Spermidine/spermine N\textsuperscript{1}-acetyltransferase overexpression in kidney epithelial cells disrupts polyamine homeostasis, leads to DNA damage, and causes G\textsubscript{2} arrest

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Submitted 23 August 2006; accepted in final form 18 October 2006

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THE EXPRESSION AND ACTIVITY of spermidine/spermine N\textsuperscript{1}-acetyltransferase (SSAT) is upregulated in kidney, brain, and liver after ischemia-reperfusion injury (IRI) (4, 21). SSAT is the rate-limiting enzyme in polyamine catabolism; it acetylates spermidine (SPD) and spermine (SPM) and leads to their export out of the cell and/or degradation by polyamine oxidase (PAO) (13). Oxidation of acetylated polyamines through the activity of PAO leads to production of potentially toxic metabolites including N-acetyl 3-aminopropanol and H\textsubscript{2}O\textsubscript{2}. The depletion of polyamines and accumulation of the by-products of polyamine catabolism also may contribute to tissue damage associated with IRI.

Polyamines (putrescine, SPD, and SPM) are aliphatic cations derived from ornithine (32, 38). Their levels are tightly regulated, and any disturbance in their homeostasis is detrimental to cell growth. These molecules play a fundamental role in the stabilization of DNA structure, regulation of gene expression, protein synthesis, and signal transduction, as well as modulation of cell growth and differentiation (20, 25, 26, 32). Polyamine depletion through inhibition of ornithine decarboxylase (ODC) leads to enhanced expression of p53, p21, and p27 cyclin-dependent kinase inhibitors and G\textsubscript{1} arrest (3, 29, 30, 39). In cultured cells, increased SSAT activity leads to depletion of SPD and SPM (53). This disturbance in cellular polyamine homeostasis is associated with an increase in the population of G\textsubscript{2} arrested cells and decreased cell growth (42, 53). The cause of decreased cell growth in SSAT-expressing cultures is not clear; however, recent studies indicate that depletion of polyamines, reduction in the cellular content of cofactors required for their biosynthesis, and accumulation of catabolic by-products of polyamine pathway are contributing factors (27, 52).

Regulation of cell proliferation is crucial to recovery from acute renal injuries (36, 37). Cell proliferation is regulated through the interaction of complex signaling pathways that affect the expression and activation of the molecular machinery of cell cycle (35–37, 41). In cells subjected to hypoxia and reoxygenation, DNA replication aberrations and double-stranded breaks are the major causes of genomic instability (18, 19). The presence of damaged DNA leads to activation of cell cycle checkpoints that slow progression through the cell cycle to allow time for DNA repair (5). The DNA repair process is activated in response to oxidative stress via the activation of ATM and ATR kinases and leads to cell cycle arrest or apoptosis in severely damaged cells (18, 19). Polyamine depletion leads to modification of chromatin and DNA structures, increases DNA susceptibility to damage by various genotoxic agents, and interferes with DNA repair (44–46). The DNA alterations in polyamine-depleted cells may be due...
to DNA strand breaks as well as inhibition of topoisomerase II function (2). Mammalian cell division also requires the activation of the Raf → MEK → ERK pathway for G₁/M progression (31, 33, 55). The role of polyamine catabolism in the mediation of DNA damage resulting from hypoxia and reoxygenation is not clear.

The role of SSAT in the pathophysiology of IRI is not understood. Previous studies have suggested that increased SSAT expression in kidney cells leads to reduced cell growth and oxidative stress (53). In current studies, we extend this observation by demonstrating that increased SSAT expression leads to DNA damage, activation of DNA repair pathway, disruption of ERK activation, and G₂ arrest. On the basis of these results, we propose that disruption of polyamine metabolism contributes to the development of DNA lesions and cell damage associated with IRI.

