Abstract. To ascertain the role of spermidine/spermine N-1-acetyltransferase (SSAT; the rate-limiting enzyme in polyamine catabolism) in cell injury, cultured kidney (HEK 293) cells conditionally overexpressing SSAT were generated. The SSAT expression was induced and its enzymatic activity increased 24 h after addition of tetracycline and remained elevated over the length of the experiments. Induction of SSAT upregulated the expression of polyamine oxidase and resulted in the reduction of cellular concentration of spermidine and spermine, increased concentration of putrescine, and inhibited cell growth. SSAT overexpression increased the expression of heme oxygenase-1 (HO-1) by 350% 24 h after addition of tetracycline, indicating the induction of oxidative stress. The presence of catalase significantly prevented the upregulation of HO-1 in SSAT overexpressing cells, indicating that generation of H2O2 is partially responsible for the induction of oxidative stress. Overexpression of SSAT caused rounding and loss of cell anchorage and significantly altered the morphology of actin-containing filopodia, suggesting an adhesion defect. SSAT upregulation may mediate majority of the oxidative stress in kidney ischemia-reperfusion injury (IRI) as manifested by decreased cell growth, generation of toxic metabolites (H2O2 and putrescine), upregulation of HO-1, disruption of cell anchorage, and defect in cell adhesion. These data point to the inhibition of polyamine catabolism as a therapeutic approach for the prevention of tissue injury in kidney IRI.

Ischemia-reperfusion injury (IRI) is the major cause of morbidity and mortality in diseases such as stroke, myocardial infarction, and acute renal tubular necrosis. Ischemic conditions result in ATP depletion and accumulation of toxic metabolites. Reperfusion results in the production of reactive oxygen intermediates (1,2). The resulting alteration in cellular metabolism and generation of toxic molecules contribute to tissue damage in IRI (1,2), which is characterized by the presence of necrotic and apoptotic areas in the affected organs (1,3). Despite important developments in our understanding of the pathophysiology of IRI in kidney, heart, and other organs, there is no specific therapy for patients except for supportive care.

Polyamines (spermidine, spermine, and putrescine) are aliphatic cations derived from ornithine (4,5). They play a fundamental role in the stabilization of DNA structure; they modulate gene expression and regulate protein synthesis, signal transduction, and cell growth and differentiation (4,6–8). Spermidine/spermine N-1-acetyltransferase (SSAT; the rate-limiting enzyme in polyamine catabolism) acetylates both spermidine and spermine. As a result, the cellular contents of spermidine and spermine are decreased and the concentrations of N-acetyl spermidine and N-acetyl spermine are increased (9). The subsequent activity of polyamine oxidase (PAO) on acetylated polyamines results in the production of spermidine or putrescine (depending on the starting substrate) and H2O2, a reactive oxygen intermediate. Figure 1 depicts the role of SSAT in polyamine catabolism.

By use of suppression subtractive hybridization, we observed a major increase in SSAT expression levels in kidneys after IRI (10). The SSAT upregulation correlated with the onset of injury and not with a significant increase in uremic toxins (10). Overexpression of SSAT was also observed in cultured cells subjected to ATP depletion, an in vitro model of IRI. To determine whether increased expression of SSAT is detrimental to cell survival in IRI, the effect of overexpression of SSAT was studied in cultured kidney cells. The results
indicated that SSAT overexpression per se generates oxidative stress and enhances susceptibility to injury in cultured cells. We propose that SSAT upregulation contributes to the tissue damage associated with IRI.

Materials and Methods

Conditional Overexpression of SSAT in Cultured Kidney Cells

HEK293 cells stably transfected with the regulatory plasmid pcDNA6/TR, which encodes the Tet repressor protein (TetR) under the control of human cytomegalovirus promoter, were purchased from Invitrogen (Carlsbad, CA). To establish the tetracycline-inducible SSAT expression, the region spanning SSAT cDNA bases 6 to 800 (containing the full coding region) was amplified by reverse transcriptase–PCR from rat kidney cDNA with sense primer 5′-CGG-GAAACGAATGAGGAACCAC and antisense primer 5′-ATTCT-GCCCTCAAACACACATAGAC, which were synthesized on the basis of the mouse SSAT cDNA sequence (accession no. NM_009121). The PCR product was gel purified, subcloned into pGEMT-Easy vector (Promega), and transformed into Escherichia coli. The insert was released by NotI digestion and ligated into pcDNA4/TO (a tetracycline-inducible mammalian expression vector). Sequencing was performed to verify the correct orientation of SSAT insert. The isolated clones were then used to transfect HEK293 cells. Several colonies of HEK 293 cells stably expressing SSAT were isolated and expanded.

