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Very low protein diets decrease cAMP-mediated cytosolic and nuclear responses in isolated rat hepatocytes

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Very low protein diets disrupt the cAMP signal transduction cascade in rat hepatocytes. After 3 days of feeding a 0.5% protein diet to weanling rats, quantity of the RI regulatory subunit of cAMP-dependent protein kinase (PKA) is decreased in the cytosol of the hepatocyte. During the second week of feeding very low protein diets, this reduction in RI quantity is sustained, but is coupled with an increase in hormone-stimulated cAMP production. We have investigated the effects of these changes in the cAMP signal transduction pathway on two downstream responses to cAMP: activation of glycogen phosphorylase and phosphorylation of cAMP response element binding protein (CREB). Isolated hepatocytes from rats fed 15% protein (control) or 0.5% protein (malnourished) diets for 3 or 14 days were treated with glucagon, dibutyryl-cAMP, an RI-specific agonist, or an RII-specific agonist pair. Activation of glycogen phosphorylase in hepatocytes from malnourished rats was lower in response to RI-agonist at days 3 and 14, whereas RII-stimulated activation did not change. Stimulation of glycogen phosphorylase by glucagon was lower in hepatocytes from malnourished rats at day 3, but not at day 14, when hormone-stimulated cAMP production is increased. After treatment with glucagon and RI agonist, quantity of phosphorylated CREB (pCREB) was lower in hepatocytes from malnourished rat; however, stimulation relative to the baseline quantity of pCREB was maintained. These data suggest that decreases in the quantity of cytosolic PKA-RI after consumption of very low protein diets impair down-stream responses to PKA activation in both the cytosol and nucleus of rat hepatocytes. (J. Nutr. Biochem. 8:172–180, 1997) © *Elsevier Science Inc. 1997*

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Introduction

cAMP is an important regulator of cellular metabolism and gene transcription.[1,2] Hormonal activation of adenylyl cyclase activity increases intracellular cAMP production; cAMP then binds to the regulatory subunits (R) of cAMP-dependent protein kinase (PKA), inducing holoenzyme dissociation and the liberation of the catalytic subunit (PKA-C). PKA-C phosphorylates target proteins in both the cytosol and nucleus, resulting in pleiotropic effects on cellular metabolism.[3] cAMP-mediated responses in the cytosol, such as the regulation of glycogen metabolism, occur within seconds of activation.[4] In contrast, the nuclear responses to cAMP are much slower. Peak rates of transcription occur 30 min after cAMP is increased.[5] Transcription of cAMP responsive genes is regulated, in part, by phosphorylation of the transcription factor CREB at Ser-133 by PKA-C.[6–8] The nuclear translocation of PKA-C is the rate-limiting step in the coupling of hormonal stimulation and CREB-dependent gene transcription.[9]

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The mechanism by which PKA can regulate such a diverse range of responses in the cell is not fully understood. Types I and II regulatory subunits (RI and RII) differ in their K_d for cAMP, tissue distribution and expression during development and differentiation.[10] RI isoforms are mainly cytosolic, whereas RII isoforms are localized to the nucleus, nucleolus, plasma membrane, Golgi apparatus, and microtubule organizing center.[11] These differences suggest that RI and RII have distinct physiologic functions and may contribute to the diversity of PKA-mediated responses.[12] However, specific functions for the two isoforms have not been fully assessed. Hormone-specific activation of RI and RII has been observed under some conditions.[13–15] Moreover, localized production of cAMP may allow for differential activation of RI and RII within the cell.[15,16] Whereas differential activation may occur under some conditions, it is clear that there is overlapping functional specificity between the two isoforms; e.g., stimulation of either RI or RII subunits with site-specific cAMP analogs results in activation of gene transcription.[17]

The proportion of RI/RII changes during development, differentiation, and transformation and has been implicated in regulation of growth in some cell types.[10] Very low protein diets decrease PKA activity and RI quantity in rat hepatocytes after 3 days of feeding.[18–20] Decreased PKA activity is localized to the cytosol and is associated with decreases in RI, but not RII, protein quantity.[19,20] In contrast, hormone-stimulated cAMP production remains at control levels until the second week of malnutrition, at which time the capacity for cAMP production increases in hepatocytes from malnourished rats.[20] These diet-induced modifications in the highly regulated cAMP signal transduction pathway are likely to disrupt cAMP-controlled cellular functions such as regulation of glycogen metabolism and/or gene expression and may contribute to the profound alterations in liver function observed during protein malnutrition. Therefore, we have investigated the effects of very low protein diets on two downstream responses to cAMP: activation of glycogen phosphorylase in the cytosol and phosphorylation of CREB in the nucleus. Using site-specific analogs of cAMP, we have demonstrated that diet-induced loss of RI quantity impairs down-stream responses to RI activation in both the cytosol and nucleus in rat hepatocytes.

Methods and materials

Materials

Rats were purchased from Harlan Sprague Dawley, Inc (Indianapolis, IN USA). [^{32}P]-ATP and enhanced chemiluminescence detection kits (ECL) were purchased from Amersham (Oakville, Ont.). Peroxidase-labeled anti-rabbit IgG antibodies (IgG-POD) were purchased from Boehringer Mannheim (Dorval, Québec). pCREB and CREB antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). PKA-C antibody was purchased from Transduction Laboratories (Lexington, KY USA). [^{14}C]glucose-1-phosphate and electrophoresis reagents were purchased from ICN (St-Laurent, Québec). Protein assay reagent, nitrocellulose and PVDF were purchased from BioRad (Mississauga, Ont). Other reagent grade chemicals were purchased from Sigma Chemical (St. Louis, MO USA).

