Biosynthetic response of mouse intermediate pituitary gland to induced drinking and dehydration

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Abstract. There are indications that the intermediate lobe peptide α-MSH is involved in the regulation of the hydromineral balance in mice and other mammals. The purpose of our studies was to determine whether manipulation of this balance in the mouse could lead to changes in either the rate of POMC biosynthesis in the pars intermedia or to changes in the direction of the processing of the precursor protein to form bioactive peptides. The results show that excess drinking, induced by substitution of drinking water by a 5% glucose solution, causes a rapid increase in POMC synthesis, whereas dehydration has the opposite effect; no evidence could be found that the above treatments have any effect on the processing of POMC, although strain differences were found in level of N-terminal acetylation of newly synthesized melanotropins and endorphins. The changes in various parameters of the hydromineral balance of the animals are consistent with the concept that peptides of the pars intermedia may be involved in regulating plasma aldosterone levels under severe conditions of low plasma sodium concentration. 

Proopiomelanocortin (POMC) is a multifunctional precursor protein that, in the cells of the pituitary pars intermedia, gives rise to a number of peptides, including the hormone α-melanocyte-stimulating hormone, α-MSH (1-4). In lower vertebrates α-MSH is involved in the regulation of pigment dispersion in dermal melanophores during the process of background adaptation (5). The physiological function of this intermediate lobe peptide in mammals is not clear; suggestions have included a role in the regulation of sebaceous gland secretion (6), hair pigmentation (7,8) and adrenal gland function, namely in the stimulation of aldosterone secretion (9,10). This latter suggestion for a function of α-MSH in hydromineral balance finds support in morphological studies concerning the pars intermedia of rodents. For example, copious water consumption, induced by offering 5% glucose drinking water, has been correlated with an increase in the volume of the rough endoplasmatic reticulum and the development of prominent Golgi apparatus in the cells of the pars intermedia of the mouse (11). In contrast, it has been shown that deprivation of water leads to a dramatic reduction in the amount of rough endoplasmatic reticulum in intermediate lobe cells (12).

The purpose of the present study was to determine whether the above-mentioned morphological changes in the intermediate lobe of rodents are reflected in changes in either the rate of biosynthesis of POMC or in changes concerning the processing of this precursor protein to produce bioactive peptides. These experiments were conducted using three genetically characterized mouse strains. POMC biosynthesis and processing was evaluated by determining the in vitro incorporation of radioactive amino acids into the precursor protein and the POMC-derived peptides. In considering processing of the precursor protein, particular attention was given to the acetylation process which gives rise to N-terminal acetylated forms of α-MSH and β-endorphin. Finally, in an attempt to attach a physiological significance to our findings, various parameters concerning the hydromineral balance of the animal, such as plasma Na⁺ and aldosterone levels and plasma hematocrit, were also measured.
Material and Methods

Animals and experimental treatments
Inbred Agouti (CN Nijm), Black (C57BL) and Tabby/+ (Ta/+) male and female mice were used in these studies. Prior to the experiments they were maintained under standard laboratory conditions (20°C; light: dark 12 h:12 h) and fed ad libitum (Hope Farms, Woerden, The Netherlands). Each experiment included three groups of animals: a control group which was given tap water and food ad libitum; a 5% glucose group in which a 5% glucose solution in tap water replaced the drinking water and food was withheld; a dehydration group which was deprived of drinking water but had food ad libitum. All treatments were started and terminated between 9.00 and 10.00 h; dehydration treatments were conducted for a maximum of 2 days and 5% glucose treatments for up to 4 days. At the end of the treatment the animals were killed by cervical dislocation and the pituitary neurointermediate lobes quickly removed for analysis of biosynthetic activity. In experiments with bromocriptine treatment, the drug was injected sc twice a day (100 μg).

