoprotective role against apoptotic cell death that is induced by IRI in renal proximal tubule cells.

**Specific Knockdown of AATF by RNAi Exacerbates Caspase-3 Activation and Increases Vulnerability of HK-2 Cells to IRI-Induced Apoptosis**

Next, we tested the hypothesis that silencing of AATF expression would confer increased sensitivity to apoptotic cell death after chemical ischemia–reperfusion. We first examined whether AATF expression could be knocked down efficiently and specifically by RNAi. The high transfection efficiency of siRNA in our preparations was documented previously (21). Cultures of HK-2 cells were either mock transfected (control) or transfected with siRNA cocktail against AATF. A nonsilencing siRNA (see Materials and Methods) was used as a negative control. Forty-eight hours after siRNA transfection, cells were subjected to IRI, and levels of AATF immunoreactivity in these cells then were examined by Western blotting. As shown in Figure 3, A and B, the early increase in AATF expression that was induced by IRI (8 h after reperfusion) largely was knocked down by siRNA that were targeted against AATF but not by a control siRNA that was targeted against GFP. For further examination of the specificity of the siRNA that were targeted against AATF, the same protein samples were probed with an antibody against the leucine zipper protein Par-4. Neither siRNA against AATF nor the siRNA against GFP altered the expression of Par-4. These results demonstrate that the AATF siRNA cocktail specifically and effectively targeted AATF or vector-transfected cell groups. (d) AATF suppresses superoxide accumulation induced by IRI. The HK-2 cell lines were subjected to IRI for the indicated time periods, and the relative levels of superoxide were quantified by imaging of hydroethidine (HE) fluorescence. Transfection of AATF ameliorated whereas AATF siRNA exacerbated the IRI-induced increase in superoxide production within 12 h. ***P < 0.001 versus corresponding values in untransfected and vector-transfected control cells. (e) AATF inhibits accumulation of reactive oxygen species induced by IRI. The indicated HK-2 cell lines were subjected to IRI for 12 h, and levels of dihydrorhodamine (DHR) fluorescence, a measure of relative levels of mitochondrial peroxynitrite, were quantified. ***P < 0.001 versus values in their respective groups before IRI; ****P < 0.001 versus corresponding values in untransfected and vector-transfected cells. (f) AATF inhibits membrane lipid peroxidation induced by IRI. The HK-2 cell lines were subjected to IRI for the indicated time periods, and relative levels of thiobarbituric acid reactive substances (TBARS), a measure of membrane lipid peroxidation, were quantified. **P < 0.01 versus corresponding values in untransfected and vector-transfected cells. All values are the means ± SE of determinations made in at least six cultures. At least 120 cells were examined per culture dish. Similar data were obtained from three separate clones of HK-2 cells transfected with AATF or AATF siRNA. ANOVA with Scheffe post hoc tests.
mRNA in HK-2 cells for degradation by RNAi. Because cysteine proteases of the caspase family have a prominent role in apoptosis, we examined levels of caspase-3 activity in HK-2 cells using DEVD, a pseudo-substrate of caspase-3. As shown in Figure 3C, transfection of AATF siRNA cocktail drastically exacerbated whereas overexpression of the full-length AATF significantly alleviated activation of caspase-3 in HK-2 cells at various time points after IRI. The inhibitory effect of AATF on IRI-induced caspase-3 activation also was confirmed by Western blotting with an antibody that detects endogenous levels of the large fragment (17/19 kD) of cleaved caspase-3 (Figure 3, D and E). For examination of the effect of RNAi silencing of AATF on apoptotic cell death that is induced by IRI, cultures of HK-2 cells were transfected with siRNA against AATF. Forty-eight hours after siRNA transfection, cells were subjected to IRI for 12 h, and cells with apoptotic nuclei were counted. As shown in Figure 3F, RNAi knockdown of AATF exacerbated whereas overexpression of AATF alleviated apoptotic cell death that was induced IRI. Collectively, our date demonstrated that AATF plays an essential role in blocking apoptotic cascades that are initiated by IRI in renal tubule cells.

