Inhibition of Extracellular Signal–Regulated Kinase in Liver of Hyperhomocysteinemic Mice

To the Editor:

We read with great interest the article by Woo et al., reporting the involvement of cAMP-dependent protein kinase A (cAMP-PKA) signaling pathway in activation of the transcription factor cAMP-response element binding protein (CREB) during hyperhomocysteinemia in the liver. Hyperhomocysteinemia, defined as elevated homocysteine (Hcy) levels in blood, is not only associated with cardiovascular and cerebrovascular disorders, but is also accompanied by hepatic steatosis.2 As CREB plays an important role in the expression of genes involved in lipid metabolism, the work of Woo et al is of great importance to show the mechanism by which Hcy induces CREB activation. Among the signaling pathways important for activation of CREB, protein kinases such as PKA, extracellular signal regulated-kinase (ERK), and p38 mitogen-activating protein (p38 MAP) kinase are able to phosphorylate CREB leading to its activation.3 Woo et al demonstrated, by incubation of hepatocytes with inhibitor of protein kinases, that Hcy-induced CREB activation is mediated by PKA signaling pathway, but not by ERK pathway or p38 MAP kinase pathway. These results are very intriguing because we have showed the involvement of ERK and PKA signaling pathways in the Hcy-induced CREB activation in an ex vivo model of hippocampal slices.4 As we have also shown an activation of ERK and the downstream nuclear target CREB in the hippocampus of cystathionine beta synthase (CBS)-deficient mice, a murine model of hyperhomocysteinemia,5 the present work was designed to investigate whether activation of ERK also occurs in liver of CBS-deficient mice.

Heterozygous CBS-deficient (Cbs+/−) mice were bred to obtain homozygous CBS deficient (Cbs−/−) mice. Genotyping for the targeted CBS allele was performed by polymerase chain reaction (PCR) analysis.6 At weaning, Cbs+/− mice were fed a standard diet supplemented with 1.592 g/kg choline chloride salt, necessary to their survival. The wild-type littermates (Cbs+/+) were also fed the same supplemented chow to avoid differences attributable to the diet. At the time of sacrifice, blood samples were collected into tubes immediately, and plasma was isolated by centrifugation at 2500g for 15 minutes at 4°C. Plasma total Hcy (tHcy), defined as the total concentration of Hcy after quantitative reductive cleavage of all disulfide bonds, was assayed by using the fluorimetric high-performance liquid chromatography (HPLC) method described by Fortin and Genest.6 The liver was harvested, snap frozen, and stored at −80°C until use. A piece of the liver was homogenized and 30 μg of protein per samples were subjected to immunoblot analysis using antibodies specific to phospho-ERK. After stripping, the membranes were reprobed with anti-ERK antibody for the control. The blots are representative of 4 independent experiments (4 mice for each genotype).

Western immunoblots showing ERK activation in liver of (a) wild-type (Cbs+/+) mice and homozygous (Cbs−/−) mice, (b) heterozygous (Cbs+/−) mice and heterozygous mice fed a hyperhomocysteinemic diet (Cbs+/− Met). Proteins were subjected to immunoblot analysis using antibodies specific to phospho-ERK. After stripping, the membranes were reprobed with anti-ERK antibody for the control. The blots are representative of 4 independent experiments (4 mice for each genotype).

<table>
<thead>
<tr>
<th>CBS Genotype</th>
<th>tHcy (mmol/L)</th>
<th>Phospho-ERK/ERK (% Wild-Type)*</th>
<th>Relative Expression of MKP-1 Gene (% Wild-Type)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (Cbs+/+)</td>
<td>4.2±0.2</td>
<td>0.92±0.08</td>
<td>1.03±0.09</td>
</tr>
<tr>
<td>Heterozygous (Cbs+/−), control diet</td>
<td>8.5±1.1*</td>
<td>0.95±0.03</td>
<td>1.02±0.1</td>
</tr>
<tr>
<td>Heterozygous (Cbs+/−), high-Met diet</td>
<td>35.8±3.7**</td>
<td>0.67±0.03*</td>
<td>1.51±0.19*</td>
</tr>
<tr>
<td>Homozygous (Cbs−/−)</td>
<td>166.3±27.1***</td>
<td>0.46±0.07*</td>
<td>1.9±0.18**</td>
</tr>
</tbody>
</table>

