Cytometric Analysis of the Cytotoxic Action of Adenosine 5'-Monophosphate on Rat Thymocytes

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The cascade of adenosine 5′-monophosphate-activated protein kinase (AMPK) is known to be a sensor of cellular energy charge. In this context, a paradigm for obesity treatment *via* the activation of AMPK has attracted the suppliers of complementary medicines and supplements. It is possible that products that increase the concentration of adenosine 5′-monophosphate (AMP) in the body would be efficacious in reducing body weight. However, since there is currently little information concerning the toxicity of AMP, the cytotoxic action of AMP on rat thymocytes was examined by flow cytometry. Incubation of cells with AMP at a concentration of 30 µM or more for 24 hr significantly increased the populations of dead cells, shrunken cells, and cells containing hypodiploidal DNA in a concentration-dependent manner. Z-VAD-FMK, a pan-inhibitor of caspases, attenuated the AMP-induced changes in cell populations. It is concluded that AMP at a concentration of 30 µM or more exerts a cytotoxic action, which is dependent on the activation of caspases.

Key words —— cytotoxicity, adenosine 5′-monophosphate, flow cytometry, thymocyte

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INTRODUCTION

Obesity has reached epidemic proportions worldwide.[1,2] Leptin, a hormone secreted by adipocytes, plays a critical role in increasing energy expenditure.[3] This hormone affects the activity of adenosine 5′-monophosphate-activated protein kinase (AMPK).[4,5] The AMPK cascade is a sensor of cellular energy charge that promote catabolic and inhibit anabolic pathways.[6,7] Consequently, since the pharmacological treatment options for obesity are limited, AMPK is considered to be one of promising candidate targets for anti-obesity drugs.[8,9]

A paradigm for obesity treatment that involves the activation of AMPK has attracted the suppliers of complementary medicines and supplements. It is possible that products that increase adenosine 5′-monophosphate (AMP) concentrations would be potentially efficacious in reducing body weight. Although a primary criterion for such products ingested by the general population is that they should exert no toxic effect, information regarding the toxicity of AMP is currently limited. AMP has been demonstrated to exert an antiproliferative action on mouse fibroblasts.[10] Furthermore, it has been shown that micromolar amounts of AMP are required for the activation of AMPK.[11–13] Therefore, in this study, the cytotoxic action of micromolar amounts of AMP was examined by flow cytometry using rat thymocytes in conjunction with appropriate fluorescent probes.

Rat thymocytes were used for following reasons. First, since single cells are prepared without enzymatic treatment, the cell membranes of thymocytes remain intact. Second, the process of apoptosis has been elucidated in murine thymocytes.[14,15] In the present study, cell shrinkage was used as parameters for cytotoxicity. Shrinkage is a precursor of certain characteristic apoptotic changes such as cytochrome C release from mitochondria, caspase activation, and DNA fragmentation.[16,17] The parameter is known to be sensitive to cytotoxic compounds.[18,19] Third, the effects of chemicals on the process of apoptosis are simultaneously estimated since some cells spontaneously undergo apoptosis.[20,21]

MATERIALS AND METHODS

Reagents —— AMP and adenosine were purchased from Wako Pure Chemicals (Osaka, Japan). Propidium iodide was obtained from Molecular Probes Inc. (Eugene, OR, U.S.A.). Z-VAD-FMK, a pan-inhibitor of caspases, was purchased from EMD Chemicals Inc. (Darmstadt, Germany).

Cell Preparation —— This study was approved by the Committee for Animal Experiments in the University of Tokushima (No. 05279 for Y. Oyama). The preparation of cell suspensions was similar to that previously reported.[22] In brief, rat thymus glands were sliced to a thickness of 400 to 500 μm. The slices were triturated in RPMI-1640 medium containing 10 % fetal bovine serum (Sigma Chemical, St. Louis, MO, U.S.A.) to dissociate thymocytes. The cell suspension was prepared to a density of approximately 10^5 cells/ml. The cells were cultured in 24-well Falcon tissue culture plates (2 ml suspension in each well) placed in a CO_2 incubator (Sanyo, Tokyo, Japan) at 37°C.