Generation and Conditional Overexpression of Inactive Mutant SSAT in Cultured Kidney Cells

To examine the role of SSAT in mediating cell injury in more detail, we generated a construct that was used to express an inactive SSAT mutant. Briefly, we used the Quick Change PCR based site directed mutagenesis method (Strategene) to develop an expression vector that codes for an inactive SSAT mutant protein that contains two mutations in residues 101 and 152 (R101 A/E152K). This mutated protein does not display any SSAT activity (9). After confirming the sequence of the insert, HEK293 cells were transfected with the tetracycline-inducible inactive SSAT construct. Stable transfectants were isolated and expanded similar to the wild type SSAT.

Stable Expression of SSAT-expressing Inducible Construct

HEK293 cells were stably transfected with the inducible construct according to established methods with Transfast Transfection reagent (Promega). The transfectants were selected in the presence of Zeocin at a final concentration of 200 μg/ml. Single clones were isolated and expanded for further studies.

RNA Isolation and Northern Hybridization

Total cellular RNA was extracted from cultured cells or kidney samples with the Tri Reagent method (MRC, Cincinnati, OH) following the manufacturer’s protocol. Total cellular RNA (30 μg/lane) was size-fractionated on a 1.2% agarose-formaldehyde gel and transferred to nylon membranes by capillary transfer with 10× SSPE buffer. Membranes were cross-linked by ultraviolet light or baked. Hybridization was performed according to Gilbert and Church (11). Membranes were washed, exposed to PhosphorImager screens at room temperature for 24 to 72 h, and scanned by PhosphorImager. A 32P-labeled cDNA fragment of the mRNA-encoding SSAT (corresponding to nucleotides 323 to 892 of a mouse SSAT cDNA; GenBank accession no. NM_009121) or PAO (corresponding to nucleotides 1 to 861 of mouse PAO cDNA (GenBank accession no. XM_113921) was used as a specific probe. For heme oxygenase-1 (HO-1), a 445-bp PCR fragment encoding nucleotides 21 to 466 of a rat HO-1 cDNA (GenBank accession no. NM_012580) was used.

Light Microscopy

Cells were exposed to tetracycline (1 μg/ml) for 24 h and light microscopic images were obtained at ×100 magnification with a Zeiss inverted microscope.

Measurement of SSAT Activity and the Intracellular Concentration of Polyamines

The enzymatic activity of SSAT and the intracellular concentration of spermine, spermidine, and putrescine were measured on cell lysate by HPLC, as described previously (12).

Cell Growth

To determine cell growth, cultured cells were plated and assayed by CellTiter 96 Non-Radioactive Cell Proliferation Assay from Promega (Madison, WI).

IRI in Rats

IRI was induced as described previously (10,13). Briefly, bilateral IRI was induced in male Sprague-Dawley rats (200 to 250 g) by occluding the renal pedicles with microvascular clamps for 15 min (mild ischemia) or 30 min (severe ischemia) under ketamine-xylazine anesthesia (150 μg/g as ketamine, 3 μg/g as xylazine). Completeness of ischemia was verified by blanching of the kidneys, signifying the
stoppage of blood flow. The blood flow to the kidneys was reestablished by removal of the clamps (reperfusion) with visual verification of blood return. Animals subjected to sham operation (identical treatment except the renal pedicles were not clamped) were used as controls. During the procedure, animals were well hydrated, and their body temperature was controlled to about 94°F with an adjustable heating pad. After ischemia, animals were kept under veterinary observation. At 12 h after ischemia, animals were killed and their kidneys harvested.

