# Increased sensitivity to oxidative injury in Chinese hamster ovary cells stably transfected with rat liver S-adenosylmethionine synthetase cDNA

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Chinese hamster ovary cells were stably transfected with rat liver S-adenosylmethionine synthetase cDNA. As a result, S-adenosylmethionine synthetase activity increased 2.3-fold, an effect that was accompanied by increased S-adenosylmethionine, a depletion of ATP and NAD levels, elevation of the S-adenosylmethionine/S-adenosylhomocysteine ratio (the methylation ratio), increased DNA methylation and polyamine levels (spermidine and spermine), and normal GSH levels. By contrast, the transfected cells showed normal growth curves and morphology. Exposure to an oxidative stress by the addition of $H_2O_2$ resulted in a greater consumption of ATP and NAD in the transfected cells than in the wild-type cells. In turn, cell killing by $H_2O_2$ was greater in the transfected cells than in the wild-type cells. This killing of Chinese hamster ovary cells by $H_2O_2$ involved the activation of poly(ADP-ribose) polymerase with the resultant loss of NAD and ATP. 3-Aminobenzamide, an inhibitor of poly(ADP-ribose) polymerase, but not the antioxidant *N*,*N*′-diphenylphenylenediamine, prevented the killing of Chinese hamster ovary cells by $H_2O_2$ and maintained the contents of NAD and ATP. The results of this study indicate that a moderate activation of the synthesis of S-adenosylmethionine leads to ATP and NAD depletion and to a greater sensitivity to cell killing by oxidative stress.

## INTRODUCTION

The methylation cycle involves the conversion of methionine into homocysteine, via S-adenosyl-L-methionine (AdoMet) and S-adenosyl-L-homocysteine (AdoHcy) (reviewed in [1,2]). This cycle serves three major cellular functions. First, it provides AdoMet for the synthesis of polyamines and for the methylation of a variety of cellular constituents, including phospholipids, methyl-accepting proteins, CpG islands in DNA, and adrenergic, dopaminergic and serotoninergic molecules. Secondly, in the liver the methylation cycle feeds the trans-sulphuration pathway that forms glutathione (GSH) from homocysteine. Finally, the methylation cycle functions in the conversion of 5-methyltetrahydrofolate to tetrahydrofolate, a necessary cofactor for the synthesis of DNA and RNA.

Several conditions and compounds impair the methylation cycle. Various chronic liver disorders, including alcoholic [3], biliary [4] and post-hepatic cirrhosis [3], decrease the activities of AdoMet-synthetase, the enzyme that makes AdoMet from methionine and ATP, and phospholipid methyltransferase [3], the enzyme that makes phosphatidylcholine from phosphatidylethanolamine. Numerous drugs, including anti-cancer and antiviral agents, inhibit AdoHcy-hydrolase [5,6], the enzyme that hydrolyses AdoHcy to produce homocysteine and adenosine. Finally, several hepatotoxins, including alcohol, carbon tetrachloride, galactosamine and acetaminophen are detoxified by GSH, and the depletion of this reducing agent inactivates liver AdoMet-synthetase (reviewed in [1]).

AdoMet plays a central role in the methylation cycle, by controlling both the remethylation of homocysteine to methionine, as well as its catabolism through the trans-sulphuration pathway [7–11]. Accordingly, the synthesis and utilization of AdoMet by the liver needs to be carefully controlled. In the present report, we have studied the effect of an increased production of AdoMet as a result of the expression of rat liver AdoMet-synthetase cDNA in Chinese hamster ovary (CHO) cells. An increased synthesis of AdoMet is accompanied by a depletion of cellular ATP and NAD, elevated AdoMet levels, and enhanced DNA-methylation and polyamine levels. Whereas overproduction of AdoMet had no effect on cellular growth, CHO cells transfected with rat liver AdoMet-synthetase cDNA were more susceptible to the development of the lethal cell injury that results from exposure to an acute oxidative stress.

## EXPERIMENTAL

### General procedures

Standard procedures were used for plasmid purification, DNA labelling, isolation of RNA, and Northern blot hybridization [12,13]. Oligonucleotide primers were synthesized on an Applied Biosystems 391 DNA synthesizer using the phosphoramidite method.

### Cell growth assay

Cell number was determined using a dye-binding assay, as described previously [14]. Briefly, cells were plated in 24-well plates at a density of approx. $10^4$ cells/well in the presence of

Abbreviations used: ABA, 3-aminobenzamide; AdoMet, S-adenosyl-L-methionine; AdoHcy, S-adenosyl-L-homocysteine; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; DPPD, diphenylphenylenediamine; LDH, lactate dehydrogenase; TCA, trichloroacetic acid.