Experimental Protocol —— The cells were incubated with AMP or adenosine at various concentrations for 24 hr in order to study cytotoxicity. Z-VAD-FMK was added to the suspension just before the application of AMP. The measurements of cellular parameters using a flow cytometer (CytoACE-150, JASCO, Tokyo, Japan) and fluorescent probes were also similar to those previously described.[22] The fluorescence was analyzed using JASCO software (Ver.3XX, JASCO). In order to assess cell lethality, propidium iodide was added to the suspension to a final concentration of 5 μM. Propidium fluorescence was measured at 2 min after the application using the flow cytometer. Since propidium stains dead cells, the measurement of propidium fluorescence provides a means of estimating cell lethality. In order to reveal the distribution of DNA content, DNA was stained with a phosphate buffer solution containing 0.1 % Triton X-100, 3 mM EDTA, 0.05 mg/ml RNase A and 100 μM propidium iodide for 1 hr at room temperature. Thereafter, the fluorescence was also measured using the flow cytometer. The excitation wavelength for propidium was 488 nm and the emission was detected at 600 ± 20 nm.

Statistics —— Statistical analysis was performed by using Tukey multivariate analysis. A p value of < 0.05 was considered significant. Values are expressed as the mean ± standard deviation (S.D.) of 4 to 5 experiments.

RESULTS

Change in the Population of Cells Induced by AMP

Cell lethality was not significantly altered when the cells were incubated with 100 μM AMP for 3–6 hr. However, the prolonged incubation with 100 μM AMP for 24 hr increased the population of cells exhibiting propidium fluorescence (Fig. 1A). Since the cells stained with propidium were dead cells, the result suggests that the incubation with 100 μM AMP increases cell lethality. The change in cell lethality induced by 1–300 μM AMP is summarized in Fig. 1A. It is suggested that AMP at a concentration of 30 μM or more (up to 300 μM) exerts a cytotoxic action on normal cells.

Figure 1B shows the effect of 100 μM AMP depicted in a cytogram (forward scatter *versus* side scatter). Incubation with AMP for 24 hr increased the population of cells with reduced intensity of forward scatter, a parameter of cell size. It is suggested that AMP induces cell shrinkage, which is one of phenomena characteristic of the early stage of apoptosis.[16,17] The concentration-dependent change in the population of shrunken cells induced by AMP is summarized in Fig. 1B. AMP at a concentration of 30 μM or more significantly increased the population of shrunken cells.

Change in the Population of Cells Containing Hypodiploidal DNA in Response to AMP

The results described above suggest that AMP increases the population of cells undergoing apoptosis. Figure 2A shows a histogram of propidium fluorescence intensity (cell cycle) monitored from the cells incubated with and without AMP for 24 hr. Incubation with 100 μM AMP increased the population of cells containing hypodiploidal DNA. The concentration-dependent change in the population of hypodiploidal cells induced by AMP is shown in Fig. 2A. When the cells were incubated with AMP at a concentration of 30 μM or more for 24 hr, the population of cells with hypodiploidal DNA was markedly increased.

On the basis of the results presented in Figs. 1 and 2, AMP is suggested to induce apoptosis in rat thymocytes. In order to determine whether caspases are involved in these AMP-induced changes, the cells were incubated with 100 μM AMP in the absence and presence of 10 μM Z-VAD-FMK, a pan-inhibitor of caspases. In the presence of Z-VAD-FMK, the statistical analysis indicated that AMP did