In vitro labelling
The isolated lobes were incubated for either 1 h in medium containing [3H]lysine (79.3 Ci/mmol, Amersham), to examine the biosynthesis of POMC using SDS electrophoretic analysis, or for 1 h in medium containing [3H]methionine (85 Ci/mmol, Amersham) followed by 4 h chase, to examine processing of POMC using HPLC analysis. The purpose of the longer incubation time in these latter experiments was to give sufficient time for processing of newly synthesized POMC, as established in earlier studies (13,14). The incubations were conducted at 37°C in a gassed (95% O2/5% CO2) medium containing 150 mmol/l NaCl, 4.8 mmol/l KCl, 2.8 mmol/l CaCl2, 1.3 mmol/l MgCl2 buffered in 15 mmol/l HEPES (Ultrol grade, Calbiochem) at pH 7.4. The incubation medium was also supplied with 11.1 mmol/l D-glucose and 0.015 mmol/l bovine serum albumin (fraction V, Sigma). Isotope concentration was 1.3 mCi/l and each lobe was incubated in a volume of 60μl. Following incubation the lobes were extracted in 0.1 mol/l HCl; extracts for electrophoretic analysis were vacuum-dried (Savant Speed-Vac) and those for HPLC were frozen immediately.

SDS electrophoretic analysis
Electrophoresis was performed according to Laemmli (15) in a Biorad vertical slab gel electrophoresis unit. The slab was fixed for 1 h in a solution of 40% methanol, 10% acetic acid, and 10% trichloroacetic acid and then treated with 2,5-diphenyloxazole (Merck) according to Bonner & Laskey (16); the radioactive proteins were detected with scintillation autoradiography (fluorography) using Kodak XR-1 film at −70°C. The film was pre-flashed in order to produce quantitative fluorograms, which were scanned (Biorad densitometer, model 1650) and peak areas determined using a MOP (Digiplan, Kontron).

HPLC analysis
HPLC of lobes extracts was conducted using a spherisorb 10 ODS-C18 column (Bischoff) on a Spectra Physics HPLC system (model SP 8700). The primary solvent was 0.5 mol/l formic acid, 0.14 mol/l pyridine (pH 3) and the gradient established with 1-propanol. HPLC fractions of 1 ml were collected, 4 ml liquid scintillation fluid (Scintillator 199, Packard) was added, and radioactivity in each fraction determined using a LKB RacBeta Scintillation analyzer.

Analysis of trunk blood
At the time of dissection trunk blood from the animals was collected to determine plasma aldosterone levels. Aldosterone was extracted from 100 μl plasma samples using 400 μl 100% ethanol, which was then dried and subsequently redissolved in citrate/phosphate buffer in preparation for aldosterone radioimmunoassay. The antisera and assay procedure used have been previously described (17); virtually no cross-reactivity to corticosterone is present. The hematocrit of trunk blood samples was also determined, as were plasma and urine sodium contents, determined by flamephotometry (Technicon).

Statistical analysis of data was conducted using analysis of variance (α=5%). For each parameter, the mean value per animal was entered. The analysis was preceded by tests for the homogeneity of variance (Bartlett’s test; 18) and the joint assessment of normality (19). For some parameters data transformation was carried out because of a weak relationship between means and variances, whereby variances were rendered homogeneous.

Results

POMC biosynthesis in 5% glucose-treated animals
Electrophoretic analysis showed that neurointermediate lobes of animals given 5% glucose treatment had higher rates of [3H]lysine incorporation into newly synthesized proteins than those of control animals. An example of a fluorogram is given in Fig. 1, together with the water consumption displayed by the animals analysed. Included among the newly synthesized products were proteins with molecular weights of 30.5 and 34 kD; these proteins have been previously characterized as two forms of mouse POMC, designated POMC I and II, which differ only in their degree of glycosylation (20) (for details see Discussion). Results shown in Fig. 1 are for Agouti female mice; analysis of Agouti male mice and males and females of the C57BL strain gave similar results. The density of
the fluorometric bands representing these POMC proteins was approximately 6 times higher in the 1-day glucose-treated animals than in the control animals, and showed further increases with longer glucose treatment (Fig. 2a).

