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Effect of exogenous *S*-adenosyl-L-methionine on phosphatidylcholine synthesis by isolated rat hepatocytes

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The possible uptake of *S*-adenosyl-L-methionine (SAM)* by rat hepatocytes has been investigated by different authors [1, 2]. While Pezzoli *et al.* [1] concluded that SAM crosses the cell membrane of isolated rat hepatocytes with an apparent K_m of *ca* 8 μM, Hoffman *et al.* [2] reported that the hepatocytes do not take up significant amounts of SAM when rat liver is perfused with 50 μM SAM.

In the present experiments we have studied the effect of the addition of SAM to isolated rat hepatocytes on the conversion of phosphatidylethanolamine to phosphatidylcholine in cells prelabelled with [^{3}H]ethanolamine. The addition of up to 5 mM SAM to isolated rat hepatocytes has no effect on the conversion of phosphatidylethanolamine to phosphatidylcholine. However, if the hepatocytes are treated with 3-deazaadenosine (C^3-Ado), adenosine (Ado) or 3-deazaaristeromycin (C^3-Ari) to inhibit phospholipid methylation by decreasing the ratio SAM/SAH, the addition of SAM restores this reaction to normal values. This effect, however, is only observed with pharmacological concentrations of SAM (concentration $\geq$ 1 mM). These results indicate that exogenous SAM can cross the cell membrane of isolated hepatocytes at pharmacological doses. At physiological doses, SAM is either unable to cross the cell membrane or is rapidly metabolized by the cell. This argues against a physiological function for the uptake of SAM by hepatocytes. Our results are consistent with the data of Hoffman *et al.* [2], who concluded that the hepatocytes do not take up significant amounts of SAM when the rat liver is perfused with micromolar concentrations of SAM.

Materials and methods

Materials. C^3-Ado, C^3-Ari and SIBA were a generous gift from Dr. Peter Chiang (Walter Reed Army Institute of Research, Washington, DC). Adenosine was from Boehringer, and homocysteine and authentic phospholipids standards were from Sigma. *S*-Adenosyl-L-methionine was from Europharma. [1-^{3}H]Ethanolamine (8.8 Ci/mmole) and [methyl-^{3}H]choline were from Amersham.

Isolation of rat hepatocytes. Hepatocytes from normally fed Wistar rats (200–250 g) were prepared by the collagenase perfusion method as previously described [3, 4]. Hepatocytes obtained under these conditions respond to a variety of hormones [3–5], indicating that these cells maintain most of their biochemical integrity.

Phospholipid methylation by isolated hepatocytes. Isolated rat hepatocytes (2–6 × 10^6 cells/ml) were incubated for 10 min at 37° with 5 μCi per ml [1-^{3}H]ethanolamine in a medium containing 118 mM NaCl, 4.75 mM KCl, 1.18 mM KPO_4H_2, 1.18 mM $MgSO_4$, 24.9 mM $NaCO_3H$, 10 mM glucose and 2.5 mM $CaCl_2$ at pH 7.4, the gas phase being 95% O_2 and 5% CO_2. At the end of this period, cells were washed three times and resuspended again at the same cell density. C^3-Ado, C^3-Ari, Ado or SIBA was then added. All these compounds were dissolved in dimethyl sulfoxide. Control cells received the same amount of dimethyl sulfoxide (final concentration 1%). C^3-Ado, C^3-Ari and Ado were added together with 200 μM homocysteine thiolactone. After 10 min (C^3-Ado, SIBA) or 30 min (Ado, C^3-Ari) incubation at 37°, SAM was added and 20 min later the cells were centrifuged for 0.5 min at 900 *g*. The cell pellet was immediately frozen in dry-ice–acetone and the phospholipids were extracted as described in ref. [3]. Control samples were obtained 30 and 50 min after the addition of dimethyl sulfoxide to account for the different incubation times applied with the different adenosine analogues. After extraction, the phospholipids were separated by thin-layer chromatography (TLC) [3, 6]. The plates were scraped into 0.5 cm bands and the amount of radioactivity incorporated into the total methylated phospholipids (phosphatidylcholine + phosphatidyl-*N*,*N*-dimethylethanolamine + phosphatidyl-*N*-monomethylethanolamine) was determined [3, 6]. Results are expressed as the amount of radioactivity incorporated into methylated phospholipids per 10^7 cells.

Incorporation of labelled choline into phosphatidylcholine. Isolated rat hepatocytes (2–6 × 10^6 cells/ml) were preincubated for 15 min at 37°. At the end of this period, C^3-Ado, Ado, C^3-Ari or SIBA was added as described above. Then SAM was added and 20 min later cells were treated with 0.2 μCi/ml [methyl-^{3}H]choline. After 15 min incubation the cells were centrifuged and the phospholipids extracted and analysed by TLC as described above. Control experiments were carried out as described above. Under these conditions, more than 90% of the radioactivity associated to lipids migrated as authentic phosphatidylcholine [6]. Results are expressed as the amount of radioactivity incorporated into phosphatidylcholine per 10^7 cells.