MATERIALS AND METHODS

Expression vectors and development of stable transfectants. Stable transfectants were developed as previously described (53). To examine the effect of SSAT in more detail, using the Quick Change PCR-based site-directed mutagenesis kit (Stratagene), we generated two constructs that were used to express an inactive SSAT and a PCR-based site-directed mutagenesis kit (Stratagene), we generated in the effect of SSAT in more detail, using the Quick Change transfectants were developed as previously described (53). To examine the effect of SSAT in more detail, using the Quick Change PCR-based site-directed mutagenesis kit (Stratagene), we generated two constructs that were used to express an inactive SSAT and a PCR-based site-directed mutagenesis kit (Stratagene), we generated in the effect of SSAT in more detail, using the Quick Change

Flow cytometry analysis of cell cycle. Cells (2 × 10⁵) were harvested, washed in PBS, fixed in 70% ethanol, and stained with propidium iodide [40 μg/ml propidium iodide (PI), 100 μg/ml RNaseA in calcium- and magnesium-free PBS]. The cells (20,000 independent events) were analyzed for their DNA content with a fluorescence-activated cell sorter.

DNA comet assay. Cells (12,000–15,000) from tetracycline-induced and control cultures were subjected to bisulfite treatment with the EZ DNA methylation gold kit (Zymo Research) following the manufacturer’s protocol. Bisulfite treatment can distinguish nonmethylated from methylated cytosine, since only nonmethylated cytosines will be deaminated. Bisulfite-treated DNA (0.25 ng) was used for PCR reactions with LINE-1 element forward 5′-TTGAGTTGTGGGTTTTATTTAG-3′ and reverse 5′-TCATCTCACTAAAAAATACCAAACA-3′ primers, using the following amplification protocol: 5 min at 95°C, followed by 35 cycles of amplification (95°C for 30 s, 50°C for 30 s, and 72°C for 30 s). Whereas bisulfite treatment leads to the formation of a Taxl site in the LINE-1 elements of unmethylated DNA, it leads to the formation of a Taxl site in the LINE-1 elements of methylated DNA. Following amplification, products were digested with Taxl and Taxl restriction endonucleases to differentiate methylated and nonmethylated DNA. Digestion of the PCR product of methylated DNA with Taxl restriction endonuclease leads to the formation of two 80-bp fragments, whereas digestion of the PCR product of unmethylated DNA leads to formation of 97- and 63-bp fragments. Restriction digest patterns were compared and quantitated densitometrically in ampiclons from SSAT-expressing and control cells. To compare the differences in DNA methylation, the ratios of methylated amplicons to total amplicon content in control and tetracycline-treated cells were compared.

Statistical analyses. Values are expressed as means ± SE. The significance of difference between mean values was examined using ANOVA. P < 0.05 was considered statistically significant.

RESULTS

Effect of SSAT overexpression on proliferation of renal epithelial cells. To determine the level of induction of SSAT in HEK cells, we examined the activity of this enzyme in control and tetracycline-induced cells. Our results indicate that the activity of SSAT is increased ~28-fold in tetracycline-induced cells (Table 1). The increase in SSAT expression is accompa-
nied by increases in the activity of ODC and SAMDC, enzymes involved in polyamine anabolism, of $\sim 17$–$25$ and $\geq 30$-fold, respectively (Table 1). These results indicate the activation of a compensatory response to counter the effect of significant reductions in the cellular content of SPD and SPM by SSAT induction. In addition to significant reductions in SPD and SPM levels, there were dramatic increases in the intracellular contents of the products of SSAT activity, $N^\text{1}$-acylspermidine (AC-SPD) and $N^\text{4}$-acetylspermidine (AC-SPM) levels, in HEK-293 cells (Table 2). Using direct cell count, we examined the effect of SSAT expression on cell proliferation. Our results indicate that disruption of polyamine homeostasis caused by the induction of SSAT expression leads to decreased cell proliferation without affecting cell viability (Fig. 1A). To determine whether the reduction in cell proliferation was due to increased SSAT activity, HEK-293 cells conditionally overexpressing a stable isoform of SSAT (LH) that is resistant to ubiquitin-mediated degradation or an inactive mutant (Mut) of SSAT were developed. The activity and properties of these proteins have been characterized in previously published studies (13, 14). Our data indicate that although the induction of wild type (Fig. 1A) and stable SSAT (Fig. 1B) reduced cell proliferation, increased expression of inactive SSAT mutant (Fig. 1C) had no effect on cell proliferation.