Analysis of plasma and urine
As expected, 5% glucose treatment led to a marked increase in water intake, which reached a maximum after 2-3 days (Fig. 2b). Occasionally, animals were found that showed little or no increase in drinking; such animals also displayed lower rates of [3H]lysine incorporation into neurointermediate lobe proteins (e.g. the 5% glucose-treated animals drinking 6.6 ml/day and 13.9 ml/day, Fig. 1). The glucose-treated animals displayed strong diuresis and the sodium content of the urine declined rapidly (Fig. 2e). Plasma sodium concentrations were maintained for the first three days of treatment (Fig. 2d), whereas blood hematocrit had increased significantly within 1 day and remained at the elevated level with longer treatment (Fig. 2f). There were no clear sex or strain differences in [3H]lysine incorporation, nor in the other parameters measured, in response to 5% glucose treatment.

POMC biosynthesis in dehydrated animals
Following two days of dehydration, the incorporation of [3H]lysine into neurointermediate lobe proteins, including the two forms of POMC, was dramatically reduced (Fig. 3). However, when such animals were given water, the rate of isotope incorporation recovered to almost control levels within 1.5 h (Fig. 3). We found no sex or strain differences in these responses (not shown).
Electrophoretic analysis of neurointermediate lobe of dehydrated and 1.5-h rehydrated Agouti-CN mice. For further details see Fig. 1.

**HPLC analysis of newly synthesized peptides**

Analysis of the radioactive peptides synthesized by neurointermediate lobes revealed that the 5% glucose-treated animals had higher rates of incorporation of [3H]methionine into peptides than the control animals, whereas the dehydrated animals had lower rates. Fig. 4 shows an example of such an analysis for C57 Black male mice. (Note difference in scale of axis.) The characterization and identification of the HPLC-resolved peptides is given elsewhere (13,14). Neither 5% glucose treatment nor dehydration appeared to have any effect on the relative pattern of peptides found in the HPLC profile. Analysis of the relative contribution of acetylated and non-acetylated forms of α-MSH and β-endorphin to the HPLC profiles showed that the experimental treatments had no effect on the amount of acetylated peptides formed (Table 1). This analysis did reveal, however, that the two mouse strains used differed in the extent to which they produced acetylated peptides. The relative contribution of des-acetylated forms of MSH to the HPLC profile of melatropins was significantly higher for Agouti mice in comparison to the C57 Black mice (Table 1). For β-endorphin related peptides Agouti CN mice again showed a significantly higher contribution of the non-acetylated form relative to the acetylated form of the peptide (Table 1).

**Effect of bromocriptine**

In order to investigate the effect of inhibition of POMC biosynthesis on the plasma aldosterone level...
Table 1.
Percentage newly synthesized melanotropins and β-endorphin in acetylated and non-acetylated forms in neurointermediate lobes of 5% glucose-treated mice, control mice, and dehydrated mice of the strains Agouti CN and C57 Black during 2 days.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>N</th>
<th>% Des-α-MSH</th>
<th>% α-MSH</th>
<th>% N-diacetyl α-MSH</th>
<th>% N-acetyl-β-endorphin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agouti CN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% glucose</td>
<td>3</td>
<td>34.3±8.0</td>
<td>30.9±4.6</td>
<td>34.9±3.7</td>
<td>56.6±3.6</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>32.5±7.9</td>
<td>31.8±7.2</td>
<td>36.5±3.8</td>
<td>59.6±6.3</td>
</tr>
<tr>
<td>Dehydration</td>
<td>3</td>
<td>37.6±2.0</td>
<td>25.7±4.3</td>
<td>36.8±4.6</td>
<td>53.5±3.5</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>34.8±6.6a</td>
<td>29.3±5.5</td>
<td>36.1±4.1b</td>
<td>56.6±4.7c</td>
</tr>
<tr>
<td>C57 Black</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% glucose</td>
<td>4</td>
<td>26.0±6.8</td>
<td>27.8±10.0</td>
<td>46.3±9.6</td>
<td>65.4±3.3</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>27.5±5.1</td>
<td>27.6±8.5</td>
<td>44.7±11.1</td>
<td>66.5±3.3</td>
</tr>
<tr>
<td>Dehydration</td>
<td>4</td>
<td>28.3±2.0</td>
<td>28.6±8.8</td>
<td>40.5±9.1</td>
<td>67.5±3.2</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>27.3±4.7a</td>
<td>28.0±7.9</td>
<td>43.8±8.6b</td>
<td>66.5±2.8c</td>
</tr>
</tbody>
</table>