**AATF Protects Proximal Tubule Cells against Ischemia-Reperfusion Injury by Preserving Mitochondrial Function and Reducing Oxidative Damage**

Apoptosis often involves mitochondrial dysfunction. Indeed, exposure of HK-2 cells to IRI led to a significant decrease in mitochondrial transmembrane potential (as measured by both rhodamine 123 and JC-1 fluorescence) 12 h after reperfusion, which was alleviated significantly by overexpression of AATF (Figure 4, A through C). Conversely, transfection of AATF siRNA significantly exacerbated the decrease in mitochondrial transmembrane potential that was induced by IRI (Figure 4, A through C). Because increased oxidative stress may be an early event in activation of apoptotic machinery, we examined whether markers of oxidative damage (mitochondrial superoxide production, peroxynitrite formation, and membrane lipid peroxidation) were altered by AATF in HK-2 cells after IRI. HE fluorescence by confocal laser scanning microscopy has been shown to be an excellent reporter of mitochondrial superoxide production (34). As shown in Figure 4D, exposure to IRI caused a significant increase in levels of HE fluorescence in untransfected and vector-transfected control HK-2 cells, whereas overexpression of AATF largely prevented superoxide accumulation that is induced by IRI. We also examined the effect of AATF on accumulation of peroxynitrite using confocal DHR fluorescence assay. As shown in Figure 4E, IRI led to a dramatic increase in the level of DHR fluorescence 12 h after reperfusion in untransfected and vector-transfected cells, which largely was blocked by AATF. Membrane lipid peroxidation is an important consequence of peroxynitrite formation. We therefore measured relative levels of lipid peroxidation by using a TBARS fluorescence-based assay. A significant increase in TBARS fluorescence was observed as early as 4 h after IRI in untransfected and vector-transfected control cells, which continued through the 24-h observation period (Figure 4F). Overexpression of AATF significantly attenuated the lipid peroxidation that was induced by IRI (Figure 4F). In contrast, transfection of AATF siRNA exacerbated the oxidative damage that was induced IRI (Figure 4).

**Overexpression of AATF Inhibits whereas Silencing of AATF Exacerbates IRI-Induced Apoptosis in Mouse PRTE Cells**

HK-2 cells are immortalized human cells and therefore may have altered apoptotic properties compared with normal renal tubule epithelial cells. To ensure that the data that were derived from HK-2 cells were pathologically meaningful and physiologically relevant, we examined whether the antiapoptotic effects of AATF also can be observed in PRTE cells after IRI. AATF overexpression in PRTE cells was achieved by infection with rAAV-AATF (Figure 5A). Furthermore, consistent with the data that were obtained from HK-2 cells, overexpression of AATF ameliorated whereas silencing AATF by RNAi exacerbated cell death that was induced by ischemia/reperfusion in PRTE cells (Figure 5B).

**Discussion**

Data presented in this study indicate that AATF is expressed in human renal proximal tubule cells and in mouse PRTE cells, wherein it plays a significant cytoprotective role against renal IRI. This notion is supported by several lines of experimental evidence obtained in this study: (1) Western blot analysis showed that not only that AATF existed in both types of cells but also that its levels of expression were altered significantly by IRI; An early increase followed by a significant decrease; (2) overexpression of AATF in transfected HK-2 cells suppressed apoptotic cell death that is induced by IRI; (3) RNAi-mediated silencing of AATF exacerbated whereas overexpression of AATF ameliorated mitochondrial dysfunction, caspase-3 activation, and oxidative damage that were induced by IRI in HK-2 cells; (4) similar antiapoptotic action of AATF also was observed in mouse PRTE cells after AAV-mediated gene transfer. These results provide convincing evidence that AATF plays an essential role in alleviating renal IRI.