Effect of SSAT overexpression on cell cycle progression. The cell cycle distribution of HEK-293 cells and HEK-293 cells expressing increased levels of SSAT were examined on days 4 and 6 after addition of tetracycline. Examination of day 4 cultures indicated the presence of an increased population of $G_2$-arrested cells in SSAT-expressing cells (Fig. 2B) but not in control cells (Fig. 2A). Examination of day 6 tetracycline-induced cultures (Fig. 2D) indicates that, in addition to having an increased population of $G_2$-blocked cells, these cultures had a significant population of endoreduplicated cells (third peak in Fig. 2D). Untreated cells did not exhibit any increase in $G_2$

Table 1. Effect of increased expression of SSAT on the activity of ODC and SAMDC

<table>
<thead>
<tr>
<th></th>
<th>SSAT</th>
<th>ODC</th>
<th>SAMDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-h Control</td>
<td>9.4±1.5</td>
<td>0.2±0.2</td>
<td>0.21±0.2</td>
</tr>
<tr>
<td>24-h Tet</td>
<td>403±116</td>
<td>7.1±0.1</td>
<td>20.2±6.8</td>
</tr>
<tr>
<td>48-h Control</td>
<td>13.6±2.5</td>
<td>0.1±0.1</td>
<td>0.29±0.2</td>
</tr>
<tr>
<td>48-h Tet</td>
<td>388±68</td>
<td>2.6±0.1</td>
<td>12.2±1.9</td>
</tr>
</tbody>
</table>

Values are means ± SE in control and tetracycline-induced (Tet) cells. Spermidine/spermine $N^\text{1}$-acetylate (SSAT) activity is expressed as pmol·min$^{-1}$·mg protein$^{-1}$. Ornithine decarboxylase (ODC) activity is expressed as nmol·h$^{-1}$·mg protein$^{-1}$. $S^\text{-adenosylmethionine}$ decarboxylase (SAMDC) activity is expressed as nmol·h$^{-1}$·mg protein$^{-1}$.

Decomposed proliferation of SSAT-expressing cells may be due to reduced intracellular content of polyamines. We therefore examined the effect of polyamine (50 μM SPD) supplementation on cell growth in normal and SSAT-expressing cells. Examination of polyamine levels in control cultured cells supplemented with SPD compared with levels in cells without the supplement indicates that the SPD content of the former was increased by $\sim 9\%$ in $24$-h, $31\%$ in $48$-h, $55\%$ in $72$-h, and $20\%$ in $96$-h control cultures (1,100 to 3,500 pmol/10$^6$ cells). Similarly, the SPM content of the control cultures was increased by $\sim 1\%$ in $24$-h, $1\%$ in $48$-h, $9\%$ in $72$-h, and $6\%$ in $96$-h control cultures (100 to 1,300 pmol/10$^6$ cells). However, SPD supplementation did not lead to increased cellular SPD or SPM content in SSAT-expressing cells and, indeed, led to increased accumulation of AC-SPM in 24-h samples ($\sim 37\%$) and increased putrescine buildup (2- to 19-fold) in samples from all time points. These results are most likely due to prompt acetylation of incoming polypeptides by SSAT such that as soon as they enter the cell, they are immediately acetylated and exported or shunted to the PAO pathway. Addition of SPD had no effect on the proliferation of normal cells and did not affect the growth of SSAT-expressing cells (Fig. 3A). Furthermore, comparison of cell cycle profiles of SSAT-expressing cells at 72 h after addition of tetracycline and time-matched control cultures in the absence or presence of SPD revealed that its supplementation does not affect the cell cycle distribution of either the control (Fig. 3B, iii) or SSAT-overexpressing cells (Fig. 3B, iv) compared with control or SSAT-expressing cells in the absence of SPD supplementation (Fig. 3B, i and ii). Other studies have indicated that supplementation with putrescine or SPM also has no effect on cell growth in normal and SSAT-expressing cells (data not shown). These results suggest that polyamine supplementation alone is not sufficient to overcome the adverse effects of SSAT expression.