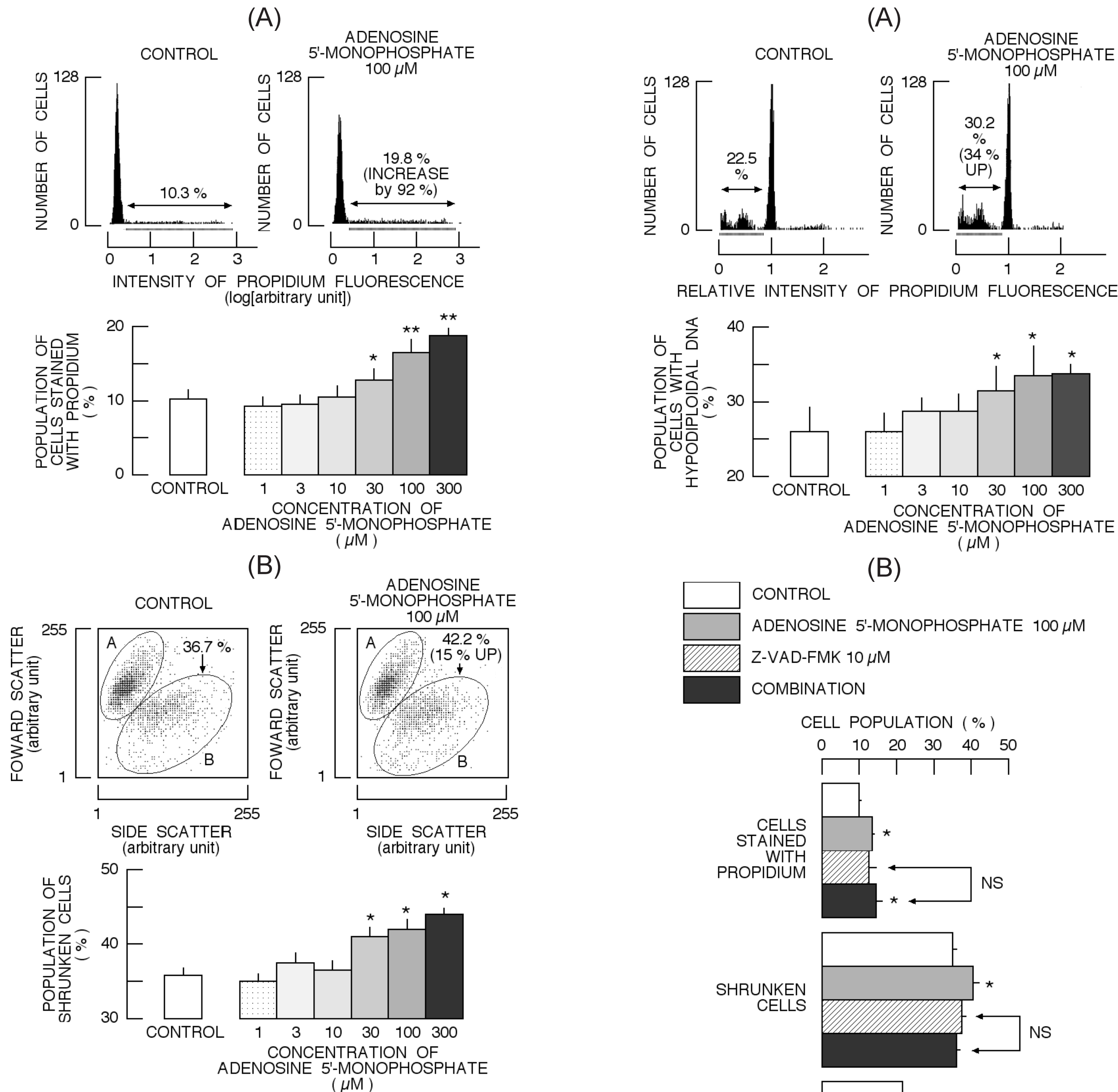

Fig. 1. AMP-Induced Changes in Cell Lethality and Population of Shrunken Cells

(A) Upper panels show the histogram of propidium fluorescence monitored from cells with and without 100 μM AMP. Dotted line indicates the intensity range where the cells were stained with propidium. Lower panel shows the concentration-dependent change in cell lethality by AMP. (B) Upper panels show the cytogram of cells incubated with and without 100 μM AMP. Lower panel indicates the concentration-dependent change in population of shrunken cells. Columns and bars indicate mean ± S.D., respectively, of 4–18 experiments. Symbols (*, **) indicate a significant difference ($p < 0.05$, $p < 0.01$) between the control group (CONTROL) and AMP-treated groups.

