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INCREASE OF ATP LEVEL IN HUMAN ERYTHROCYTES INDUCED BY *S*-ADENOSYL-L-METHIONINE

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Abstract—The effect of *S*-adenosyl-L-methionine (SAM) on the ATP level, the morphology and the deformability of human erythrocytes was investigated and compared with that of adenosine. (i) Upon incubation with SAM, the ATP level increased considerably in fresh cells (in both young and old cells in similar extent) and in stored (partially ATP-depleted) cells. But the incubation with adenosine increased ATP level to a lesser extent. (ii) The incubation of stored cells with SAM hardly affected (or rather decreased) the IMP level, while that with adenosine remarkably increased IMP (and ITP). (iii) The morphology and the deformability of stored erythrocytes were well conserved in spite of the treatment with SAM, as compared with the treatment with adenosine. The echinocytic transformation was induced in old cells to some extent by SAM, while did not in young cells.

It is now known that *S*-adenosyl-L-methionine (SAM)* enters into the intact erythrocytes through the membrane [1, 2] and induces the methylation of membrane proteins [1–4], phospholipids [5–7] and cytosolic proteins [8, 9]. Therefore, the functional and metabolic properties of intact erythrocytes alter upon incubation with SAM [7, 10], and the deformability may be modified by the methylation of cytoskeletal proteins [11]. However, the methylation of membrane proteins does not appear to be dependent on the drug-induced shape change [12], though the level of intracellular SAM was varied with shape-modifying compounds or with Ca^{2+} content.

It is well known that adenosine is incorporated into the purine moiety of ATP in erythrocytes and is used for the restoration of ATP level [13–15]. The fate of adenosine in erythocytes is dependent on the metabolic state (thus dependent on various metabolites and inorganic ions) and the metabolism is regulated by the activities of enzymes, such as adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4), adenosine kinase (ATP: adenosine 5′-phosphotransferase, EC 2.7.1.20), AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) and so on [16–19]. Therefore, the metabolism of adenosine in fresh erythrocytes is different from that in and stored erythrocytes. Since the energy metabolism, the cell shape and the deformability (thus, the survival of cells) are, in some way, closely correlated to the ATP level, it is important to investigate the influence of SAM on the adenylate metabolism of human erythrocytes.

In this study, the effect of SAM on human erythrocytes is investigated in relation to ATP level, cell shape and deformability, and is compared with those of adenosine. Upon incubation with SAM, the ATP level increased considerably in partially ATP-depleted cells (stored in the state of heparinized whole blood) and in both young and old cells (separated by the density gradient centrifugation from fresh erythrocytes). Adenosine also increased the ATP level, but the degree of increment was less than with SAM. SAM and adenosine presumably permeated the erythrocyte membrane and supplied the source of ATP, but the metabolism of these compounds differed distinctly from each other, i.e. adenosine increased IMP (and ITP slightly), while SAM did not. Also, their effects on the cell shape and deformability were different. The possible mechanisms of these phenomena will be discussed.

* Abbreviation used: SAM, *S*-adenosyl-L-methionine.

MATERIALS AND METHODS

Erythrocytes. The fresh blood was drawn from antecubital vein of healthy male donor and heparinized (10 units heparin/ml blood were used). (i) Stored (partially ATP-depleted) cells: the fresh heparinized blood was stored at 0° for 17 hr, in order to reduce the ATP level. (ii) Fractionated cells with respect to age: The fresh erythrocytes were fractionated by the density gradient centrifugation with Percoll [20, 21], in order to obtain the light (rich in young cells) and heavy (mostly old cells) fractions; they were denoted as young and old cells, respectively. The percentage of each fraction was determined on the basis of hemoglobin concentration.

Treatment with SAM and adenosine. The erythrocytes, after washing with an isotonic phosphate-buffered saline (90 mM NaCl, 5 mM KCl, 50 mM Na–phosphate, 5.6 mM D-glucose, pH 7.4; 285 mOsM), were incubated with *S*-adenosyl-L-methionine-HCl (gift of Fuji Chemical Industry Co., Tokyo, Japan) or adenosine (purchased from Wako Chem. Co., Osaka, Japan) at 37°. The final hematocrit was adjusted to 10%.