SSAT-expressing cells produce increased $H_2O_2$ levels. Previous studies in our laboratory (53) indicated that increased expression of SSAT is accompanied by increased expression of PAO. Oxidation of the products of SSAT reaction (acylated SPD and SPM) by PAO may contribute to cell damage through the production of cytotoxic compounds such as $H_2O_2$. We therefore compared the production of $H_2O_2$ by SSAT-expressing cells with that by control cells. Examination of $H_2O_2$ concen-
trations in culture supernatants indicated that the production of H$_2$O$_2$ by tetracycline-induced SSAT-expressing cells was 2.5- and 1.5-fold higher than control cells at 24 (0.56 ± 0.02 vs. 0.26 ± 0.02 μmol/10$^6$ cells) and 48 h (0.31 ± 0.02 vs. 0.17 ± 0.01 μmol/10$^6$ cells) after induction of SSAT (Fig. 4). Furthermore, studies qualitatively comparing control and SSAT-expressing cells also revealed the latter to produce higher levels of H$_2$O$_2$ at both 24- and 48-h time points (data not shown).

We next examined the effect of inhibition of PAO, the activity of which leads to the production of H$_2$O$_2$ and other toxic molecules such as acetylaminopropanol and putrescine, on cell proliferation. The activity of PAO was inhibited with the use of MDL 72527 (50 μM), a proven inhibitor of polyamine and spermine oxidases (SMO) (43). The comparison of cell proliferation in control and SSAT-expressing cells revealed that treatment with MDL 72527 does not affect the SSAT-mediated reduction in cell proliferation (data not shown), suggesting that the inhibition of PAO per se does not reverse the reduction in cell growth. These results suggest that products of PAO-catalyzed reactions are not the primary cause of cellular injury brought about by SSAT overexpression, but rather they may contribute to increased injury in cells that are physiologically compromised due to polyamine depletion.

SSAT overexpression leads to DNA damage and activates the DNA repair response. Polyamines are crucial to the maintenance of DNA structure and integrity (5, 19, 20). We therefore hypothesized that G$_2$ arrest in SSAT-expressing HEK-293 cells might be due to changes in the DNA integrity. To determine the effect of SSAT overexpression on DNA integrity, we subjected control cells and cells expressing high levels of SSAT to neutral comet assay. Comparison of the tail moments (quantitative measure of DNA integrity) indicated that the degree of DNA damage was significantly higher ($P < 0.01$) in SSAT-expressing cells than in time-matched control cells. Our results indicate that the DNA of cells overexpressing SSAT is damaged (Fig. 5, a and b). In comparison, the DNA of control cells remained intact as evidenced by the absence of a comet tail (Fig. 5, c and d).

Cells with damaged DNA initiate DNA repair process and, if the damage is too extensive, may undergo apoptosis. The DNA repair process is initiated via the activation of a cascade of kinases that ultimately affect the cell cycle and apoptosis machinery. HEK-293 cells used in these studies have a defective p53 pathway (48, 49, 58). In support of this observation, our results indicate that the expression of p53 and p21 was unaffected in control and SSAT-overexpressing cells (data not shown). The role of p53 in SSAT-mediated inhibition of HEK-293 cell proliferation was further studied by examining the effect of pifithrin-α, a p53 inhibitor, on cell proliferation. The addition of pifithrin-α did not affect the proliferation rate of control or SSAT-overexpressing HEK-293 cells (data not shown).

Alterations in DNA structure and integrity activate the cell cycle checkpoints (5, 18, 19). Since SSAT-overexpressing cells have increased DNA damage (Fig. 5), we examined the effect of elevated SSAT expression on the activation of DNA repair response. DNA damage in response to oxidative stress leads to activation of ATM and ATR kinases. Activated ATM/ATR kinases in turn phosphorylate and activate Chk1 and Chk2 kinases. The activation of Chk1 and Chk2 leads to cell cycle arrest by regulating the activities of Cdc25 phosphatase and Cdc2 kinase (5, 18, 19). Our results demonstrate that
SSAT expression led to an increase in the levels of phosphor-
ylated Chk1 and Chk2 (Fig. 6). In comparison, activated Chk1
and Chk2 levels remained very low in the control cultures (Fig.
6). The increase in the active (phosphorylated) form of these
kinases was apparent as early as 12 h after induction of SSAT.
Our data indicate that the peak activation of these enzymes
was observed by day 3 following induction of SSAT and remained
elevated for the duration of our studies. These results suggest
that ATM/ATR DNA repair pathways are activated as the
result of SSAT expression in HEK-293 cells.