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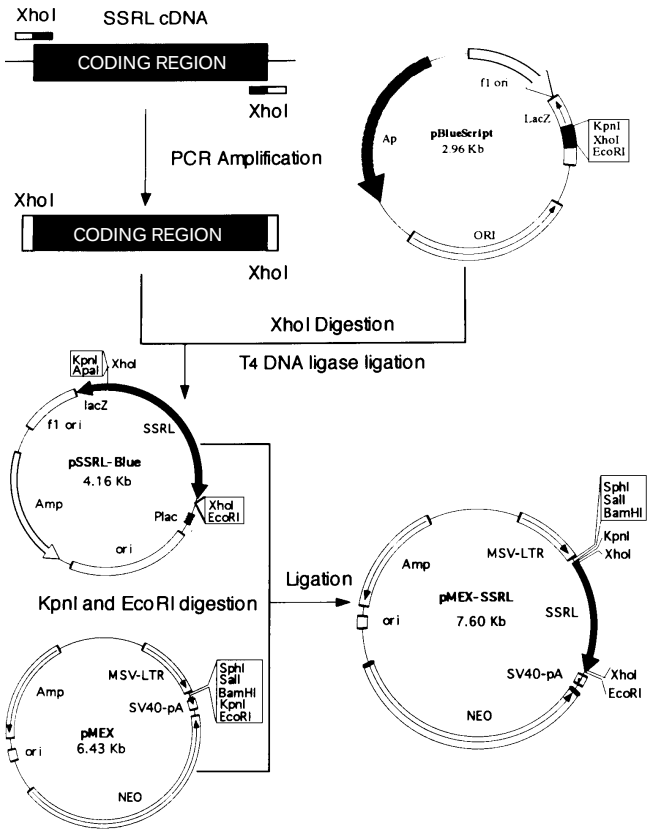


**Figure 1 Construction of the AdoMet-synthetase expression vector**

A PCR fragment containing the coding region of rat liver AdoMet-synthetase (SSRL) was ligated to Bluescript to be then subcloned into the eukaryotic expression vector pMEX-neo. The resulting recombinant plasmid was designated pMEX-SSRL. Details are given in the Experimental section.

10 % or 0.5 % fetal-calf serum. At the times indicated, cells were fixed with 1 % glutaraldehyde and stained with 0.1 % Crystal Violet. Subsequently, plates were washed with a continuous stream of deionized water and air-dried. Cell numbers were estimated by solubilizing the dye in 10 % acetic acid and measuring absorbance at 590 nm.

### Construction of the AdoMet-synthetase expression vector

A 2.3 kb *Eco*RI fragment of the rat liver AdoMet-synthetase clone pSSRL [15] was initially adapted for cloning into the *Xho*I site of the vector Bluescript by means of the PCR (Figure 1). The 5′ PCR primer (5′-GCACTCGAGATGAATGGACCTGTG-3′) consisted of an unannealed sequence containing a *Xho*I restriction site at the 5′-end (underlined) and a 15-nucleotide sequence homologous to bases 1–15 of the rat liver AdoMet-synthetase clone pSSRL. The 3′ PCR primer (5′-GCACTCGAGGCTCTAAAACACAAG-3′) had a *Xho*I site at the 5′-end (underlined) and a 15-nucleotide sequence complementary to bases 1183–1197 of the pSSRL clone. The PCR product was purified, digested with *Xho*I and ligated to the plasmid Bluescript to allow subsequent subcloning into the *Kpn*I–*Eco*RI sites of the eukaryotic expression vector pMEX-neo. The resulting construct was designated pMEX-SSRL. The identity of the entire coding region was confirmed by sequencing using the dideoxynucleotide chain-termination method [16].

### Isolation of stable transfected cells

CHO cells ($10^6$) were plated on 100-mm-diam. dishes and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum. Duplicate plates were then transfected with 20 $\mu$g of either pMEX-neo or pMEX-SSRL by the calcium phosphate precipitation method [13]. After transfection, cells were cultured for 18 h at 37 °C in a humidified 2 % $CO_2$ atmosphere. Cells were then rinsed and incubated in serum-containing medium for 24 h before neomycin (G418) was added in fresh medium at 0.7 mg/ml. The medium containing decreased concentrations of antibiotic was changed every 3 days. After 15 days, colonies of cells resistant to G418 developed. For each plasmid transfected, 24 individual colonies (identified by a letter) were isolated using cloning cylinders and trypsin. These colonies were transferred to 35-mm-diam. dishes, cultured to near confluence in transition to 35- to 60- to 100-mm-diam. plates, and assayed for AdoMet-synthetase expression.