*Relative enzyme activation was determined by normalization of the density of images from phosphorylated ERK with that of the total inactive ERK on the same blot [4]. The values of active phospho-ERK of 4 Cbs+/−, Cbs−/− fed a hyperhomocysteinemic diet (high-Met diet) and Cbs−/− mice was normalized to the mean of 4 Cbs+/− mice.

**Q-PCR values of 4 Cbs+/−, Cbs−/− fed a high-methionine diet (high-Met diet) and Cbs−/− mice were normalized to the mean of Cbs+/− mice. Primer sequences specific for MKP-1 transcripts are: 5′-TGCCCTGAGATGGAGATC-3′ for left primer and 5′-TCCTGGAGAGGCTGTAAG-3′ for right primer. Primer sequences specific for mouse SOD1 transcripts are 5′-GGGACACATAGAACAGC-3′ for left primer and 5′-TGTGACACCATGGC-3′ for right primer. Data correspond to means±SEM, and the statistical analysis was done with ANOVA followed by Student unpaired t test using Statview software. *P<0.05; **P<0.004; ***P<0.001.
Table), showing a 40-fold increase in tHcy (Table). To determine whether this decrease was attributable to the loss of CBS expression or to the resultant hyperhomocysteinemia, the activation of ERK was also examined in liver of Cbs+/− mice fed a hyperhomocysteinemic diet compared with the activation in liver of Cbs−/− mice. At weaning, mice were fed a standard diet supplemented with 0.5% L-methionine in drinking water for 3 months. Such a diet led to a 4-fold increase in tHcy (Table) and decreased ERK activation by 30% (Figure, b; Table). These results show that hyperhomocysteinemia inactivates ERK in mouse liver.

Activated MAP kinases can be inactivated by MAP kinase phosphatases (MKPs). Among these MKPs, MKP-1 is inducible by L-methionine inactivates ERK in mouse liver.

MKP-1 were assessed by real-time quantitative reverse transcription-PCR (Q-PCR), using Light Cycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics), as described. The primer-annealing temperature was 65°C. The mouse superoxide dismutase-1 (SOD1) mRNA was used as an endogenous control. Activated MAP kinases can be inactivated by MAP kinase phosphatases (MKPs). Among these MKPs, MKP-1 is inducible by a variety of cellular stresses, including oxidative stress, at mRNA levels. As oxidative stress has been found to be promoted by hyperhomocysteinemia in liver of CBS-deficient mice, we have also analyzed the gene expression of MKP-1. The mRNA levels of MKP-1 were assessed by real-time quantitative reverse transcription-PCR (Q-PCR), using Light Cycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics), as described. The primer-annealing temperature was 65°C. The mouse superoxide dismutase-1 (SOD1) mRNA was used as an endogenous control. ΔΔCp analysis of the results allows to assess the ratio of the target mRNA versus SOD1 mRNA. MKP-1 mRNA levels in livers of Cbs+/− mice were similar from Cbs−/− mice (Table), in agreement with ERK activation. However, MKP-1 mRNA levels not only in the livers of Cbs−/− mice but also in the liver of Cbs+/− mice fed a hyperhomocysteinemic diet were higher than that of Cbs+/− mice and Cbs−/− mice (Table). These results emphasize the role of hyperhomocysteinemia on MKP-1 expression, which may be contributing to the inhibition of ERK phosphorylation in liver.

Taken together, our results support the view of Woo et al. that ERK kinase signaling pathway is not involved in Hcy-induced CREB activation in hepatocytes. Moreover, our results demonstrate that some signal transduction pathways, like ERK, can be differentially regulated according to the tissue which is affected in hyper-homocysteinemic mice.

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None.

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