Control of Spermidine and Spermine Levels in Rat Tissues by trans-4-Methylcyclohexylamine, a Spermidine-Synthase Inhibitor

Masaki KOBAYASHI,a Toshiko WATANABE,a Yong Ji XU,b Minoru TATEMORI,a Hitomi GODA,a Masaru NIITSU,a Akira SHIRAHATA,a and Keijiro SAMEJIMA.*,a

a Faculty of Pharmaceutical Sciences, Josai University; I–1 Keyakidai, Sakado, Saitama 350–0295, Japan; and b Institute of Chemical & Molecular Technology, Qingdao University of Science and Technology; 53 Zhongzhou Road, Qingdao 266042, Shandong, P.R. China. Received November 18, 2004; accepted January 13, 2005

In rat tissues, a decrease in spermidine, accompanied by an increase in spermine was induced by the oral administration (once daily for either 1 week or 1 month) of trans-4-methylcyclohexylamine (4MCHA), a spermidine synthase inhibitor. This is similar to the changes observed in polyamine content when cell growth is arrested. The body-weight gain of the rats tended to decrease with increasing doses of 4MCHA. A decrease in spermidine, combined with a moderate increase in spermine, was observed dose-dependently in all of the tissues tested, with a relatively fast clearance of 4MCHA. Manipulating the polyamine content of tissues, by daily administration of 100 μmol 4MCHA for 1 week, made it possible to estimate the effects of simultaneously added spermidine or spermine on endogenous polyamine contents. The altered polyamine levels, obtained after daily administration for 1 week, were maintained during the extended 1-month period, with growth-dependent alteration. The results show it is possible to produce experimental rats with a higher spermine:spermidine ratio than control rats to investigate the physiological significance of spermidine downregulation and spermine upregulation in vivo.

Key words putrescine aminopropyltransferase; inhibitor; oral administration; 15N-labelled polyamine; ionspray ionization-mass spectrometry; spermine : spermidine ratio

The polyamines, putrescine, spermidine (spd) and spermine (spm), present in mammalian tissues, are regulated under a variety of physiological conditions. Their tissue concentrations are controlled by a number of factors: (1) polyamine biosynthetic enzymes, such as ornithine decarboxylase (ODC), S-adenosyl-l-methionine decarboxylase (AdoMetDC), spermidine synthase (spd-syn) and spermine synthase (spn-syn); (2) polyamine catabolic enzymes, such as spd/spn N4-acetyltransferase (SSAT), polyamine oxidase (PAO), spermine oxidase (SMO) and diamine oxidase (DAO); and (3) the polyamine transport system on the cell membrane. The polyamines metabolic pathways catalyzed by these enzymes have been previously established; however, it is unclear how these factors function in vivo to regulate each polyamine under specific physiological conditions. A variety of inhibitors of these enzymes have been used, not only to elucidate the physiological significance of polyamines, but also to regulate cell growth, as polyamines are involved, either directly or indirectly, in many cellular events through their interactions with macromolecules and other cellular components. In liver regeneration, when cells are growing actively, putrescine and spd levels remain high, and the spm level is lower; this trend is reversed when cell growth is arrested. This suggests that downregulation of spd concentration concomitant with spm upregulation, might be associated with a reduced cell growth rate. In cultured cells, inhibition of spd-syn led to a decrease of spd and an increase of spm, but in short-term experiments, using the inhibitors, S-adenosyl-1,8-diamino-3-hiooctoane (AdoDato) and trans-4-methylcyclohexylamine (4MCHA), little or no change in growth was observed. We obtained similar results in a study using rats administered 4MCHA, dissolved in drinking water and available ad libitum, for a period of 10 d. The basic response of cells treated with spd-syn inhibitors was elucidated from these studies. However, no detailed quantitative study has been performed in vivo on the effect of spd-syn inhibitors, although some studies have used cyclohexylamine, which is an order of magnitude weaker than 4MCHA. Recently, we discovered a new inhibitor, 5-amino-1-pentene (APE), which showed a similar IC50 value to that of 4MCHA against purified spd-syn from rat prostate. The present work investigated spd downregulation and spm upregulation in rat tissues during continuous oral administration of 4MCHA over a 1-week or 1-month period. We predicted that normal physiological conditions would be disturbed if the manipulated polyamine contents were maintained in the rat tissues.