### Western blot analysis of extracts from wild-type and pMEX-SSRL-transfected CHO cells

Wild-type CHO cells, stable transfectants, and rat liver were homogenized in 10 mM Tris/HCl buffer, pH 7.5, containing 0.3 M sucrose buffer. Aliquots of 20 $\mu$l of the cytosolic fractions were loaded on to SDS/10 %-polyacrylamide gels and electrophoresed as described [17]. Proteins were electrotransferred to nitrocellulose membranes using 20 mM Tris/HCl buffer, pH 7.5, containing 20 % (v/v) methanol. AdoMet-synthetase was detected using a rabbit antiserum raised against the rat liver purified enzyme [18] and goat anti-(rabbit IgG) horseradish peroxidase-conjugated antibody. Blots were developed by chemiluminescence using DuPont New England Nuclear Renaissance reagents.

### Determination of AdoMet-synthetase activity

AdoMet-synthetase activity was measured using the cytosolic fraction of the wild-type and stable transfectants. Briefly, $6 \times 10^6$ cells were homogenized in 800 $\mu$l of 10 mM Tris/HCl buffer, pH 7.5, containing 0.3 M sucrose, 1 mM benzamidine, 0.1 mM PMSF, 10 $\mu$g/ml soybean trypsin inhibitor and 0.1 % 2-mercaptoethanol. The cytosol was obtained by centrifugation. The activity was measured as described by Cabrero et al. [19] using saturating concentrations of each of the substrates, 5 mM methionine and 5 mM ATP.

### Determination of AdoMet and AdoHcy

Cells ($6 \times 10^6$) were labelled with 3 $\mu$Ci of [$^{35}$S]methionine (1225 Ci/mmol) for 30 min before being scraped and deproteinized by homogenization in 1 ml of 10 % trichloroacetic acid (TCA)/0.05 M HCl at 4 °C as described previously [20]. TCA was eliminated by three successive washes with diethyl ether saturated with 0.05 M HCl, and the samples were lyophilized. Samples were then resuspended in 100 $\mu$l of 10 mM ammonium formate, pH 4.0, and analysed by HPLC using an Ultrasil CX column (46 mm × 250 mm; particle size 10 $\mu$m) as described previously [20]. The AdoMet and AdoHcy content was calculated by measuring their absorbance at 254 nm, and their recovery after TCA/diethyl ether extraction. The AdoMet/AdoHcy ratio was determined by measuring the amount of radioactivity associated with both fractions.

### DNA methylation assay

Differences in the extent of *in vivo* DNA methylation in CHO wild-type cells and stable transfectants were determined following the method of Christman et al. [21], which quantifies *in vitro* transfer of radiolabelled methyl groups from AdoMet to sites in DNA that were not methylated *in vivo*. DNA was extracted by a standard technique [12]. The reaction mixture, containing 2 $\mu$g of DNA, 5 $\mu$Ci of [$^3$H-*methyl*]AdoMet and 2 units of *Sss*I methylase from *Escherichia coli* (New England Biolabs, Beverly, MA, U.S.A.), a C5-DNA methyltransferase that acts at the same site of cytosine residues in both hemimethylated and fully methylated CpG sites, was incubated for 3 h at 37 °C.

To explore also possible differences in specific DNA methylation, samples of DNA were subjected to digestion with either 25 units of *Msp*I, which is insensitive to CC$^+$GG methylation, or *Hpa*II, which is sensitive to methylation, for 8 h at 37 °C. The relative degree of methylation at CCGG sequences was assessed by comparing the restriction patterns from total DNA after fractionating on 1 % agarose gel. At least six different DNA preparations isolated from six independent passages were assayed.

### Determination of glutathione, ATP and NAD levels

Cells were washed twice with PBS before being scraped and resuspended with PBS. Perchloric acid 20 % was added immediately to a final concentration of 2 %. Samples were centrifuged for 10 min at 3500 rev./min and the acidic supernatants were used for GSH measurements. GSH was measured by the GSH-transferase method [22]. ATP content was determined with the use of luciferin–luciferase as described previously [23]. The content of NAD was determined by the method described previously [24].