Determination of purine nucleotides. A known amount of erythrocytes (0.2–0.5 ml in packed cells) was washed twice with cold isotonic phosphate-buffered saline, and purine nucleotides were extracted by treating with 0.4–1.0 ml of cold tri-

chloroacetic acid (10 g/dl). Clear supernatant was collected by centrifugation at 3000 rpm for 5 min at 4°. The brown precipitate was treated with the same amount of trichloroacetic acid once more to extract the residual nucleotides. Pooled extracts were treated with ether three times to remove trichloroacetic acid. The residual ether was removed by flashing with nitrogen. The volume of the extract was measured for the calculation of the amount of nucleotides.

The quantitative measurement of purine nucleotides was performed by liquid chromatography (Shimadzu Manuf. Co., Model LC-3A, Kyoto, Japan); with Permaphase AAX, an anion exchanger, column 2.1 mm × 100 cm operated at 40°. After applying a known amount of extract (20–50 μl) to the column equilibrated with 0.5 mM KH_2PO_4 (pH 3 adjusted with phosphoric acid), nucleotides were eluted at the flow rate of 1.2 ml/min by the two step linear gradient programmed as follows: the initial linear gradient of 0.5–30 mM KH_2PO_4 (pH 3) up to 6 min, and the following linear gradient of 30–300 mM KH_2PO_4 (pH 3) from 6 min to 15 min. The eluent was monitored at 254 nm (the representative elution patterns were shown in Fig. 1).

Measurement of cellular deformability. The erythrocytes were suspended in 20% Dextran T-40 (Pharmacia Fine Chem., Uppsala, Sweden; dissolved in the phosphate-buffered saline and adjusted to pH 7.4 and 285 mOsM; the viscosity, 18.6 cP at 25°), containing SAM or adenosine in the same concentration as those in the incubation medium. The deformation in a uniform shear flow was observed by using a rheoscope [22, 23] (consisting of an inverted microscope, a transparent cone-plate viscometer, a flash lamp and a camera). The erythrocytes were deformed to ellipsoidal disc by the applied shear stress, and the ratio of short radius to long radius was adopted as a measure of cellular deformability [24]. The increase of the ratio shows the impairment of deformability.

Hematological examination. Mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were calculated from hematrocit (measured by a microhematocrit centrifuge), cell count (measured by an automatic cell counter) and hemoglobin concentration (measured by CN-methemoglobin method). The erythrocyte shape was observed by a scanning electron microscope (Hitachi Manuf. Co., Model S-500A, Hitachi, Japan), after fixing with 1% (v/v) glutaraldehyde and then with 1% (w/v) OsO_4. The morphological index (M.I.) of Fujii *et al.* [25] was adopted to express the degree of transformation to echinocytes [26]. The increase of the index shows the progress of echinocytic transformation: actually, M.I. = 0, 0.5, 1.0, 2.0, and 3.0 corresponds to discocyte, echinocyte I, echinocyte II, echinocyte III and spheroechinocyte in Bessis' classification [27], respectively.

Statistical analysis. Data are presented as mean ± standard deviation. The statistical significance is evaluated by the Student's *t*-test.

RESULTS

Effect of SAM and adenosine on partially ATP depleted erythrocytes

(1) *On the purine nucleotide level.* During storage of heparinized blood for 17 hr at 0°, the ATP level of erythrocytes decreased *ca.* 50% of the fresh erythrocytes, and the IMP level increased by more than 10 times that of the fresh cells (see Fig. 2). The representative patterns of chromatographic separation of purine nucleotides are shown in Fig. 1, in order to show the differences among the samples incubated with (a) 5.6 mM glucose, (b) 1.25 mM SAM (with 5.6 mM glucose) and (c) 1.25 mM adenosine (with 5.6 mM glucose), respectively. The separation was good and reproducible. It was recognized that the changes in ATP and IMP levels was remarkable among these samples.

The effect of SAM and adenosine on ATP and IMP levels of stored erythrocytes is shown in Fig. 2.

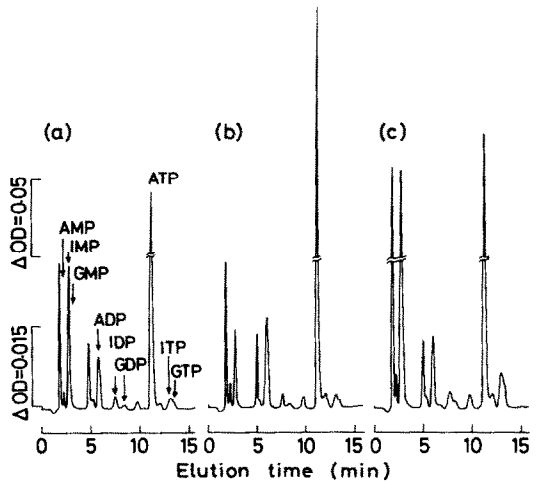

Fig. 1. Representative chromatographic separation of purine nucleotides. (a) Treated with 5.6 mM glucose, (b) treated with 1.25 mM SAM and 5.6 mM glucose, and (c) treated with 1.25 mM adenosine and 5.6 mM glucose, at 37° for 180 min. The pattern was obtained for the extract from 5 μl of packed erythrocytes. Identified nucleotides were shown in the figure.