**Cell Attachment Assay**

HEK cells, stably transfected with tetracycline inducible wild-type SSAT expression vector, were seeded at $1.0 \times 10^5$ cells per well in six-well tissue culture plates (Falcon, Franklin Lakes, NJ). Forty-eight hours after seeding, the growth medium was removed and replaced with fresh growth medium with or without tetracycline. Twenty-four hours after changing the medium, light microscopic images of the cells were obtained. To examine the effect of SSAT induction on cell attachment, the unattached cells were harvested by aspirating the culture medium, and the attached cells were harvested by treating the adherent cells with trypsin-EDTA solution (Life Technologies BRL, Grand Island, NY). The number of nonadherent and adherent cells was determined by performing cell counts with a hemacytometer.

**Filamentous Actin Staining**

Flasks of cells expressing SSAT under the control of the tetracycline transactivator were induced with tetracycline for approximately 24 h, then trypsinized and replated on coverslips coated with fibronectin ($10 \mu g/ml$ for 2 h at 37°C). After 3 h or overnight incubation, the cells were fixed with 3% formaldehyde in PBS, permeabilized with 0.1% Triton-X100 in PBS, and stained with a 1:100 dilution of Alexafluor 488–phalloidin (Molecular Probes) for 30 min to visualize filamentous actin according to established methods (14,15). Coverslips were mounted in Vectashield Hard Set mounting medium containing DAPI (Vector Laboratories) as a nuclear counterstain and viewed in a Zeiss confocal fluorescence microscope with a ×63 PlanApo lens. Both confocal and Nomarski differential interference contrast images were digitally photographed.

**Materials**

$^{32}$P-dCTP was purchased from New England Nuclear (Boston, MA). Nitrocellulose filters and chemicals were purchased from Sigma Chemical (St. Louis, MO). High Prime labeling kits were purchased from Roche Diagnostics Gmbh (Mannheim, Germany).

**Statistical Analyses**

Values are expressed as mean ± SEM. The significance of difference between mean values was examined by ANOVA. $P < 0.05$ was considered statistically significant.

**Results**

**Conditional Overexpression of SSAT Leads to PAO Upregulation**

Figure 2 depicts a Northern blot analysis for the expression of SSAT mRNA in several clones stably transfected with an SSAT inducible construct. Our results indicate that addition of tetracycline induces the expression of SSAT mRNA. To determine whether SSAT induction upregulates the expression of PAO, the same blot was stripped and reprobed with radiolabeled PAO cDNA. PAO mRNA levels increase in tetracycline-treated cells. Taken together, these data indicate that increased SSAT expression causes the induction of PAO. For the purpose of the following studies, one of the clones expressing high levels of SSAT (clone 1) was chosen and studied in detail.

To determine the optimal time course of SSAT induction by tetracycline, cells from clone 1 were exposed to tetracycline for various time intervals (1, 2, 3, and 4 d) and processed for RNA isolation. As indicated in Figure 3, the expression of SSAT peaks at 24 h and remains elevated at 96 h after the addition of tetracycline versus time-matched controls.
Measurement of Enzymatic Activity of SSAT and Concentration of Polyamines

The overexpression of SSAT is associated with a dramatic induction of SSAT enzymatic activity (Figure 4a), induction of PAO (Figure 2), and a significant reduction in the intracellular concentration of spermidine and spermine throughout the experiment (Figure 4b). The decrease in the concentration of polyamines was also associated with a significant reduction in cell growth (Figure 4c).

Upregulation of SSAT and HO-1 In Vivo Is an Early Event and Correlates with the Severity of Kidney Ischemic Injury

In vivo kidney IRI results in the upregulation of SSAT and causes the generation of H$_2$O$_2$ and putrescine, which are both toxic metabolites (10). HO-1 is upregulated in nephrotoxicity, and its enhanced expression is regarded as a marker of oxidative stress (16–22). It is increased in response to a number of injury-causing chemicals, including H$_2$O$_2$, and may provide protection against cell injury (23,24). The purpose of the next series of experiments was to examine the expression of SSAT and HO-1 with respect to each other in two groups of rats with mild and severe renal ischemic injury. Animals were subjected to 15 min (mild injury) or 30 min (severe injury) of ischemia and killed after 12 h of reperfusion. As demonstrated in Figure 5, the onset and the magnitude of expression of HO-1 and SSAT are similar and correlate with the severity of injury.