Determining the effect of SSAT expression and the onset of
polyamine anabolic pathway on DNA methylation. The inducti-
on of synthesis of polyamines to compensate for the increase
in their catabolism leads to increased consumption of S-adeno-
sylmethionine (SAM) and increased production of decarboxy-
lated SAM (dcSAM). The former is a substrate needed for
DNA methyltransferases, and the latter is an aminopropyl
group donor for polyamine synthesis as well as an inhibitor of
DNA methyltransferases. A compensatory increase in poly-
amine synthesis as evidenced by increased ODC and SAMDC
activity leads to depletion of SAM and an increase in dcSAM
levels; together, these may contribute to a reduction in DNA
methylation and the induction of DNA anomalies (10, 15, 34).
To test the above suggestion, we examined the effect of
SSAT expression on global genomic DNA methylation
using COBRA. Comparing the amplification products of
LINE-1 elements from control and SSAT-expressing cells for
their sensitivity to digestion with TaqI and TasI indicates that
the methylation of LINE-1 elements is not different in SSAT-
expressing cells and control cells (data not shown). These
results seem to rule out the role of reduced DNA methylation
as the primary mechanism of DNA damage in SSAT-overex-
pressing cells in our system.

MAPK signaling is disrupted in SSAT-expressing cells. To
further decipher the signaling pathway involved in SSAT-
methyl mediated inhibition of cell proliferation, we decided to exa-
mine the activation status of ERK. We chose to examine the
ERK activation pathway, since the Raf → MEK → ERK
pathway has been implicated in the upregulation of expression
of cell cycle machinery and promotion of cell division (33, 55).
Examination of extracts obtained from control and SSAT-
overexpressing HEK cells (Fig. 7) indicates that both popula-
tions express equal amounts of ERK1 and ERK2 proteins.

Fig. 2. Effect of SSAT on cell cycle progression of HEK cells. SSAT expression was induced in semiconfluent monolayers by treatment with tetracycline (1
μg/ml in 70% ethanol). Control cultures were treated with identical volume of solvent (70% ethanol). Flow cytometric analysis of DNA content of the cells was
used to examine the cell cycle distribution of control HEK cells on days 4 (A) and 6 (C) and of HEK cells that overexpress SSAT on days 4 (B) and 6 (D). The
first (left) peak indicates the number of cells with 2n DNA content (G1 population), the second peak represents the cells with 4n DNA content (G2 population),
and the third peak (far right, only present in B and D) represents cells with 8n DNA content (endoreduplicated cells). The increase in the height of the second
and third peaks observed in the SSAT overexpressers (B and D) indicates that SSAT overexpression, possibly through polyamine depletion and/or increased
expression of toxic metabolites, leads to G2 block and cell cycle arrest. Similar results were obtained in 3 independent experiments.
Examination of the same extracts for the presence of the active (phosphorylated) form of ERK1 and ERK2 indicates that SSAT overexpression leads to a reduction in ERK activation as early as day 3 postinduction and a drastic reduction in the cellular levels of activated ERK1 and ERK2 by days 6 and 9 post-SSAT induction. These results suggest that the ERK signaling pathway, which plays an important role in regulation of cell proliferation, is disrupted in cells that express SSAT; however, the lateness of disruption in ERK signaling suggests that it is not the primary mechanism involved in the mediation of G2 arrest of SSAT-expressing cells.

**Effect of SSAT expression on cyclin B1 levels and Cdc2 activation.** The transition from G2 to M and completion of mitosis depends on the activity of cyclin B-Cdc2 complex (40,
Activated as the result of ATM and ATR activation, Chk kinases directly modulate the function of Cdc25 through phosphorylation of its Ser-216 and prevent it from dephosphorylating Cdc2 (1, 8, 56, 57). ERK activity, on the other hand, is involved in the regulation of a number of events that are required for G2/M transition and completion of mitosis (31, 33, 55). These events include, but are not limited to, the regulation of cyclin B-Cdc2 activity via the modulation of Wee1 and Myt1 (11, 57). To determine whether activation of ATM/ ATR → Chk1/2 and disruption of ERK signaling due to expression of SSAT affect cyclin B-Cdc2 signaling, we examined the levels of cyclin B1, Cdc, and activated Cdc2 (Thr-161 phosphorylated) in cell extracts obtained from control and SSAT-expressing HEK-293 cells. Our results indicate that the induction of SSAT reduces the expression of cyclin B1 and interferes with the activation of Cdc2 (Fig. 8).