### Determination of polyamine levels

Cells [$(15–20) \times 10^6$] were homogenized in 1 ml of 10 mM Tris/HCl buffer, pH 7.5, containing 0.3 M sucrose. An equal volume of 10 % TCA/20 % methanol solution (v/v) was added, as well as 50 nCi of [$^{14}$C]putrescine, 50 nCi of [$^{14}$C]spermine and 50 nCi of [$^{14}$C]spermidine. The samples were centrifuged at 3000 ***g*** for 10 min, and the supernatant extracted three times using water saturated with diethyl ether before lyophilization. The samples were dissolved and derivatized as described by Malaisse et al. [25] prior to HPLC separation using an Ultrasphere ODS column (46 mm $\times$ 250 mm; particle size 10 $\mu$m), using a flow rate of 1.5 ml/min as described by Brown et al. [26]. The polyamine content was determined by measuring the fluorescence emitted at 518 nm using an excitation wavelength of 347 nm.

### Killing of CHO cells by $H_2O_2$

$H_2O_2$ (Sigma) was dissolved in the culture medium and added to the wild-type and transfected CHO cells at the concentrations indicated in the text. Diphenylphenylenediamine (DPPD) was dissolved in DMSO, diluted with medium, and added to the cultures at a final concentration of 1 $\mu$M (final DMSO concentration was 0.005 %). 3-Aminobenzamide (ABA) was dissolved in medium and added to the cultures at a final concentration of 10 mM. Viability was defined as the ratio of the lactate dehydrogenase (LDH) released into the medium to the total that could be released from the culture by Triton X-100. A correction was made for any effect of the treatment on the growth of the cells or on the content of LDH [27].

## RESULTS

### Generation and selection of CHO stable cell lines expressing hepatic AdoMet-synthetase

CHO cells were transfected with the pMEX-SSRL vector containing a cDNA insert encoding rat liver AdoMet-synthetase. Stable transfectants were selected in the presence of neomycin. As revealed by Northern blot analysis (results not shown) 16 out of 24 randomly picked transfectants accumulated an mRNA species of the size expected for the AdoMet-synthetase transcript. However, only seven of the 16 contained a protein that cross-reacted with an antibody raised against rat liver AdoMet-synthetase and that was identical in size to the native hepatic protein (Figure 2). Figure 2 is representative of six independent determinations with each of the stable transfectants and with the wild-type CHO cells. The reaction between the antibody to liver AdoMet-synthetase and the AdoMet-synthetase endogenous to the CHO cells was always very faint or undetectable. This was the case even when the concentration of cytosolic protein from the wild-type CHO cells loaded on to the SDS/PAGE was increased 4-fold. When cytosolic proteins derived from rat kidney or spleen were subjected to immunoblotting with the antibody raised against rat liver AdoMet-synthetase, no signal from either tissue was detected (results not shown). Since the enzyme expressed in the liver is tissue specific [28], these results suggest that the differences in Figure 2 between the transfectants and the wild-type CHO cells are mainly a specificity difference and not a concentration effect. One stable transfected clone (ST-C) that expressed the highest levels of hepatic AdoMet-synthetase was selected for functional studies. In order to test the stability of the expression level, stable transfected cells were maintained for 60 passages (up to 7 months). As assessed by Western analysis, the amount of the expressed protein remained constant for the above-mentioned period.

The growth curves of the stably transfected clone ST-C and of wild-type CHO cells were identical, both in the presence of high (10 %) or low (0.5 %) fetal-calf serum (Figure 3). By phase-

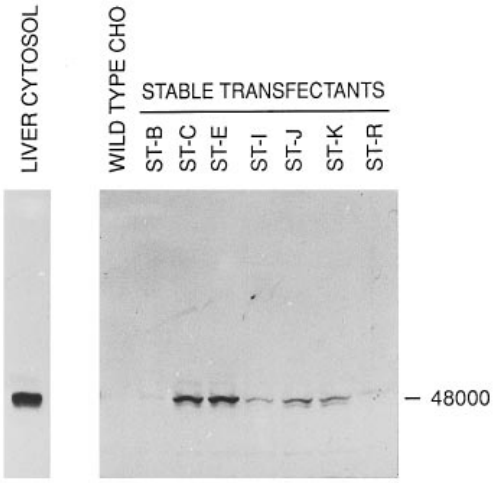


**Figure 2 Western blot analysis of extracts from wild-type and pMEX-SSRL-transfected CHO cells**

Approx. 15 $\mu$g of cytosolic protein from wild-type and stably transfected (ST) CHO cells were resolved on SDS/10 %-polyacrylamide gels. As a control, 2 $\mu$g of cytosolic protein from rat liver were included. Immunoblotting was performed on nitrocellulose membranes, using an antibody raised against rat liver AdoMet-synthetase. Molecular mass is given in daltons. The Figure shown is representative of six independent determinations.