* Abbreviations: C^3-Ado, 3-deazaadenosine; Ado, adenosine; C^3-Ari, 3-deazaaristeromycin; SIBA, 5′-deoxy-5′-isobutylthioadenosine; SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine.

Results and discussion

In our studies the addition of up to 5 mM SAM to isolated rat hepatocytes had no significant effect on phospholipid methylation (Fig. 1). These results indicate that either SAM cannot cross the cell membrane of the hepatocyte or that the intracellular concentration of SAM is high enough to maintain the conversion of phosphatidylethanolamine to phosphatidylcholine at a rate close to V_{max}. The addition of 10 μM C^3-Ado to isolated rat hepatocytes, as previously reported [7, 8], inhibited phospholipid methylation *ca* 50% (Fig. 1). The addition of 5 mM SAM to cells treated with C^3-Ado restored phospholipid methylation to normal values (Fig. 1). Similar results were obtained with cells treated with Ado or C^3-Ari, which are both also known to inhibit phospholipid methylation by decreasing the intracellular ratio of SAM/SAH [2, 7, 8]. These results indicate that the addition of SAM to hepatocytes can restore the ratio SAM/SAH to normal values.

As shown in Fig. 1, the situation with SIBA was different from that observed with other inhibitors of phospholipid methylation. The inhibition by SIBA of phospholipid methylation was not restored by the addition of SAM. Furthermore, while C^3-Ado, Ado and C^3-Ari had no effect on the synthesis of phosphatidylcholine by the CDP-choline pathway, the addition of SIBA to rat hepatocytes strongly inhibited this pathway (Fig. 2). The addition of SAM to rat hepatocytes under the present conditions had no effect on the incorporation of choline into phosphatidylcholine. SIBA has been previously found to inhibit the synthesis of phosphatidylcholine by the CDP-choline pathway in rat basophilic leukemia cells [9]. These results indicate that the mechanism by which SIBA perturbs phosphatidylcholine synthesis is different from that of C^3-Ado, Ado and C^3-Ari. Other compounds, like dansylcadaverine, are also known to inhibit phosphatidylcholine synthesis by both the CDP-choline and the transmethylation pathways without affecting the ratio SAM/SAH or the levels of CDP-choline [10].

The effect of exogenous SAM on phospholipid methylation of cells treated with C^3-Ado was dose-dependent (Table 1). The concentration of SAM necessary to observe a significant increase in phospholipid methylation was *ca* 1 mM. These results argue against any physiological role for the uptake of SAM by hepatocytes. However, if used at pharmacological doses, enough SAM may enter the hepatocyte to restore a previously decreased transmethylation reaction to normal levels. A similar situation is known to occur with cAMP, where the effects of hormones which use this nucleotide as a second messenger are mimicked by extracellular addition of millimolar con-

Table 1. Effect of different doses of SAM on phospholipid methylation by isolated rat hepatocytes

Addition	% of control
C^3-Ado (10 μM)	45.1 ± 6.6
C^3-Ado (10 μM) + SAM (0.5 mM)	56.9 ± 2.9
C^3-Ado (10 μM) + SAM (1 μM)	66.1 ± 7.9
C^3-Ado (10 μM) + SAM (5 mM)	117.4 ± 9.6

Rat hepatocytes were treated with C^3-Ado in the presence of 200 μM homocysteine as described in Materials and Methods. SAM was then added and the conversion of phosphatidylethanolamine to methylated phospholipids was determined as described in Materials and Methods. Results are the mean ± S.E.M. of at least three independent experiments, each carried out in triplicate.

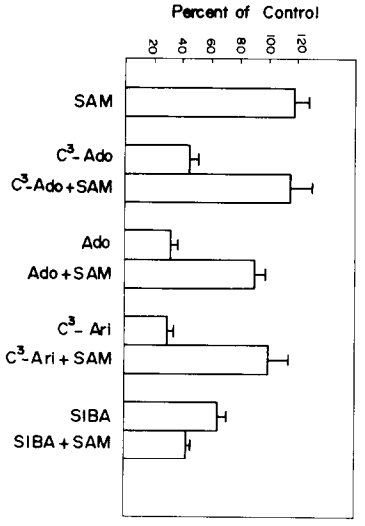

Fig. 1. Effect of the addition of SAM to isolated rat hepatocytes on phospholipid methylation. Rat hepatocytes, prelabelled with [^{3}H]ethanolamine, were treated with C^3-Ado (10 μM), Ado (1 mM), C^3-Ari (500 μM) or SIBA (500 μM) in the presence or absence of 5 mM SAM as described in Materials and Methods. Twenty minutes after the addition of SAM, the incorporation of radioactivity into methylated phospholipids was measured as described in Materials and Methods. One hundred per cent corresponds to 50,809 ± 5576 dpm/10^7 cells (cells preincubated 10 min before the addition of SAM) or 48,481 ± 7547 dpm/10^7 cells (cells preincubated 30 min before the addition of SAM). Results are the mean ± S.E.M. of at least three independent experiments, each carried out in triplicate.