DISCUSSION

Expression of SSAT increases significantly in organs (e.g., brain, liver, and kidney) subjected to IRI and in cells after ATP depletion and metabolic poisoning (4, 21, 59). We previously demonstrated that increased expression of SSAT can lead to oxidative stress, reduced cell proliferation, and impaired cell adhesion (53). In the current study, we have examined the effect of SSAT on polyamine homeostasis and determined how its increased levels affect cell proliferation. Our results demonstrate that SSAT expression disrupts the polyamine homeostasis in HEK-293 cells, leading to the depletion of SPD and SPM and a concomitant increase in the acetylated forms of these molecules that are either excreted or shunted to the PAO pathway for degradation (Table 2). Furthermore, we observed a compensatory response in SSAT-expressing cells indicating the activation of polyamine anabolic pathways aimed at reestablishment of normal polyamine levels (Table 1). Comet assay data indicate that SSAT-expressing cells show DNA damage (Fig. 5). The patterns of the comet assays for SSAT-expressing cells are similar to those of viable cells with DNA damage and not apoptotic cells (7, 24). Our studies also indicate that increased SSAT expression leads to the activation of ATM/ ATR pathway 12 h postinduction (Fig. 6), suggesting that DNA damage is detected by checkpoint machinery early after SSAT induction. In addition to activation of the ATM/ATR DNA repair response, SSAT overexpression disrupts the activation of ERK1 and ERK2 (Fig. 7). The aforementioned
signaling cascades are known to affect the cell cycle (1, 8, 31, 33, 55–57) and mediate the onset of G2/M transition arrest in SSAT-expressing cells.

Polyamines play an important role in the regulation of DNA structure, modulation of signal transduction, gene expression, and protein synthesis (20, 25, 26, 32). They are necessary for cell growth, and their depletion reduces cell proliferation. Previous studies indicated that inhibition of polyamine synthesis by α-difluoromethylornithine (DMFO), an inhibitor of ODC, leads to G1 arrest (3, 29, 30, 39). Cells treated with DMFO exhibit increased expression levels of p53 as well as cyclin-dependent kinase inhibitors p16, p21, and p27 (3, 29, 30, 39). In contrast to DFMO treatment, increased expression of SSAT leads to G2 arrest (42). A number of factors may account for the growth arrest induced by SSAT, including depletion of polyamines, SAM, and acetyl-CoA or increased production of toxic by-products of polyamine catabolism (e.g., H₂O₂ and aldehydes). Examination of cell lines from different origins indicates that all of the aforementioned factors contribute to reduced proliferative activity of LN-CaP prostate carcinoma cells after induction of SSAT appears to be due to depletion of metabolic precursors needed for polyamine synthesis (27, 52).

Our studies indicate that increased SSAT expression leads to DNA damage (Fig. 5). The SSAT-induced DNA damage may be mediated through multiple mechanisms including polyamine depletion and/or increased production of H₂O₂. Our results demonstrating that the addition of exogenous polyamines had no effect on the proliferative activity of SSAT-expressing HEK-293 cells (Fig. 3) indicate that polyamine supplementation alone does not overcome the effects of increased SSAT activity. This is most likely due to immediate acetylation of incoming polyamines that are then either shunted to the PAO pathway or exported out of the cell. This possibility is supported by our data indicating that polyamine supplementation leads to increased cellular content of polyamines in control but not SSAT-expressing cells. The H₂O₂ generated during polyamine catabolism results from the activity of PAO and SMO. Shunting of acetylated polyamines to the PAO or SMO pathway leads to increased production of H₂O₂ and other toxic metabolites that may play a significant role in the mediation of DNA damage and reduction in cell proliferation. Previous studies by our group (51) using catalase suggested...
that H$_2$O$_2$ does not play a significant role in cell growth and proliferation. We therefore examined the effect of inhibition of PAO on cell proliferation in SSAT-expressing cells. The inhibition of PAO fails to restore the proliferative activity in SSAT-expressing HEK cells (data not shown), confirming published reports (24) and supporting the possibility that the PAO-catalyzed reaction that leads to the production of H$_2$O$_2$ and other toxic metabolites does not play a primary role in the induction of DNA damage and reduction of cell proliferation, at least in vitro. Transcript levels of the polyamine oxidases PAO and SMO increase in kidneys subjected to IRI (Ref. 55; unpublished data). The inhibition of these enzymes leads to reduced tissue damage in cerebral IRI, suggesting that they play a significant role in the induction of tissue damage in ischemic conditions in vivo (16). Coupled to studies that indicate the induction of PAO (49) but not SMO (data not shown) in SSAT-expressing HEK cells, we propose that the dramatic reduction in polyamine levels plays a primary role in the mediation of cell damage. It is very plausible that PAO activation exacerbates the cell damage by increasing the production of H$_2$O$_2$ and other toxic metabolites.