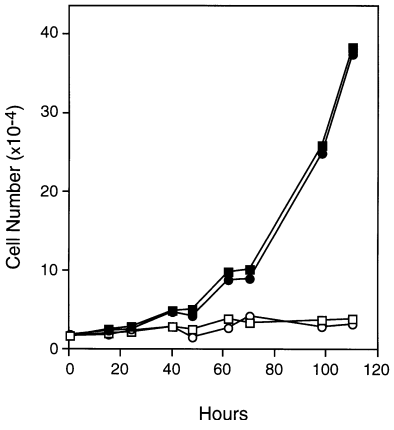


**Figure 3 Growth curves of wild-type and ST-C CHO cells**

Approx. $10^4$ cells from wild-type (circles) or ST-C (squares) CHO cells were plated on 24-well plates in DMEM supplemented with 10% fetal-calf serum (closed symbols) or 0.5% fetal-calf serum (open symbols). At the time indicated, cells were fixed with 1% glutaraldehyde and stained with 0.1% Crystal Violet. Values reported are representative of three independent measurements.

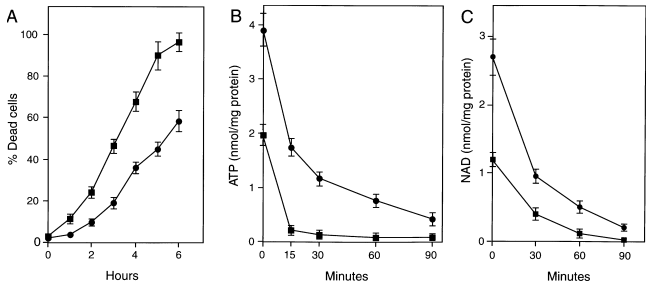


**Figure 4 Time course of the changes in viability and the contents of ATP and NAD in wild-type and ST-C CHO cells treated with $H_2O_2$**

Wild-type (circles) or ST-C (squares) CHO cells were exposed to 10 mM $H_2O_2$ and the changes in viability (**A**) and the contents of ATP (**B**) and NAD (**C**) determined at different intervals. Results are the means ± S.D. of the determinations of three separate cultures.

contrast microscopy, the morphologies of both types of cells were also indistinguishable.

## AdoMet synthesis and metabolism in wild-type and transfected CHO cells

At saturating levels of methionine and ATP, AdoMet-synthetase activity in ST-C cells was 2.3-fold higher than that in the wild-type CHO cells (Table 1). In turn, the levels of AdoMet were also 3.3-fold higher in the transfected cells than in the wild-type cells. The ratio AdoMet/AdoHcy was increased 3-fold, from 5.3 in the wild-type cells to 15.8 in the ST-C cells. Higher AdoMet content may lead to greater methylation of the DNA. To test this hypothesis, DNA methylation in ST-C cells and wild-type CHO cells was compared. The amount of [$^3$H]methyl groups incorporated into DNA isolated from ST-C cells by *Sss*I methylase was half that incorporated into DNA isolated from wild-type cells (Table 1). The degree of incorporation of exogenous labelled methyl groups into isolated DNA is inversely related to the extent of *in vivo* global DNA methylation, since the methyl groups (unlabelled) already in the DNA do not allow *de novo* methylation by *Sss*I methylase to take place. Therefore, the results in Table 1 indicate that DNA isolated from ST-C cells was hypermethylated as compared with the DNA isolated from wild-type CHO cells. Site-specific differences in DNA methylation were investigated by using the isoschizomers *Hpa*II and *Msp*I. The restriction patterns of DNA samples derived from wild-type CHO cells and ST-C cells were very similar (results not shown), thus suggesting that DNA hypermethylation in AdoMet-synthetase-transfected cells is a random event.

The levels of polyamines, ATP, NAD and GSH in ST-C cells and wild-type CHO cells are shown in Table 2. Polyamine levels (spermidine and spermine) in ST-C cells were approx. 2-fold higher than in wild-type cells. By contrast, the concentrations of ATP and NAD were reduced by more than 50% in the transfected cells. GSH levels were identical in both types of cells. Neither wild-type nor ST-C CHO cells synthesized GSH from methionine. Thus, neither cell type has an active trans-sulphuration pathway and synthesizes GSH from the cysteine in the incubation medium (results not shown).