Fig. 2. AMP-Induced Increase in Population of Cells with Hypodiploidal DNA

(A) Upper panels indicate the relative content of DNA (relative intensity of propidium fluorescence) obtained from cells incubated with and without 100 μM AMP. Lower panel shows the concentration-dependent change in population of cells containing hypodiploidal DNA. (B) Effects of Z-VAD-FMK on AMP-induced changes in the percentage of propidium-stained cells, shrunken cells, and the cells containing hypodiploidal DNA. Columns and bars indicate mean ± S.D., respectively, of 4 experiments. Symbol (*) indicates significant difference ($p < 0.05$) between the control group and AMP-treated groups. NS indicates no significance.

not increase the populations of propidium-stained cells, shrunken cells, or hypodiploidal cells [Fig. 2B as shown with NS (no significance)]. Thymocytes spontaneously underwent apoptosis during the incubation. In the presence of Z-VAD-FMK, the spontaneous increase in population of cells with hypodiploidal DNA was greatly attenuated. Under the suppression of caspase-dependent increase in population of cells with hypodiploidal DNA, the incu-

bation with AMP did not increase the population. Thus, it is suggested that the activation of caspase is involved in the AMP-induced increase in the population of cells with hypodiploidal DNA (presumably AMP-induced apoptosis).

Effects of Adenosine on Rat Thymocytes

It is possible that AMP is decomposed to adenosine and that this decomposition product affects the cells *via* the activation of adenosine receptors. However, incubation of cells with 30 μM adenosine for 24 hr failed to induce significant changes in populations of propidium-stained cells, shrunken cells, and the cells with hypodiploidal DNA. Therefore, it is unlikely that adenosine is involved in the AMP-induced changes in cellular parameters.

DISCUSSION

It is likely that the incubation of rat thymocytes with AMP at a concentration of 30 μM or more for 24 hr induces caspase-dependent apoptosis, a process that is independent of adenosine receptors. This contention is based on following observations. First, incubation with AMP increased the populations of shrunken cells (Fig. 1B) and cells containing hypodiploidal DNA (Fig. 2A). The process of spontaneous apoptosis in murine thymocytes has been extensively studied.[14, 15] Cell shrinkage and exposure of phosphatidylserine occur prior to certain characteristic apoptotic changes such as cytochrome C release from mitochondria, caspase activation, and DNA fragmentation.[16, 17] Second, Z-VAD-FMK completely attenuated the AMP-induced increase in the population of the cells containing hypodiploidal DNA (Fig. 2B). Finally, adenosine at a concentration of 30 μM did not significantly affect the populations of propidium-stained cells, shrunken cells, or cells containing hypodiploidal DNA.

It remains questionable, however, whether the activation of AMPK is involved in AMP-induced cytotoxicity in rat thymocytes. To the best of our knowledge, there has been only one published study concerning the role of AMPK in thymocytes;[23] this study indicated that the activation of AMPK inhibits the activation of a caspase 3-like enzyme, resulting in protection against thymocyte apoptosis. Therefore, it appears unlikely that the activation of AMPK is involved in the AMP-induced cytotoxicity in rat thymocytes. However, it is noted that the activation of AMPK is involved in the pathway of controlling apoptosis and interleukin-2 production in the case of lymphocytes such as B-cell chronic lymphocytic leukemia cells and Jurkat T cells, respectively.[24, 25]

Obesity has currently reached global epidemic levels. It has been estimated that over 22 million children under the age of 5 years are severely overweight.[26] Efforts to reverse this trend by dietary or behavioral counseling have thus far been unsuccessful.[27] From the perspective of the market for supplements and complementary medicines aimed at obesity treatment and/or prevention, supplements, including AMP itself, that increase intracellular AMP concentrations can be considered as potential candidates. AMPK is fully activated by concentrations of AMP at 5–10 μM,[13] 10–30 μM,[12] and 50 μM or more.[11] In order to achieve such concentrations of AMP within cells, one possible solution might be the exogenous application of high micromolar AMP. However, AMP at a concentration of 30 μM or more exerts a cytotoxic action on rat thymocytes (Figs. 1 and 2). If so under *in vivo* condition, there would be a health concern regarding the therapeutic use of AMP.

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