The depletion of SAM levels also may contribute to reduced growth rate in SSAT-expressing cells (27). It is plausible that the upregulation of polyamine synthesis (increased activity of ODC and SAMDC) to compensate for the increase in their catabolism leads to increased consumption of SAM and increased production of dcSAM (17, 22, 23, 27, 51). The former is a substrate needed for DNA methyltransferases, and the latter is an aminopropyl group donor for polyamine synthesis as well as an inhibitor of DNA methyltransferases (17, 22, 23). Therefore, a compensatory increase in polyamine synthesis through depletion of SAM and an increase in dcSAM levels may lead to a reduction in the methylation levels of cellular DNA (17, 22, 23), potentially leading to increased DNA damage, genomic instability, and growth arrest (10, 15, 34). However, our data indicate that increased SSAT expression in HEK cells does not affect the state of DNA methylation (Fig. 7) and that DNA methylation anomalies are not involved in the mediation of DNA damage in our model system.

The depletion of polyamine content and increased H$_2$O$_2$ production correlate closely with the activation of DNA repair...
checkpoint enzymes. However, since PAO inhibition does not lead to normalization of growth rate, we propose that the induction of PAO and increased production of H$_2$O$_2$ in SSAT-expressing cells are not of primary importance and, in isolation, do not account for the DNA damage and reduction in cell growth in the current model. Our results support the hypothesis that polyamine depletion is the primary factor that affects DNA integrity and cell proliferation in SSAT-overexpressing cells. Previous studies demonstrating that depletion of SPM leads to DNA strand breaks and that polyamine depletion leads to modification of chromatin and DNA structure, increases susceptibility to damage by genotoxic agents, and interferes with DNA repair support our hypothesis that depletion of SPD and SPM is the primary factor involved in the induction of DNA damage and growth arrest in SSAT-expressing cells (2, 45, 47, 54).

SSAT levels increase in tissues (e.g., kidney, brain, and liver) subjected to hypoxia reoxygenation injuries (4, 21, 59). A possible effect of SSAT expression in IRI may be the induction of DNA lesions. In our model, increased SSAT expression leads to DNA damage and cell cycle arrest as evidenced by cell cycle analysis and comet assay results. It is therefore possible that SSAT production partially contributes to the induction of DNA lesions in IRI. The mechanisms and signaling cascades induced by increased DNA damage due to elevated levels of SSAT and polyamine depletion involved in G$_2$/M transition arrest were therefore examined. Our findings indicate that the DNA damage repair response (ATM/ATR → Chk1/Chk2) is activated in SSAT-expressing cells. The role of ATM/ATR signaling in DNA damage repair subsequent to hypoxia reoxygenation has been examined (18, 19). The activation of ATM/ATR → Chk1/Chk2 cascades in response to DNA damage leads to inactivation of Cdc2, disruption of Cdc2 activity also compromises the proliferative activity of SSAT-expressing cells. Our results suggest that in our system, the primary driving mechanism behind the increased DNA damage and growth reduction is the depletion of cellular polyamines, whereas increased H$_2$O$_2$ production may play a secondary role and DNA methylation plays no role in the induction of DNA damage and reduced cell proliferation. The components of the proposed scheme that contribute to reduced cell proliferation in our model system are shown in boxes with bold outlines.