MATERIALS AND METHODS

Chemicals 4MCHA was purified by repeated recrystallization (at least three repetitions) of the hydrochloride of cis/trans-4-methylcyclohexylamine (Tokyo Kasei Kogyo, Tokyo, Japan) from ethanol/ethyl acetate, to confirm the complete elimination of the cis-isomer by NMR. trans-4-Ethylcyclohexylamine (4ECHA) and APE were prepared according to our recent report. The following stable isotope-labeled polyamines were prepared in our laboratory: [1,4-15N2]putrescine 2HCl (15N-put); [1,4,8-15N3]spermidine 3HCl (15N-spd); [1,4,8,12-15N4]spermine 4HCl (15N-spm); [1,4-13C4,1H-15N3]putrescine 2HCl (13C,15N-put); [5,8-13C3,1,4,8-15N4]spermidine 3HCl (13C,15N-spd); and [5,8-13C3,1,4,8,12-15N5]spermine 4HCl (13C,15N-spm). Standard polyamines were purchased from Sigma (Japan). Perchloric acid (PCA), CM-cellulose and heptafluorobutyric (HFB) anhydride (GC grade) were purchased from Wako Pure Chemical (Tokyo, Japan). Dansyl chloride was obtained from Kanto Chemical (Tokyo, Japan). Physiological saline was obtained from Ohtsuka Pharmaceutical (Tokyo, Japan). Dansyl chloride was obtained from Kanto Chemical (Tokyo, Japan). All other chemicals and organic solvents were of the purest grade available.

* To whom correspondence should be addressed. e-mail: keisame@josai.ac.jp © 2005 Pharmaceutical Society of Japan
Double-distilled deionized water (Milli Q; Millipore, Milford, CT, U.S.A.) was used throughout the experiments.

**Animal Care and Tissue Samples** Male Donryu rats (5 weeks old) were purchased from Saitama Experimental Animals (Saitama, Japan) and housed under standard animal laboratory conditions (25 °C; 12-h light/dark cycle; diet and water available ad libitum). Certified diet MF was obtained from Oriental Yeast (Tokyo, Japan). All experiments began when the rats were 6 weeks old (body weight: 170—200 g).

Sample solutions, dissolved in 1 ml of physiological saline, were orally administered to the rats once daily, and their body weights and the remaining diet were measured daily. Each group contained five rats housed in one cage. At the end of each experiment (after 1 week or every week during 1 month), the rats were orally administered 1 ml of saline solution containing a mixture of 15N-labelled spd and spm (20 µmol of each). After 3 h, when the 15N-labelled spd and spm content of the tissues had reached a maximum, the rats were sacrificed by cervical dislocation. The small intestine (approximately 10 cm next to the duodenum), liver, kidney and spleen were removed and washed at least three times with fresh physiological saline. The tissue samples were immediately frozen in liquid nitrogen and stored at −30 °C until analysis. In this study, 15N-labelled spd and spm were measured, as marker compounds, to detect any unusual absorption over time from the digestive tract exposed daily to a higher concentration of 4MCHA. No significant changes in polyamine uptake were observed during the present experiments, therefore, the data were omitted.

**Preparation of PCA Deproteinized Solution** Frozen organs were cut into fine pieces with scissors onto ice. Aliquots of the resulting paste (0.3 g) were placed in a Potter–Elvehjem homogenizer and homogenized with 1.0 ml of 0.1 M hydrochloric acid. The homogenates were mixed with 1.0 ml of 0.5 M PCA and re-homogenized. The supernatants were separated by centrifugation and stored at 4 °C.