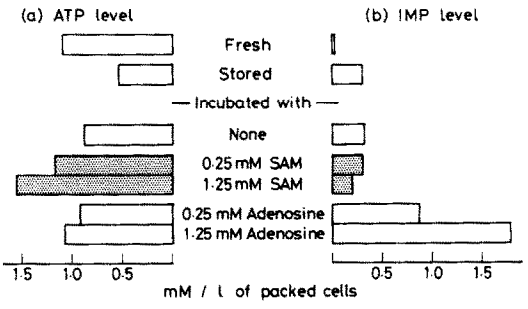

Fig. 2. Effect of SAM and adenosine on the ATP and IMP levels in stored erythrocytes. Fresh erythrocytes were stored as heparinized whole blood at 0° for 17 hr. The stored erythrocytes were incubated with various concentrations of SAM and adenosine in isotonic 50 mM Na–phosphate-buffered saline containing 5.6 mM glucose at 37° for 180 min. (a) Effect on the ATP level, (b) effect on the IMP level.

Table 1. Effect of S-adenosyl-L-methionine (SAM) and adenosine on the shape and the deformability of stored erythrocytes

Treatment	Incubation time (min)	Morphological index	Deformability of deformable cells (N)
Fresh	—	0.01	0.40 ± 0.04 (58)
Stored	—	0.68	0.41 ± 0.04 (63)
Incubated with			
5.6 mM glucose			
	20	0.20	0.42 ± 0.05 (64)*
	180	0.05	0.43 ± 0.04 (57)**
0.25 mM SAM + 5.6 mM glucose			
	20	0.28	0.40 ± 0.04 (61)*
	180	0.07	0.42 ± 0.03 (59)*
1.25 mM SAM + 5.6 mM glucose			
	20	0.23	0.41 ± 0.04 (71)*
	180	0.16	0.41 ± 0.04 (62)*
0.25 mM adenosine + 5.6 mM glucose			
	20	0.25	0.41 ± 0.04 (61)*
	180	0.27	0.42 ± 0.05 (42)*
1.25 mM adenosine + 5.6 mM glucose			
	20	0.27	0.43 ± 0.04 (58)**
	180	0.11	0.45 ± 0.05 (60)***

Stored, heparinized whole blood was kept at 0° for 17 hr. Incubated, stored erythrocytes were incubated at 37° in isotonic 50 mM Na–phosphate-buffered saline containing compounds shown in the table. Morphological index, according to Fujii *et al.* [25]. Deformability, expressed by the ratio of the short radius to long radius (±S.D.) of ellipsoidal disc deformed at the shear stress of 70 dyn/cm^2. (N) cell number measured on the flash photographs by the rheoscope. The statistical significance of deformability was evaluated by comparing with stored cells, using the Student's *t*-test (expressed by P-value): *, not significant; **, $P < 0.05$; ***, $P < 0.001$.

(i) The ATP level in erythrocytes increased slightly without noticeable change of IMP by incubating with 5.6 mM glucose in isotonic phosphate-buffered saline, and the ADP level slightly decreased. (ii) Upon incubation with SAM (with 5.6 mM glucose), the ATP level of stored cells increased considerably and specifically, as increasing SAM concentration, e.g. by treating with 1.25 mM SAM for 180 min at 37°, the ATP level of stored cells increased by 3 times the original level (by *ca.* 140% of fresh cells). The IMP level slightly decreased and no significant change of ITP and GTP was observed (also shown in Fig. 1). (iii) By treating with 1.25 mM adenosine (with 5.6 mM glucose), the ATP level increased only by twice the level of stored cells (the level was about the same as that of fresh cells). However, IMP accumulated considerably, and ITP and GTP also increased (Figs 1 and 2). In all cases, no appreciable differences in other purine nucleotides and several unidentified peaks in the chromatograms were observed between SAM-and adenosine-treated cells (Fig. 1). Total purine nucleotides clearly exceeded the level of fresh erythrocytes (e.g. 1.98 mM and 3.20 mM in cells incubated with 1.25 mM SAM and adenosine for 180 min at 37°, respectively, while 1.40 mM in fresh cells).