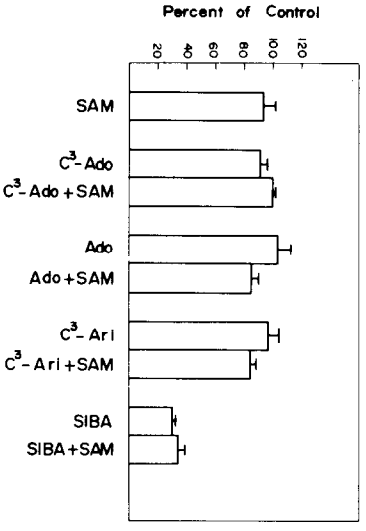

Fig. 2. Effect of the addition of SAM to isolated rat hepatocytes on the incorporation of labelled choline into phosphatidylcholine. Rat hepatocytes were treated with C^3-Ado (10 μM), Ado (1 mM), C^3-Ari (500 μM) or SIBA (500 μM) in the presence or absence of 5 mM SAM as described in Materials and Methods. Twenty minutes after the addition of SAM, 0.2 μCi/ml [methyl-^{3}H]choline was added and 15 min later the incorporation of radioactivity into phosphatidylcholine was measured as described in Materials and Methods. One hundred per cent corresponds to 7931 ± 120 dpm/10^7 cells (cells preincubated 10 min before the addition of SAM) or 7331 ± 133 dpm/10^7 cells (cells preincubated 30 min before the addition of SAM). Results are the mean ± S.E.M. of at least three independent experiments, each carried out in triplicate.

centrations of cAMP. Since SAM is currently being used to treat certain hepathopaties on the basis that this condition is accompanied with inhibition of the synthesis of SAM [11], the present results support the pharmacological use of SAM.

In summary, exogenous SAM can cross the cell membrane of isolated hepatocytes at pharmacological doses (concentrated ≥ 1 mM). At physiological concentrations SAM is either unable to cross the cell membrane or is rapidly metabolized. This argues against any physiological role for the uptake of SAM by hepatocytes. Our results are consistent with the data of Hoffman *et al.* [2], who concluded that the hepatocytes do not take up significant amounts of SAM when rat liver is perfused with 50 μM SAM.

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The effect of urine pH on the reduction of urinary PGE_2 excretion by indomethacin

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Urinary prostaglandin excretion has been regarded as an index of renal prostaglandin synthesis [1], and the urinary excretion of prostaglandin E_2 (PGE_2) has recently been used as a means of comparing the inhibition of prostaglandin synthesis elicited by different non-steroidal anti-inflammatory drugs [2]. There are several reasons why such comparisons between different agents can be misleading: it is widely recognized that changes in urine flow can influence prostaglandin excretion [3] but there are also other important determinants of prostaglandin excretion, notably the urinary pH [4, 5]. Thus if different non-steroidal anti-inflammatory agents change urine pH to different extents, this could alter urinary prostaglandin excretion in a manner not necessarily related to the extent of prostaglandin synthesis inhibition. In the present paper, we report the effects of a single non-steroidal anti-inflammatory agent, indomethacin, on the urinary excretion of PGE_2 at different urinary pH values.

Materials and methods

PGE_2 was obtained from the Upjohn Co. (Kalamazoo, MI) and [^{3}H]PGE_2 from Amersham International. (Amersham, Bucks., U.K.). PGE_2 antiserum was from Miles-Yeda (Rehovot, Israel). Silicic acid for column chromatography was from the Sigma Chemical Co. (St. Louis, MO).

Experimental protocol. All experiments were performed on conscious female Wistar rats (weight range 200–350 g), which were allowed free access to food and water prior to the experiments. Rats were placed in individual metabolic cages at the same time on each of 2 days, a control day and an experimental (indomethacin) day, and urine was collected over a 3 hr period. Three groups of animals were used; the animals in each group received, by stomach tube on each of the 2 days, the following solutions at the start of the urine collection period: group A, 0.9% NaCl (2 ml/100 g body wt); group B, 1% $NaHCO_3$ (2 ml/100 g body wt); group C, 1.3% NH_4Cl, 0.3% NaCl (3 ml/100 g body wt). On the experimental day each animal received indomethacin (10 mg/kg body wt, i.p.) at the start of the urine collection period.

Assays. The pH of each urine sample was measured, and the urine flow was determined. The samples were stored at −20° while awaiting radioimmunoassay for PGE_2. The assay procedure has been described elsewhere [5, 6].

Statistical methods. The significance of differences in the measured variables between the control and experimental days was assessed using Student's *t*-test. Results are presented as means ± S.E.

Results

The three groups of rats (A, B, C) were intended to have normal, alkaline and acidic urines, respectively (in the rat, urine pH is typically approximately 6.0). The urine pH values achieved are shown in Fig. 1. It is clear that on the control days, urine pH correlates well with PGE_2 output (Fig. 1), PGE_2 output being high at high pH (group B) and low at low pH (group C), with group A being intermediate for both pH and PGE_2 output.

Indomethacin administration had no significant effect on the pH of any of the groups. However, indomethacin did elicit a marked reduction in PGE_2 output in the group with alkaline urine (B) and the group with normal urine (A), but in group C (acid urine), where PGE_2 excretion was