**Polyamine Determination by Ionspray-Mass Spectrometry (IS-MS)** The supernatants (0.1 ml) were mixed with internal standards containing 2 nmol of 13C,15N Put, 15 nmol of 13C,15N-spd and 15 nmol of 13C,15N-spm, with the addition of a standard mixture of 0.5 nmol putrescine, 3 nmol spd and 3 nmol spm to one tube in duplicate. The mixtures were fractionated by CM-cellulose column (0.2 ml) chromatography. The polyamine fractions were collected and derivatized to HFB-polyamines, which were determined using an API 300 mass spectrometer (PE-SCIEX, Thornhill, Canada) with an attached IS-ionization interface, as described previously. In this experiment, an auto-injector (SIL-10AD VP, Shimadzu, Kyoto, Japan) was attached to make it possible to measure many samples accurately and simultaneously (three determinations per sample tube). It took approximately 20 min to complete the measurements of each pair of duplicate samples.

**Determination of 4MCHA** The supernatants (0.4 ml), with 2 nmol 4ECHA added as internal standard, were alkalinized with 2 ml of 1 M sodium hydroxide solution and extracted with 2 ml of ethyl acetate. After removal of the slightly acidified ethyl acetate with hydrochloric acid under a nitrogen stream, the residue was dissolved in 0.6 ml of acetone and 0.4 ml of a saturated sodium carbonate solution, and reacted with 0.1 ml of an acetone solution of dansyl chloride (10 mg/ml) under stirring for 1 h. Then, 0.1 ml of an aqueous solution of proline (150 mg/ml) was added to the mixture and stirring was continued for 30 min. The reaction mixture was extracted with 0.5 ml of toluene, and 5 µl of the extract was analyzed using HPLC. The conditions were as follows: column, CAPCELL PAK C18 (Type UG 120; I.D., 4.6×250 mm; SHISEIDO, Tokyo, Japan); elution, 75% acetonitrile/water; flow rate, 0.6 ml/min; detection, fluorescence excitation at 360 nm and emission at 510 nm. Retention times of dansyl derivatives of 4MCHA and 4ECHA were 15 and 17 min, respectively.

**RESULTS AND DISCUSSION**

**Selection of Inhibitor** To reveal any unknown side-effects, different types of inhibitor might help to analyze the results obtained from in vivo experiments. Two inhibitors, 4MCHA and APE, with similar inhibitory activities against purified spd-syn, were initially examined for their effects on spd and spm levels in rat tissues after oral administration of 100 µmol once daily for 1 week. The results from liver and kidney tissue are summarized in Table 1. A significant decrease in spd was observed in the two tissues treated with 4MCHA, whereas spd and spm levels in liver tissue treated with APE were similar to controls. On the basis of these results, 4MCHA (which is the best inhibitor for in vivo studies at present) was used in the following experiments. To obtain quantitative data, a once daily oral administration regime was employed.

**Administration of Different Doses of 4MCHA for 1 Week** Rats were separated into four groups (five rats per group) with similar average body weights. Once daily, each group was administered a 1-ml physiological saline solution orally containing 4MCHA (0, 30, 50 or 100 µmol). The body-weight gains of the four groups are summarized in Table 2. The body-weight gain tended to decrease with increasing doses of 4MCHA. A temporary retention of body-weight observed on day 2 with high doses (e.g. 50 or 100 µmol) of 4MCHA might indicate side-effects, such as the metabolic adaptation of rats to the drug. On day 8, the rats were sacrificed 24 h after the final administration of

### Table 1. Effects of Orally Administered APE and 4MCHA Once Daily for 1 Week on Polyamine Contents in Rat Liver and Kidney

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Kidney</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>spd</td>
<td>spm</td>
</tr>
<tr>
<td>Control</td>
<td>904±52</td>
<td>682±125</td>
</tr>
<tr>
<td>APE (100 µmol/d)</td>
<td>967±99</td>
<td>793±82</td>
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<tr>
<td>4MCHA (100 µmol/d)</td>
<td>542±141*</td>
<td>1002±158*</td>
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</tbody>
</table>