To determine the SSAT enzymatic activity in kidneys with IRI, tissue samples (cortex or medulla) from sham-operated animals and rats with 30 min ischemia and 12 h of reperfusion were harvested, homogenized in ice-cold isolation solution, and centrifuged at low speed as described. The extracts were processed for SSAT activity as described (above) (10). SSAT enzymatic activities were 1.96 ± 0.21 and 2.36 ± 0.23 pmol/mg protein/min in cortex and medulla, respectively, in sham-operated animals and increased to 37.5 ± 2.4 and 47.6 ± 2.7 pmol/mg protein/min, respectively, at 12 h of reperfusion ($P < 0.01$ in both cortex and medulla versus sham). These results demonstrate that IRI is associated with an approxi-
mately 20-fold increase in SSAT activity in kidney tissue in \textit{in vivo} IRI.

**Overexpression of SSAT Upregulates the Expression of HO-1 in Cultured Cells**

On the basis of the time course of HO-1 upregulation with respect to SSAT in kidney IRI (Figure 5), and on the basis of polyamine catabolic pathway (schematic diagram) indicating that SSAT upregulation increases the generation of \( \text{H}_2\text{O}_2 \), we hypothesized that SSAT overexpression increases the expression of HO-1 via the release of \( \text{H}_2\text{O}_2 \). Accordingly, we first determined whether SSAT overexpression \textit{in vitro} results in the upregulation of HO-1. Toward this end, we examined the expression of HO-1 in HEK cells in response to SSAT overexpression after 24 h of exposure to tetracycline. Figure 6 is a representative Northern hybridization and demonstrates that the overexpression of SSAT for 24 h upregulates the expression of HO-1, with mRNA levels increasing by approximately 3.5-fold \((P < 0.02, n = 4)\). To examine the time course of upregulation of HO-1, membrane from Figure 3 was stripped and reprobed with radiolabeled HO-1 cDNA. As demonstrated in Figure 7a, HO-1 expression peaked at 24 h after SSAT induction and gradually returned toward baseline after 96 h. These results indicate that SSAT induction \textit{per se} (and in the absence of IRI) increases the expression of HO-1. The induction of SSAT directly correlated with putrescine concentration, which peaked at 24 h and returned toward baseline levels at 96 h after the addition of tetracycline (Figure 7b).

**Effect of Catalase on HO-1 Upregulation in Response to SSAT Overexpression**

To determine whether the upregulation of HO-1 in response to SSAT overexpression (Figures 6 and 7) is due to the generation of \( \text{H}_2\text{O}_2 \), the effect of catalase on the induction of HO-1 was examined. Cells were exposed to tetracycline in the presence or absence of catalase (500 units/ml, added to the media 1 hr before the addition of tetracycline) and examined 24 h later. As shown in Figure 8, presence of catalase significantly inhibited the upregulation of HO-1, with HO-1 mRNA levels decreasing by 63% in the catalase treated cells \((P < 0.05, n = 4)\). The above studies demonstrate that the upregulation of HO-1 in cultured cells with conditional overexpression of SSAT is partly mediated via \( \text{H}_2\text{O}_2 \) release (Figure 8) and directly correlates with the concentration of putrescine (Figure 7), the toxic metabolic product of polyamine catabolism generated by SSAT overexpression.

**Effect of SSAT Overexpression on Cell Attachment, Morphology, and Cytoskeletal Structure**

In the next series of experiments, we examined and compared the effect of overexpression of wild-type and inactive SSAT mutant on cell morphology. The inactive SSAT mutant was generated as described in Materials and Methods. The
expression of SSAT mRNA increased in both the wild-type and inactive SSAT transfectants in response to tetracycline treatment. After 24 h of stimulation by tetracycline, putrescine concentration increased by sevenfold in cells expressing the wild-type SSAT and by only 0.8-fold in cells expressing the inactive mutant SSAT, a more than 80% inhibition in polyamine catabolic pathway activity.