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Fig. 9. Schematic of potential pathways affected by SSAT that mediate its antiproliferative activity. In our proposed scheme, increased SSAT expression leads to enhanced catabolism of SPD and SPM (polyamine depletion). Catabolism of polyamines through polyamine oxidase (PAO) activity leads to enhanced production of H$_2$O$_2$. In addition, the onset of a compensatory response aimed at reestablishing polyamine homeostasis may lead to a reduction in S-adenosylmethionine (SAM) levels and a buildup of decarboxylated SAM (dcSAM) and reduced DNA methylation. These alterations may lead to DNA damage, ATM/ATR → Chk1/2 pathway activation, and G$_2$/M arrest. Polyamine depletion also disrupts the Raf → MEK → ERK pathway; based on the role of Raf → MEK → ERK in G$_2$/M transition, the disruption of this pathway may also adversely affect cell proliferation. In addition, downregulation of cyclin B1 expression and Cdc2 activity also compromises the proliferative activity of SSAT-expressing cells. Our results suggest that in our system, the primary driving mechanism behind the increased DNA damage and growth reduction is the depletion of cellular polyamines, whereas increased H$_2$O$_2$ production may play a secondary role and DNA methylation plays no role in the induction of DNA damage and reduced cell proliferation. The components of the proposed scheme that contribute to reduced cell proliferation in our model system are shown in boxes with bold outlines.

SSAT EXPRESSION CAUSES DNA DAMAGE AND BLOCKS CELL PROLIFERATION

ATM/ATR signaling in DNA damage repair subsequent to hypoxia reoxygenation has been examined (18, 19). The activation of ATM/ATR → Chk1/Chk2 cascades in response to DNA damage leads to inactivation of Cdc2, disruption of Cdc2 activity, and G$_2$ arrest (1, 8, 56, 57). Our data also indicate that ERK activation is disrupted in cells that express SSAT. The Raf → MEK → ERK pathway plays an integral role in the modulation of inhibitory phosphorylation of Cdc2 by Wee1 and Myt1 and in G$_2$/M transition (11, 57). Dysregulation of Cdc2 activity may be an additional mode of induction of G$_2$ arrest in cells that express SSAT. In our studies, we observed a decrease in the cellular content of cyclin B1 and Cdc2 in SSAT-expressing cells. Hence, depletion of polyamines can potentially modulate both the expression of cyclin B1 and the activation of cyclin B1-Cdc2 complex.

Increased SSAT levels seem to affect multiple pathways that regulate cell division. The schematic in Fig. 9 outlines the potential mechanisms that may be involved in the mediation of the effect of SSAT on cell proliferation (i.e., depletion of cellular contents of precursors of polyamines and polyamines, changes in DNA, and increased levels of toxic metabolites). Our results suggest that SSAT expression enhances the catabolism of SPD and SPM, leading to depletion of polyamine levels, which may lead to changes in chromatin structure and increased DNA instability through a reduced capacity to buffer the repulsion of negatively charged DNA backbone. In addition, in SSAT-expressing cells, the DNA integrity may be compromised through the production of toxic metabolites such as peroxides and free radicals, leading to DNA damage and cell cycle arrest.

**C1213**

**AJP-Cell Physiol • VOL 292 • MARCH 2007 • www.ajpcell.org**
as H$_2$O$_2$. However, the latter mechanism is at best of secondary importance in our model system. The DNA damage in SSAT-expressing cells leads to activation of ATM/ATR → Chk1/Chk2 pathways and G2/M arrest. The mechanism by which SSAT expression downregulates MAPK activation and the importance of disruption of this signaling cascade to early events involved in the modulation of cell proliferation remain to be elucidated. However, based on the role of Raf → MEK → ERK in G2/M transition (11, 57), it is not surprising that the disruption of this pathway also adversely affects cell proliferation.

The current studies begin to address how SSAT contributes to the pathophysiology of renal IRI at the molecular level. We propose that in IRI, one of the mechanisms through which increased SSAT expression contributes to tissue damage is disruption of polyamine homeostasis and induction of DNA damage. Furthermore, it is possible that reduction in cell proliferation due to enhanced expression of SSAT can interfere with the tubular repair process and contribute to ongoing renal damage. Other potential effects of increased SSAT levels and disturbances in polyamine homeostasis such as disruption of cell-cell and cell-matrix interactions as well as metabolic anomalies are currently being studied and need to be considered when examining the role of SSAT in IRI.

GRANTS

These studies were supported by National Institutes of Health Grants CA-22153 and CA-76428 (to C. W. Porter), DK061458 (to J. J. Bissler), and DK-66589 and Department of Veterans Affairs Merit Review Award (to M. Soleimani).

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