Conditions are described in the text. Values in the table show nmol/g wet tissue, mean±S.D. of 5 rats. Significance between 4MCHA-treated and controls (* p<0.05).
moderately rising spm levels with increasing doses of 4MCHA. The pattern of falling spd and spm contents in tissues show nmol/g wet tissue, mean ± S.D. of 5 rats. Significance between 4MCHA-treated and controls (∗∗p<0.001, ∗∗∗p<0.0001). Table 3. Effects of spd and spm Administered Simultaneously with 4MCHA Once Daily for 1 Week on Polyamine Contents in Rat Tissues

<table>
<thead>
<tr>
<th>Administered (μmol)</th>
<th>Liver</th>
<th>Kidney</th>
<th>Intestine</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>4MCHA</td>
<td>spd</td>
<td>spm</td>
<td>spd</td>
<td>spm</td>
</tr>
<tr>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1295±114</td>
<td>599±35</td>
</tr>
<tr>
<td>100</td>
<td>—</td>
<td>—</td>
<td>455±52**</td>
<td>974±62**</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
<td>—</td>
<td>588±88**</td>
<td>745±49**</td>
</tr>
<tr>
<td>100</td>
<td>50</td>
<td>—</td>
<td>711±47**</td>
<td>720±61**</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>—</td>
<td>832±149**</td>
<td>769±123</td>
</tr>
<tr>
<td>100</td>
<td>—</td>
<td>20</td>
<td>495±28**</td>
<td>1063±28**</td>
</tr>
</tbody>
</table>

Values in the table show body weight (g), mean ± S.D. of 5 rats. Significance between 4MCHA-treated and controls (p<0.05).

Fig. 1. Changes in Polyamine Levels in Rat Tissues Administered Different Doses of 4MCHA Once Daily for 1 Week

Putrescine (○), spd (□), spm (△). The experimental conditions are described in the text. Significance between 4MCHA-treated and controls (0 μmol) (p<0.01).

4MCHA and the polyamine contents of the liver, kidney, intestine, and spleen were measured (Fig. 1). The polyamine levels were tissue-dependent. The pattern of falling spd and moderately rising spm levels with increasing doses of 4MCHA was common to all these tissues. Putrescine levels were low in the liver and kidney, and were moderate in the intestine and spleen, which have an active turnover. In the latter tissue samples, a moderate rise in putrescine was observed with increasing doses of 4MCHA. The weak increase in putrescine might be due to a limitation of the use of putrescine produced with a normal induction of ODC to spd. From these data, no particular induction of ODC seemed to occur with 4MCHA. The total polyamine content of the intestine, liver and spleen tended to decrease with increasing doses of 4MCHA, whereas it was unchanged in the kidney. The decrease in total polyamine content might be explained by a deficiency of newly biosynthesized spd, which should have been liberated and used for the biosynthesis of spm, even though decarboxylated AdoMet (dcAdoMet) was available through the induction of AdoMetDC as demonstrated in cultured cells. The concentration of 4MCHA in liver was also measured 24 h after final administration; however, at a detection limit of 3 nmol/g wet tissue, 4MCHA was not identified, even in the 100 μmol dose group. These results suggested a rapid clearance of 4MCHA.