**Table 1 Characteristics of wild-type and ST-C CHO cells**

Results are the means ± S.D. of the determinations carried out on three separate cultures. Details are given in the Experimental section.

| Clone type | AdoMet-synthetase activity (pmol/min per mg) | AdoMet content (pmol/$10^6$ cells) | AdoMet/AdoHcy ratio | [$^3$H]Methyl group incorporation into DNA (d.p.m./$\mu$g of DNA) |
|---|---|---|---|---|
| Wild type | 2.47 ± 0.18 | 255 ± 48 | 5.35 ± 0.71 | 32671 ± 2715 |
| ST-C | 5.76 ± 0.91 | 845 ± 99 | 15.76 ± 0.93 | 15981 ± 1550 |

**Table 2 Levels of ATP, NAD, GSH and polyamines in wild-type and ST-C CHO cells**

Results are the means ± S. D. of the determinations carried out on three separate cultures. Details are given in the Experimental section.

| Clone type | ATP content (nmol/mg) | NAD content (nmol/mg) | GSH content (nmol/mg) | Spermidine content (pmol/$10^6$ cells) | Spermine content (pmol/$10^6$ cells) |
|---|---|---|---|---|---|
| Wild type | 3.91 ± 0.25 | 2.84 ± 0.17 | 26.1 ± 2.4 | 142.9 ± 25.2 | 412.8 ± 59.5 |
| ST-C | 1.95 ± 0.15 | 1.20 ± 0.14 | 26.1 ± 4.6 | 246.2 ± 53.6 | 772.7 ± 58.8 |

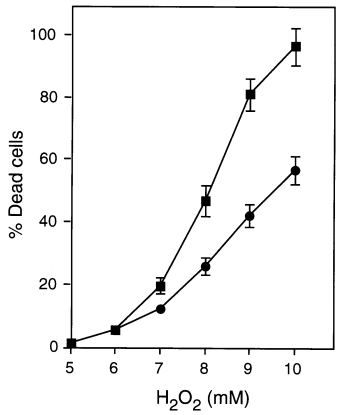


**Figure 5 Effect of different doses of $H_2O_2$ on the viability of wild-type and ST-C CHO cells**

Wild-type (●) or ST-C (■) CHO cells were exposed to increasing concentrations of $H_2O_2$ and the viability of the cells determined after 6 h. Results are the means ± S.D. of the determinations of three separate cultures.

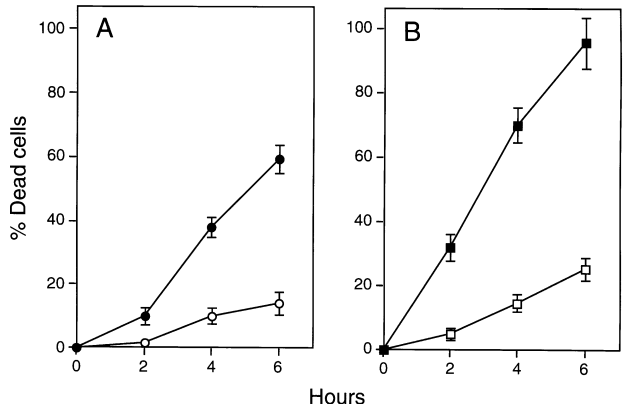


**Figure 6 Effect of ABA on the killing of wild-type and ST-C CHO cells by $H_2O_2$**

Wild-type (**A**) or ST-C (**B**) CHO cells were exposed to 10 mM $H_2O_2$ in the presence (open symbols) or absence (closed symbols) of 10 mM ABA and the viability of the cells determined at different intervals. Results are the means ± S.D. of the determinations of three separate cultures.

### Killing of CHO cells and ST-C cells by $H_2O_2$

Figure 4 shows the killing of wild-type and ST-C CHO cells by $H_2O_2$. Within 3 h, cell killing induced by 10 mM $H_2O_2$ in the transfected cells was about 50 % and in the wild-type cells 18 %. After 5 h exposure to the oxidative agent, the killing in ST-C cells was 95 % and in wild-type cells 57 %. Figure 4 also shows that the exposure of both types of cells to $H_2O_2$ was accompanied by the rapid depletion of NAD and ATP. However, the basal levels of both ATP and NAD were substantially reduced in the ST-C cells, and the reduction of ATP and NAD observed in ST-C cells was more pronounced than that observed in wild-type CHO cells. In fact, even after 90 min exposure to 10 mM $H_2O_2$ the concentration of ATP in the wild-type cells was still higher than that observed in the ST-C cells 15 min after exposure to $H_2O_2$. As shown in Figure 5, the killing of both wild-type and ST-C cells by $H_2O_2$ was dose-dependent. At doses above 6 mM $H_2O_2$ the killing of the transfected cells was greater than that of wild-type cells.