(2) *On the morphology and the hematological indices.* The erythrocytes slightly transformed to echinocytes during 17 hr storage. These echinocytes were remarkably reduced by the incubation not only with 5.6 mM glucose (as control experiment) but also with SAM and adenosine with 5.6 mM glucose (Table 1). Furthermore, the hematological indices (such as MCV, MCHC and MCH) were not altered by the incubation with SAM and adenosine.

(3) *On the deformability.* During storage at 0° for 17 hr, the percentage of undeformable cells at the shear stress of 70 dyn/cm^2 was slightly increased (in less than 3%), but the deformability of deformable cells was not significantly altered (Table 1). The deformability of stored cells tended to decrease upon 180 min incubation with glucose or adenosine (with glucose), as shown in Table 1. However, SAM (with glucose) well conserved the deformability, i.e. the ratio of short radius to long radius did not alter.

In short, the ATP generation was the greatest with SAM, while it was moderate with adenosine accompanying remarkable increase of IMP. The cell shape was restored to discocytes by SAM or adenosine. The deformability of SAM-treated erythrocytes was well conserved, while that of adenosine-treated cells slightly decreased.

Effect of SAM on young and old erythrocytes

For the sake of the differences in the ATP content [21] and in the mode and activity of protein methylation [11, 12], the response to SAM was compared for two distinct young and old cells separated from fresh blood (*ca.* 10% of total erythrocytes, respectively).

(1) *On the purine nucleotide level.* The ATP level of old cells fractionated by the density gradient centrifugation of Percoll was *ca.* 30% less than that of young cells. As shown in Fig. 3a, the amount of ATP increased *ca.* 35% for both young and old cells upon incubation with 1.25 mM SAM for 180 min at 37°,

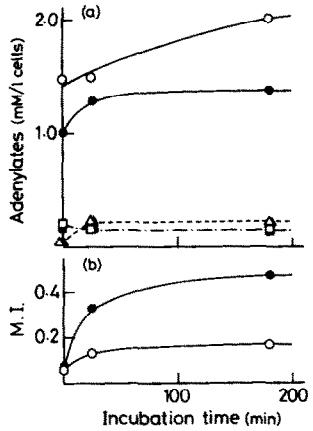

Fig. 3. Effect of *S*-adenosyl-L-methionine on the adenylate level and the morphology of erythrocytes fractionated with respect to cell age. Young cells (11.4% of total cells, MCV= 91.7 μm^3, MCHC = 35.2 g/dl, MCH = 32.2 pg) and old cells (10.7% of total cells, MCV = 79.1 μm^3, MCHC = 42.7 g/dl, MCH = 33.7 pg) were incubated with 1.25 mM SAM in isotonic 50 mM Na–phosphate-buffered saline containing 5.6 mM glucose (pH 7.4) at 37°. (a) Changes of adenylate level: ATP (○,●), ADP (□,■) and AMP (△,▲) in young (open symbols) and old (closed symbols) cells, respectively. (b) Morphological changes: M.I., the morphological index; young cells (○), old cells (●).

and the amount of AMP slightly increased. The IMP level for both young and old cells was not altered by the incubation with SAM.

(2) *On the morphology and the hematological indices*. The young cells were larger (*ca.* 15% larger in the present preparation) in MCV and lower (*ca.* 15% lower) in MCHC than the old cells, as previously reported [21]. The old cells tended to transform to echinocytes during incubation with SAM, in spite of the increment of ATP level, as shown in Fig. 3b. However, the echinocytosis of young cells by SAM was hardly induced. The treatment of both young and old cells with SAM did not alter the hematological indices (the variations of MCV and MCHC during incubation with SAM were less than 2% for young cells and 3% for old cells).

(3) *On the deformability*. The deformability of old cells was originally reduced, compared with that of young cells [21]; the ratio of short radius to long radius of old cells at the shear stress of 70 dyn/cm^2 (0.44 ± 0.06, N = 26) was significantly larger than that of young cells (0.37 ± 0.04, N = 19) ($P < 0.001$). The echinocytes became frequently undeformable, thus the percentage of undeformable cells increased parallelly with the morphological index. However, the deformability of the deformable cells was not significantly altered by SAM.