To examine the effect of SSAT overexpression on cell morphology, light microscopic analysis of HEK cells was performed in the absence (control) or presence of tetracycline (SSAT induction). Figure 9a is a light microscopy image of live HEK cells before (control) and after the induction of wild-type SSAT with tetracycline for 24 h. Low and high magnification images clearly indicate that conditional overexpression of SSAT causes the rounding and loss of cell attachment. Morphologic examination of the cells revealed that the induction of SSAT leads to loss of cell adhesion processes even in the cells that remain attached to the substratum. Comparison of induced and uninduced cultures (cell attachment assay studies in Materials and Methods) revealed that overexpression of SSAT disrupts cell attachment and leads to a significant increase in the number of nonadherent cells (Figure 9b). As demonstrated, the number of adherent cells significantly decreased (from 98% to 32%, \( P < 0.001, n = 3 \) separate experiments involving a total of nine dishes) and the number of nonadherent cells significantly increased (from 2% to 68%, \( P < 0.001, n = 3 \) separate experiments involving a total of nine dishes) in response to SSAT induction. These effects were specific to the wild-type SSAT because the overexpression of the inactive SSAT mutant had no significant effect on cell attachment or morphology (data not shown).

Defects in cell anchorage (Figure 9) point to the cytoskeleton as a major cell structure affected by SSAT overexpression. In the last series of experiments, we examined the arrangement of filamentous actin, a major cell cytoskeletal structure, in response to conditional overexpression of SSAT in HEK 293 cells. Control and tetracycline-treated cells (Materials and Methods) were plated on fibronectin-coated coverslips for 3 h, then fixed and stained with fluorescence phalloidin (green) to visualize filamentous actin. Nomarski images of cells with or without tetracycline induction are depicted in Figure 10 (taken with Zeiss Axioskop, \( \times 63 \) PlanApo lens). As demonstrated, cells are well spread in the control group but are significantly less spread in SSAT overexpressing cells. The confocal fluorescence images of cells with or without tetracycline induction were stained with Alexafluor 488–phalloidin to visualize filamentous actin. The results are depicted in Figure 10 and demonstrate that the thick peripheral band of actin and the filopodia in the noninduced cells are absent in cells with SSAT induction. These pictures were taken with a Zeiss Confocal 510, \( \times 63 \) PlanApo lens. Figure 10, C and D, are projections of a complete through-focus series of images.

**Discussion**

Polyamines play a fundamental role in cell growth and proliferation. Under the effect of SSAT, polyamines are catabolized to putrescine. The reactive oxygen intermediate \( \text{H}_2\text{O}_2 \) is also generated as a toxic metabolic byproduct. The reduction in polyamines decreases cell proliferation, whereas generation of putrescine and \( \text{H}_2\text{O}_2 \) causes cell injury and ultimately death. As a result, various laboratories have focused on SSAT as a novel target for the treatment of solid tumors, including breast cancer, where the desired reduction in cell growth can be achieved by SSAT upregulation. Enhanced expression of SSAT by spermine analogue in the breast cancer cell line L56Br-C1 resulted in the depletion of the cellular pools of polyamine within 24 to 48 h (25). Cell proliferation appeared to be totally inhibited, and within 48 h of treatment, there was an extensive apoptotic response (25). In *Ehrlich ascites* tumor cells, treatment with the antitumor drug 1’-acetoxychavicol acetate resulted in increased activity of SSAT with subsequent lowering of intracellular polyamines (26). Apoptosis immediately followed. Administration of exogenous polyamines prevented acetoxychavicol acetate–induced apoptosis (26). A similar observation has been documented in human leukemic cells, where depletion of intracellular polyamines secondary to the overexpression of SSAT resulted in decreased cell growth and caused the induction of apoptosis (27). These studies indicate that enhanced expression of SSAT depletes the cellular polyamine pools, decreases cell growth, and leads to cell injury.