The total radioactivity in HPLC profiles associated with the melanotropins or with the endorphins was determined; the radioactivity associated with the various forms of these peptides was then expressed as a percentage of the above totals. No significant differences exist within the treatment groups. Data with a common superscript differ significantly (p<0.01). N=number of animals analysed.

we injected bromocriptine, a dopamine receptor agonist which inhibits pars intermedia function (8,21) in Tabby/+ mice. In contrast to in the Agouti CN mouse, the Na⁺ levels in Tabby/+ mice were strongly reduced within 2 days on 5% glucose drinking water, and aldosterone levels were strongly elevated within 2 days (Table 2). These levels were reduced to control values in bromocriptine-treated mice, as were the levels of POMC biosynthesis.

Table 2.
Effects of drinking a 5% glucose solution during 2 days, either with or without treatment with bromocriptine on various parameters for MSH cell activity and internal homeostasis of Tabby/+ mice. Mean values are given ± SEM. Superscript indicates group with which a significant difference exists (p<0.01). Parameter 1: N=3 (animals), other parameters: N=5 (animals). Arb U: arbitrary units; OD: optical density.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (a)</th>
<th>5% Glucose (b)</th>
<th>5% Glucose/bromocriptine (c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Biosynthesis (OD in Arb U)</td>
<td>6.5b±1.0</td>
<td>13.6e±1.14</td>
<td>4.0b±1.0</td>
</tr>
<tr>
<td>2. Water intake (ml/day)</td>
<td>6.1bc±0.3</td>
<td>68.5b±2.7</td>
<td>45.5b±1.7</td>
</tr>
<tr>
<td>3. Plasma Na⁺ (mmol/l)</td>
<td>154.4bc±1.8</td>
<td>129.0a±4.0</td>
<td>138.4b±3.9</td>
</tr>
<tr>
<td>4. Aldosterone (pmol/l)</td>
<td>1903b±75</td>
<td>3881bc±510</td>
<td>1510b±399</td>
</tr>
<tr>
<td>5. Hematocrit (%)</td>
<td>49.7bc±0.5</td>
<td>54.6a±0.8</td>
<td>54.0a±0.2</td>
</tr>
</tbody>
</table>

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Discussion

Our results show that 5% glucose treatment leads to an increase in biosynthetic activity of the mouse pars intermedia. Two prominent bands of the fluorometric analysis, the 30.5 and 34 K products, have been extensively characterized in a previous study using selective amino acid incorporation in combination with tryptic mapping (20). These products represent two forms of mouse POMC which differ only in their degree of glycosylation, with the 34 K form of POMC (POMC I) being glycosylated in the γ-MSH region and corticotropin-like intermediate lobe peptide (CLIP) region, while glycosylation of the 30 K precursor (POMC II) was restricted to the γ-MSH region. In the present study long pulse incubations in radioactive amino acids were employed and thus labelling was observed not only in the bands representing POMC, but also in other protein bands. The latter bands are likely to represent structural proteins and intermediates in the processing of POMC. Densitometric analysis of the POMC bands revealed that there was a progressive increase in the rate of biosynthesis of the precursor proteins over the 4-day treatment period. Moreover, there was a strong correlation between the volume of liquid consumed relative to the level of precursor synthesis achieved when individual results were considered. Altogether, it can be concluded that 5% glucose treatment activates the mouse pars intermedia cells to produce POMC.