**Table 3 Prevention by ABA of the killing of wild-type and ST-C CHO cells**

Cell death was determined after 6 h incubation. Results are the means ± S.D. of the determinations performed on three separate cultures. Details are given in the Experimental section.

| | Dead cells (%) | |
|---|---|---|
| Treatment | Wild-type | ST-C |
| $H_2O_2$ (10 mM) | 52.2 ± 5.7 | 80.6 ± 1.2 |
| $H_2O_2$ + ABA (10 mM) | 9.6 ± 0.9 | 20.0 ± 1.5 |
| $H_2O_2$ + DPPD (1 mM) | 51.5 ± 2.3 | 78.4 ± 1.2 |
| $H_2O_2$ + ABA + DPPD | 11.4 ± 0.4 | 22.6 ± 3.2 |
| ABA (10 mM) | 3.7 ± 1.3 | 6.6 ± 0.9 |
| DPPD (1 μM) | 2.5 ± 0.5 | 3.8 ± 1.4 |

**Table 4 Prevention by ABA of $H_2O_2$ induced ATP and NAD depletion in wild-type and ST-C CHO cells**

NAD and ATP levels were determined after 6 h incubation. Results are the means ± S.D. of the determinations made on three separate cultures. Details are given in the Experimental section.

| | NAD content (nmol/mg) | | ATP content (nmol/mg) | |
|---|---|---|---|---|
| Treatment | Wild type | ST-C | Wild type | ST-C |
| None | 2.91 ± 0.23 | 1.21 ± 0.13 | 3.87 ± 0.32 | 1.92 ± 0.15 |
| $H_2O_2$ (10 mM) | 0.21 ± 0.08 | 0.04 ± 0.02 | 0.36 ± 0.08 | 0.09 ± 0.04 |
| ABA (10 mM) | 2.84 ± 0.21 | 1.24 ± 0.09 | 3.80 ± 0.19 | 1.79 ± 0.11 |
| $H_2O_2$ + ABA (10 mM) | 2.86 ± 0.18 | 1.24 ± 0.07 | 3.80 ± 0.26 | 1.79 ± 0.19 |

Two main mechanisms have been implicated in cell killing induced by $H_2O_2$: activation of poly(ADP-ribose) polymerase, and lipid peroxidation. To distinguish between the two mechanisms, the effect of ABA, an inhibitor of poly(ADP-ribose) polymerase, and of the antioxidant DPPD on the killing of CHO cells by $H_2O_2$ was studied. The effect of 10 mM ABA on the killing of CHO cells and ST-C cells by $H_2O_2$ is illustrated in Figure 6 and in Table 3. ABA prevented the killing of both types of cells by $H_2O_2$. In addition, ABA maintained the contents of NAD and ATP (Table 4). It is of note in Table 4 that the reduction in the basal contents of NAD and ATP in the ST-C cells was not prevented by ABA. Thus, the constitutive decreases in ATP and NAD in the ST-C cells are not the consequence of an increased activity of poly(ADP-ribose) polymerase.

The cell killing by $H_2O_2$ occurred largely in the absence of lipid peroxidation. This was shown by the fact that the killing of both types of cells by $H_2O_2$ was not affected by the presence of 1 μM of the antioxidant DPPD and by the fact that ABA protected against the cell killing in the absence or presence of DPPD (Table 3).

## DISCUSSION

Expression of rat liver AdoMet-synthetase cDNA in CHO cells increased the activity of the enzyme 2- to 3-fold, an effect that resulted in an accumulation of AdoMet and a depletion of ATP and NAD. The depletion of ATP is most probably the metabolic consequence of the higher AdoMet-synthetase activity, as ATP is a substrate of the enzyme. The active site of AdoMet-synthetase

catalyses two reactions [29]. The first is the formation of AdoMet; the second is the hydrolysis of the tripolyphosphate moiety of ATP ($PPP_i$) into pyrophosphate ($PP_i$) and orthophosphate ($P_i$). The synthesis of AdoMet is, therefore, unusual among ATP-dependent reactions in that the three phosphates of ATP are utilized to yield $PP_i$ and $P_i$. Obviously, the effect of AdoMet-synthetase on the cellular stores of ATP is greater than that produced by the more common kinases that convert ATP into ADP and $P_i$. The depletion of NAD is probably the metabolic consequence of the reduced concentration of ATP, as ATP is required for NAD synthesis.