The precise mechanisms by which AATF confers a cytoprotective role against IRI-induced renal injury still needs to be investigated further. Increased production of ROS and activation of apoptotic pathways have been shown to contribute to the pathogenesis of renal IRI (2,9,36–41). Proximal tubule epithelial cells are significantly more sensitive than distal tubule cells to IRI because they tend to produce more ROS and tend to sustain more severe oxidative and apoptotic damage than distal tubule cells do. In addition, levels of the cytoprotective bcl-2 often were kept at low levels in proximal cells after IRI. We demonstrate that AATF significantly suppresses mitochondrial dysfunction, oxidative stress, and caspase activation that are induced by IRI. These results suggest that AATF functions to protect against IRI-induced renal cell death during early phases of apoptotic activation, before mitochondrial dysfunction and caspase-3 activation.

One potential mechanism by which AATF confers the cytoprotective actions that were observed in this study is by blocking the apoptotic cascades that are activated by Par-4. Par-4 is
and nonsilencing siRNA groups. Values are the means ± SE of at least 120 cells were examined per culture dish. ANOVA with post hoc Scheffe testing was used to identify the significance of differences between groups. Values in untransfected cells were significantly different (*P < 0.05, **P < 0.01, and ***P < 0.001, respectively, versus untransfected controls). Values in non-silencing siRNAs and silencing AATF siRNAs were significantly different (**P < 0.01 and ***P < 0.001, respectively, versus non-silencing siRNAs and non-silencing AATF siRNAs). These studies identify Par-4 as a critical link in the chain of events that lead to the initiation of apoptosis in human kidney proximal tubular cells after IRI. Of importance, AATF has been shown to bind to Par-4 via the leucine zipper domain, leading to a complete blockade of Par-4 activity (20,35). In renal proximal tubular cells, increased AATF might become physically associated with Par-4 and thereby block the proapoptotic signaling that is initiated by Par-4.

The antiapoptotic actions of AATF were observed consistently in both transduced HK-2 cells and normal renal tubule epithelial cells, indicating that the cytoprotective effect of AATF against IRI is physiologically relevant and pathologically meaningful. The in vitro model of IRI used in this study reproduces several key hallmark features of IRI, including a profound intracellular ATP depletion and a fall in tissue oxygen and glucose content with a concomitant rise in intracellular calcium. HK-2 is a proximal tubular cell (PTC) line that is derived from normal human kidney. These cells were immortalized by transduction with human papillomavirus 16 E6/E7 genes and retain many functional characteristics of proximal tubular epithelium, including a phenotype that is indicative of well-differentiated PTC (52). Previous studies demonstrated that, without calcium ionophore, combination of ATP and glucose depletion with antimycin A and 2-deoxyglucose, respectively, produces approximately 90% ATP depletion but fails to kill HK-2 cells effectively (30,53). Addition of the calcium ionophore (A23187) mimics the rise in intracellular calcium load seen in ischemic renal tubule cells in vivo and facilitates HK-2 cell death in vitro (30). It was shown previously that addition of high concentrations of the calcium ionophore (>2 μM), combined with ATP and glucose depletion with antimycin A and 2-deoxyglucose, induced rapid necrotic cell death in HK-2 cells (30), and we have found that concentrations of the calcium ionophore <1 μM failed to facilitate significantly HK-2 cell death that was associated with ATP and glucose depletion (data not shown). The 1-μM calcium ionophore (A23187) concentration was chosen for our studies because, at this concentration, a significant amount of apoptosis of HK-2 cells was induced during a period of 48 h after in vitro reperfusion.

**Conclusion**

We have identified AATF as a novel cytoprotective factor against oxidative and apoptotic damage in kidney tubule cells. Targeted enhancement of AATF activity in the kidney by pharmacologic and/or genetic manipulations may prove to be useful in preventing and treating renal IRI.

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