Simultaneous Administration of 4MCHA and spd or spm for 1 Week

We examined the effects of exogenous spd or spm, administered with 4MCHA, on body-weight gain and the polyamine content of rat tissues. The dose of 4MCHA was set at 100 μmol, expecting a distinct role of ex-
ogenous spd or spm under the significantly altered spd and spm contents. The dose of exogenous spd was 20, 50 or 100 μmol, whereas that of exogenous spm was 20 μmol. Each mixture, in 1 ml saline, was administered orally once daily for 1 week (as described above). The simultaneous administration of spd or spm had no significant effect on body-weight gain, showing similar body-weight gains to those in Table 2 (4MCHA, 100 μmol). On day 8, the rats were sacrificed 24 h after the final administration and the polyamine content was measured in the four tissues, as described above (Table 3). The significant decrease of spd and the increase of spm in the four tissues administered 4MCHA alone compared with control were the basic responses seen in the four groups administered 4MCHA plus spd or spm. However, there were some new observations regarding the in vivo behavior of exogenous spd and spm. In the groups administered exogenous spd, the spd content in the four tissues tended to increase gradually with increasing doses of exogenous spd, and the spm content remained higher than that of control but lower than that of the group receiving 4MCHA alone, irrespective of the increasing doses of spd. This indicated that exogenous spd could inhibit the de novo synthesis of spm in the tissues. It is well known that adding polyamines to the medium immediately suppresses polyamine biosynthesis in cultured cells by inhibiting the induction of ODC and AdoMetDC.17) It is possible, therefore, that the inhibition of spm synthesis with exogenous spd is due to a deficiency of deAdoMet, suggesting the repression of AdoMetDC, which should be induced by the administration of 4MCHA. In the group administered exogenous spm, the spd and spm contents were similar to those of the group receiving 4MCHA alone, thus showing a distinct difference between the effects of exogenous spd and spm. These data suggest that the altered polyamine levels with 4MCHA will be restored gradually to normal values with the addition of exogenous spd and the inhibition of spm biosynthesis, perhaps caused by the inhibition of AdoMetDC induction.

Extended Administration of 4MCHA for 1 Month To investigate the maintenance of the altered polyamine levels caused by 4MCHA, the period of daily administration was extended to 4 weeks. Experimental conditions were the same as those described above for the 1-week experiments, with the exception of the doses of 4MCHA, which were increased weekly in proportion to the average body-weight gain as follows: 30, 38, 45 and 53 μmol in the 30-μmol daily-administration series, and 50, 63, 75 and 88 μmol in the 50-μmol daily-administration series, during the first, second, third and fourth weeks, respectively. The tendency of the dose-dependent reduction of body-weight gain in the rats administered 4MCHA was observed over the 4 weeks with no apparent loss of appetite and little change in the behavior of the rats. The average body weights before sacrifice after 4 weeks were 390, 380 and 340 g for the control, 30- and 50-μmol series, respectively. Rats were sacrificed at the end of each week, and the spd and spm content of the liver, kidney, intestine and spleen measured. A downregulation of spd and upregulation of spm was observed in the four tissues in the 50-μmol daily-administration series (Fig. 2a), but was marginal in the 30-μmol daily-administration series; the results of this series were therefore omitted from Fig. 2. The spleen showed a significant decrease in spd content, which corresponded to normal growth (Fig. 2a). The spm : spd ratios in all tissues, with the exception of the intestine, tended to increase during the 4 weeks, in accordance with growth. In the four tissues, the ratios in the 50-μmol daily-administration series were maintained at higher levels than control, and seemed to parallel those of the control during the 4 weeks (Fig. 2b). These data suggest that continuous administration of 4MCHA, at least at the 50-μmol series dose, can generate experimental rats with an elevated spm : spd ratio, and might be useful for elucidating the physiological significance of this elevated ratio.

Limited increases in spm content were observed throughout the present study. These results are consistent with the
limited increase of spm observed in transgenic mice that overexpress spm-syn. 18) In vivo, spm levels (mostly in a bound form 6)), compared with spd, must be regulated normally over a relatively narrow range (Fig. 2a) by various factors, such as depletion of liberated spd, inhibition of AdoMetDC induction and catabolic enzymes, including SSAT, PAO and SMO. Changes in total polyamine levels in tissues seem to depend largely on the spd content. Undoubtedly, spd is essential for cell growth, as was illustrated in SSAT-transgenic mice by a report on the importance of spd for liver regeneration after partial hepatectomy. 19) In future studies, the artificial manipulation of the spm : spd ratio with 4MCHA might provide additional information on the physiological significance of polyamines, as well as cell growth.

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