The accumulation of AdoMet and the concomitant increase in the AdoMet/AdoHcy ratio (the methylation ratio) in ST-C cells led to DNA hypermethylation and to elevation of polyamines, effects that are consistent with our present understanding of the regulation of the methylation cycle. The main biological functions of AdoMet are the transfer of the propylamine group for the synthesis of polyamines and the transfer of the methyl group to a large variety of substrates, including nucleic acids, proteins and phospholipids (reviewed in [1,2]). Whereas only DNA methylation was considered in the present experiments, it is likely that other transmethylation reactions are also increased in ST-C cells. Interestingly, despite these large changes in DNA methylation and polyamine levels, the growth of the transfected cells was not altered.

The hypermethylation of DNA in ST-C cells suggests that either the concentration of AdoMet is insufficient to saturate DNA methyltransferases *in vivo* or that these enzymes are partially inhibited by AdoHcy at physiological values of the AdoMet/AdoHcy ratio. Together with our previous study showing that a reduction in the content of AdoMet and/or in the AdoMet/AdoHcy ratio leads to DNA hypomethylation [30], the present data indicate that the levels of AdoMet and/or the methylation ratio are important components of the regulatory mechanisms of DNA methylation. Similarly, the present results suggest that in CHO cells the concentration of AdoMet is a limiting factor for polyamine synthesis.

CHO did not synthesize GSH from methionine, a result that is consistent with the concept that the trans-sulphuration pathway is present almost exclusively in the liver (reviewed in [1,2]). As a consequence, GSH levels were the same in the wild-type and transfected cells. This is important, since in CHO, as in most if not all cell types, cellular GSH concentration is an important component of the cell's defence system to oxidative stress [31].

In the absence of exposure to $H_2O_2$, the levels of ATP and NAD in ST-C cells were unaffected by ABA, an inhibitor of poly(ADP-ribose) polymerase. This result suggests that poly(ADP-ribose) polymerase is not activated as a consequence of the DNA hypermethylation induced by overexpression of AdoMet-synthetase. The basal reductions in ATP and NAD are, therefore, readily attributable to the overexpression of AdoMet-synthetase. Upon response to $H_2O_2$, poly(ADP-ribose) polymerase was activated, as indicated by the finding that ABA not only preserved the viability of the cells, but also maintained the contents of NAD and ATP of both wild-type and ST-C cells. By contrast, the antioxidant DPPD was without effect, a result that indicates that in response to $H_2O_2$ the killing of CHO cells does not occur by lipid peroxidation, a situation similar to that previously observed in L929 fibroblasts [27]. When $H_2O_2$ was used to injure these fibroblasts, little cell killing occurred by lipid peroxidation and ABA completely protected these cells in the presence or absence of DPPD [27]. The precise relationship between the activity of poly(ADP-ribose) polymerase and cell death is not entirely clear (reviewed in [32]). As previously shown in cultured cells [27,33,34], inhibition of poly(ADP-ribose) polymerase not only preserves viability, but also maintains the contents of NAD and ATP. The depletion of these constituents is readily attributable to an exaggerated ADP-ribosylation of proteins, and the loss of viability has been attributed to the depletion of energy stores.

Exposure of ST-C cells to oxidative stress produced a dramatic depletion of cellular ATP and NAD within 15–30 min of the addition of the oxidative agent. The depletion of ATP and NAD levels following $H_2O_2$ addition in ST-C cells was greater than in the wild-type CHO cells. As mentioned above, the depletion of ATP and NAD and concomitant mitochondrial de-energization have been proposed as the main causes of cell death from oxidative injury [27,33,34]. The present results support this hypothesis by showing that ST-C cells are more sensitive to $H_2O_2$-induced oxidative death than wild-type CHO cells.

The present experiments argue that the expression and activity of AdoMet-synthetase have to be maintained within strictly controlled limits. An uncontrolled cellular rise in the specific activity of this enzyme, owing to either its activation or an increased gene expression, may lead to an increased consumption and depletion of ATP and NAD, which under conditions of oxidative cell injury could be damaging to the cell. Thus, and although it is difficult to extrapolate the results of this study to events that take place in the liver, the well documented reduction of AdoMet-synthetase activity observed in liver disease (reviewed in [1]) may represent an adaptive mechanism to spare ATP and maintain NAD levels in order to combat oxidant-induced cell injury.

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