The question arises whether activation of the intermediate lobe by 5% glucose treatment is a consequence of high glucose intake or, alternatively, a consequence of the copious drinking associated with this treatment. We believe the latter to be the case because it has been reported that in mice maintained on 2% NaCl drinking water, a treatment that also leads to copious drinking, the pars intermedia displays morphological characteristics of hyperactivity (12). Indeed, in preliminary experiments with the Agouti CN mouse we found that treatment with 2% NaCl drinking water may lead to copious drinking and an elevation of POMC biosynthesis, although not as dramatic or as reproducible as observed after 5% glucose treatment. Treatment with 2% NaCl occasionally leads to a decrease in water consumption, and we found in such cases a decrease in POMC synthesis. Caution should be taken when considering results of Na⁺ loading experiments, in that Elkahes & Loh (22) recently reported that treatment of mice with NaCl leads to a decrease in POMC biosynthesis in the pars intermedia.

Our data show that dehydration, in sharp contrast to 5% glucose treatment, results in an inhibition of POMC biosynthesis in the mouse neurointermediate lobe. This extends an earlier observation showing that such treatment leads to an involution of the pars intermedia, which includes a reduction in the amount of rough endoplasmatic reticulum and Golgi (12). In view of these morphological observations it is somewhat surprising that rehydration causes a rapid return in POMC biosynthesis (i.e. to levels approaching that of the control, within 1.5 h). Possibly, dehydration in the mouse strains used is not accompanied by the drastic morphological and structural changes reported above.

The observations concerning the overall level of POMC biosynthesis are reflected in the results of experiments where processing of the precursor proteins were examined using HPLC analysis. These results showed that the pars intermedia of 5% glucose-treated animals produced more than twice the amount of newly synthesized peptides as compared with control animals and displayed an approximately 4-fold difference when compared with the dehydrated animals. The HPLC-resolved peptides have been extensively characterized in previous studies utilizing a number of techniques, including selective amino acid incorporation, tryptic and chymotryptic mapping, and immunoprecipitation (13,14). In the present study, [3H]-methionine has been used for labelling to focus our analysis at newly synthesized α-MSH-related peptides and endorphins; both mouse CLIP and γ-LPH lack this amino acid and, therefore, it is not present in the profiles. Mouse γ-MSH elutes before 10 min from the HPLC column and is excluded from our analysis. The results show that the relative profile of α-MSH- and β-endorphin-related peptides are remarkably similar among the experimental groups. This holds for both mouse strains. Therefore, we conclude that the experimental treatments had no effect on either the processing of POMC to produce these peptides, or on the post-translational acetylation process to produce acetylated forms of the peptides. There were clear strain differences in the degree to which MSH and β-endorphin were acetylated within the time limits of the analysis. Previous biosynthetic studies have shown that the acetylated endorphin is processed further to form C-terminal-truncated peptides (14). As this cleavage step occurs only very slowly
(taking several days), analysis of this aspect of processing was not within the time frame of our pulse-chase analysis.

It is difficult to attach any physiological significance to the observation that dehydration leads to an inhibition of the POMC cells of the pars intermedia and that augmented drinking results in a stimulation of the activity of these cells. One of the POMC-derived peptides, α-MSH, has been reported to function as an aldosterone secretagogue (9), and more recently Hinson et al. (23) have shown that there may be up to a 1000-fold increase in adrenal sensitivity to α-MSH under low Na⁺ conditions. A Na⁺-induced modulation of adrenal tissue sensitivity to regulatory factors might explain some of our results. In Agouti CN mice, where plasma Na⁺ levels were maintained for 3 days, there was only a very slow rise in plasma aldosterone. In contrast, in Tabby/+ mice, in which plasma Na⁺ level was decreased drastically within 2 days, there was a dramatic increase in aldosterone levels. That peptides from the pars intermedia might play a role in inducing aldosterone release is demonstrated by the observation that bromocriptine treatment, which appeared extremely effective in blocking POMC biosynthesis, is equally effective in blocking the increase in plasma aldosterone.

Finally, it should be mentioned that our results are reminiscent to those of Legait & Legait (24) who showed that desert rodents have involuted intermediate lobes under conditions of extreme dehydration and acquire large lobes when given free access to water. Possibly, studies of these desert rodents, which must make drastic physiological and environmental adaptations for survival, might help in defining the physiological function of the pars intermedia in mammals.

Acknowledgments

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