In short, the ATP level in both young and old erythrocytes increased after incubation with SAM, but the echinocytosis in old cells was induced, while the young cells resisted to the echinocytic transformation by SAM.

DISCUSSION

(1) *On the specific generation of ATP by SAM*

Adenosine penetrates rapidly inside the erythrocytes by facilitated diffusion [28] and is phosphorylated to ATP; thus, adenosine is frequently used for the functional restoration of ATP-depleted, stored erythrocytes [13–15]. The present study shows that SAM is more effective for the specific augmentation of the ATP level of stored erythrocytes (and also of fresh cells) than adenosine (Figs 1 and 2). The amount of total nucleotides in cells increased over the level of fresh cells by incubating with SAM and adenosine. The results clearly showed that SAM and adenosine were incorporated into the purine nucleotide pool in erythrocytes.

In the metabolic process of incorporated SAM and adenosine, the remarkable difference was observed in the formation of inosine nucleotide: the incubation with 0.25–1.25 mM adenosine (in the presence of 5.6 mM glucose and 50 mM inorganic phosphate) increased IMP (and also ITP) considerably besides the ATP generation, while the incubation with SAM increased ATP specifically and decreased ADP and IMP (Figs 1 and 2). Guanine nucleotide is normally present in much lower concentration than adenine nucleotide in erythrocytes [29]. The amount of GTP in erythrocytes treated with 1.25 mM adenosine was more than those treated with 1.25 mM SAM (Fig. 1), and was probably produced from IMP. These results show that the metabolic pathways and the related enzymatic activities in producing ATP from SAM are distinctly different from those of adenosine. Adenosine undergoes two main processes: (i) the direct phosphorylation to AMP by adenosine kinase, then to ADP and ATP, and (ii) the deamination to inosine by adenosine deaminase [16, 19], leading the synthesis of IMP and ITP via hypoxanthine [14, 15, 19, 30]. Furthermore, AMP is deaminated to produce IMP by AMP deaminase [18]. The activities of these enzymes are controlled by various organic phosphates (adenylates, 2,3-diphosphoglycerates), inorganic phosphate, adenosine itself, Mg^{2+}, and so on [18, 19]. The effective regeneration of ATP by SAM may be explained by the changes of these enzymatic activities: probably the activation of adenosine kinase and the inhibition of adenosine deaminase (and additionally the inhibition of AMP deaminase) by SAM itself and/or its metabolites or by the methylation of these enzymes in the metabolic process of SAM. Agarwal *et al.* [31] have reported that adenosine analogues inhibit adenosine deaminase.

(2) *On the morphological restoration and the conservation of deformability of stored erythrocytes*

The echinocytic transformation is induced during storage, perhaps due to the ATP depletion [32], and the deformability is impaired [33, 34]. ATP is important for the viability and the deformability of erythrocytes. The incubation of stored erythrocytes with 1.25 mM adenosine (with 5.6 mM glucose) or 5.6 mM glucose alone slightly decreased the deformability in spite of the restoration of ATP and cell shape (to discocytes) (Table 1). On the other hand, SAM was superior in both the restoration of cell shape and the conservation of deformability, as well as the remarkable augmentation of ATP (Table 1). Such different behavior of two ATP sources, SAM and adenosine, on the deformability of erythrocytes may come from (i) the accelerated methylation of

proteins and phospholipids with SAM [1–5] and/or (ii) the different metabolism of adenosine and SAM to produce adenine nucleotides.

(3) *On the behavior of SAM to aged erythrocytes.* The metabolism in erythrocytes is altered during *in vivo* aging and thus the rheological and oxygen transporting functions are influenced [21, 35]. The amount of ATP increased for both young and old cells during incubation with SAM (Fig. 3a). However, the transformation to echinocytes occurred inevitably in old cells by the incubation with SAM, while young cells resisted to the echinocytosis (Fig. 3b). In this connection, Barber and Clarke [36] have shown the increase of sites for methylation in old cells with L-[methyl-^{3}H]methionine, and Galletti *et al.* [11] have demonstrated more rapid turnover of methylation in old cells, compared with that in young cells. These cell age-dependent difference in protein methylation may lead the undesirable effect of SAM to old cells, with respect to cell shape. Or, the methylation to some unbarried portion of membrane proteins (which causes the increase of sites for methylation in old cells) may accelerate the echinocytosis.

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