Enhanced expression of SSAT in kidney IRI was recently reported (10). It was postulated that the upregulation of SSAT in IRI is likely detrimental to cell survival by decreasing growth and causing the release of toxic metabolic products such as \( \text{H}_2\text{O}_2 \). This was tested in the current studies by developing cell lines that conditionally overexpress SSAT in response to tetracycline. The most salient feature of the study presented here was the upregulation of HO-1 and alteration in cell morphology in response to overexpression of SSAT. It is


well known that the expression of HO-1 is enhanced in conditions associated with oxidative stress (23,24). HO-1 is involved in the degradation of heme molecules to iron, carbon monoxide, and biliverdin, products that provide protection against the injury through their antioxidant, antiinflammatory, and cytoprotective activities (17,28). The upregulation of HO-1 in response to overexpression of SSAT directly correlated with putrescine concentration, which is increased as a result of the sudden breakdown of polyamines (Figure 7). The reduction in HO-1 levels, and presumably oxidative stress, after its peak at 24 h may be the result of a decline in the catabolic reaction of PAO that yields putrescine and H2O2, most likely as a result of the depletion of polyamines, which are the substrates for SSAT and PAO activities. Alternatively, it is plausible that the return of HO-1 to its baseline after its peak at 24 h is the result of the generation of antioxidant products of HO-1 activity such as bilirubin or carbon monoxide (17,28).

In addition to putrescine, SSAT overexpression results in the generation of H2O2, a toxic byproduct of polyamine catabolism. Several studies have demonstrated that the addition of H2O2 in cultured cells upregulates the expression of HO-1 (23). We tested the possibility that the generation of H2O2 by SSAT overexpression (Figures 6 and 7) was responsible for the upregulation of HO-1 by incubating the HEK cells with catalase at the time of addition of tetracycline. The results demonstrated that the presence of catalase decreased the expression of HO-1 by 63% in cells overexpressing SSAT (Figure 8). The above experiments demonstrate that the generation of H2O2 and putrescine by SSAT overexpression causes oxidative stress in cultured kidney cells.

The light microscopic analysis of live HEK 293 cells that are transfected with SSAT demonstrate that conditional overexpression of SSAT significantly altered the morphology of HEK cells (Figure 9a, right panels) when compared with control (Figure 9a, right panels). The addition of tetracycline for 24 h caused rounding up and detachment of cell anchorage in cells overexpressing the SSAT (Figure 9a, a and b). This time point (24 h) correlates with the highest concentration of putrescine and H2O2 (Figure 7). No significant alteration in cell morphology was observed in HEK cells overexpressing the inactive SSAT mutant (data not shown). Cell attachment assays demonstrated increased number of non adherent and floating cells in response to SSAT induction (Figure 9b). The rounding of the HEK cells and their loss of anchorage after induction of SSAT expression resembles the detachment of kidney tubular epithelial cells from the basement membrane observed in IRI (29–31).

Interestingly, cell cytoskeleton staining revealed significant alterations in assembly of actin (Figure 10). Cells are well spread in the control group but are significantly less spread in SSAT-overexpressing cells (Figure 10, A and B). The confocal fluorescence images of cells stained with Alexafluor 488–phalloidin to visualize filamentous actin demonstrated that the thick peripheral band of actin and the filopodia in the noninduced cells are absent in cells with SSAT induction (Figure 10, C and D). In additional studies not shown, we observed the rearrangement of focal adhesion molecule paxicillin in re-
response to SSAT overexpression. In summary, tetracycline induction of SSAT expression rendered cells less able to spread over a fibronectin substratum and altered the morphology of actin-containing filopodia, suggesting an adhesion defect.

In addition to the kidney, IRI in heart is a major cause of morbidity and mortality. We have examined the expression of SSAT in mice subjected to cardiac IRI. Our results indicate that the occlusion of left anterior descending artery for 30 min causes significant upregulation of SSAT expression in the injury zone at 6 h after reperfusion (data not shown), indicating that the activation of SSAT and polyamine catabolism may be a universal pathway in IRI. As such, SSAT upregulation may play an important role in mediating tissue injury in IRI in a number of organs in mammals. It is therefore intriguing to speculate that prevention or inhibition of polyamine catabolic pathway is a potential target for the treatment of IRI in kidney and heart, which are major causes of morbidity and mortality in the United States.

In conclusion, conditional overexpression of SSAT in cultured kidney cells resulted in decreased cell growth, altered cell morphology, and upregulated HO-1 expression, indicating that SSAT overexpression per se generates cellular phenotypic changes that mimic those that are observed in IRI. We propose that enhanced SSAT expression in kidney mediates majority of cell damage in IRI. Future studies on the role of SSAT and polyamine catabolic pathway may provide novel treatments for kidney failure in IRI.

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