Animal and diets

Male weanling Wistar rats (initial body weight, 45 to 50 g) were housed individually in plastic, closed-bottom cages in a temperature- and humidity-controlled room with lights on from 0800 to 2000 hr. After a 3-day acclimatization period to a semipurified casein-based diet containing 15% protein, rats were allowed free access to either 15% protein (control) or 0.5% protein (malnourished) diets for 3 or 14 days.[21] Rats were allowed free access to water throughout the feeding period. The initial body weight was 55.0 ± 1.7 g. After consuming the 15% and 0.5% protein diets for 3 days, rats weighed 87.9 ± 3.4 and 57.6 ± 3.0 g, respectively. At day 14, control and malnourished rats weighed 157.9 ± 5.6 and 46.5 ± 1.1 g, respectively. The animal care protocol for these experiments was approved by the University of Guelph Animal Care Committee under the guidelines of the Canadian Council on Animal Care.

Hepatocyte isolation and stimulation

Hepatocytes were isolated by collagenase perfusion, as described previously.[22] Both a control and malnourished rat were perfused simultaneously before 10:00h; the cells from these rats were paired throughout the incubation process. The cell suspension was centrifuged at 50 × g for 5 min and hepatocytes resuspended in William's E medium. Cell count and viability was determined by trypan blue exclusion. Cells were divided into 50 mL conical tubes (5 mL @ 2.5 × 10^6 cells/mL) and incubated at 37°C for 15 min in a shaking waterbath before the addition of agonists.

Hepatocytes were incubated with media alone (basal), or with added glucagon, dibutyryl-cAMP (db-cAMP), 8-Cl-cAMP or N^6-benzoyl-adenosine cAMP (N^6-BZL-cAMP) and 8-thiomethyl adenosine cAMP (8-SCH_3-cAMP), at concentrations indicated in the figure legends. The combination of N^6-BZL-cAMP and 8-SCH_3-cAMP selectively activates RII,[23] whereas 8-Cl-cAMP activates RI.[10] After 1 min, 100 µL of the cell suspension was removed and added to 300 µL ice-cold stopping buffer (50 mM NaF, 25 mM β-glycerophosphate, 10 mM morphaline ethanosulfonic acid (MES), 2 mM EDTA, and 0.2 mM digitonin; pH 7.0) and frozen for subsequent assay of glycogen phosphorylase activity.

At 30 min, cells were centrifuged at 50 × g for 5 min at 4°C. Nuclear extracts were prepared following the method of Dignam et al.[24] with the addition of phosphatase inhibitors (40 µM sodium vanadate, 20 µM phosphoserine, and 20 µM phosphothreonine) to buffers B and D. Pellets were incubated in 2 mL of ice-cold buffer B (10 mM Tris-HCl, 10 mM KCl, 1.5 mM $MgCl_2$; pH 7.9) and incubated on ice for 15 min. Cells were lysed with a Dounce homogeniser (Pestle B) and centrifuged at 4000 × g for 8 min at 4°C. The pellet was resuspended in 200 µL of buffer D (50 mM Tris-HCl, 0.42 M KCl, 5 mM $MgCl_2$, 10% sucrose, 0.1 mM 20% glycerol, 1 mM dithiothreitol, 1000 units/mL aprotinin; pH 7.5) and incubated on ice for 30 min with shaking. The samples were centrifuged at 16,000 × g for 30 min at 4°C. The supernatant was removed, and mixed to 53% with saturated ammonium sulphate and centrifuged at 16,000 × g for 30 min, at 4°C. Pellets were resuspended in 100 µL TM buffer (50 mM Tris-HCl, 0.1 M KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1000 units/mL aprotinin, 20% glycerol; pH 7.9) and protein concentration was measured using the Bradford protein assay.

Western blotting

Nuclear protein was diluted to 1 g/L in Laemmli sample buffer and 30 µg of protein was separated by 10% SDS-PAGE using the Laemmli buffer system.[25] Nuclear protein from each pair of control and malnourished rats was separated on the same gel.

Proteins were transferred to nitrocellulose or PVDF and stained with fast green to confirm equal protein loading. Membranes were blocked with 4% BSA in Tris-buffered saline (20 mM Tris-base, 137 mM NaCl; pH 7.6) with 0.1% tween (TBS-T) for 2 hr and incubated overnight at 4°C with antibody to CREB (produced against the first 205 amino acids of CREB; 1:1000 in 4% BSA in TBS-T) or pCREB (produced against a synthetic phosphopeptide of residues 123–136 of rat CREB; 1:10,000 in 4% BSA in TBS-T). Membranes were washed for 15 min and then 3 times for 5 min each in TBS-T. Membranes were then incubated with anti-rabbit IgG-POD (1:40,000) for 1 hr at room temperature, washed as above, and bound IgG detected by ECL.

For detection of the catalytic subunit, membranes were incubated in stripping buffer (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5 mM Tris-HCl; pH 6.7) at 50°C for 30 min, washed in TBS-T for 20 min, and blocked with 5% nonfat dry milk in TBS-T for 1 hr at room temperature. Membranes were incubated overnight at 4°C with PKA-C antibody (1:1000 in 5% nonfat dry milk in TBS-T). The washes and incubation with anti-rabbit IgG-POD were performed as described for pCREB.

Bands for control and malnourished hepatocytes were compared by scanning densitometry. Comparisons were made for control and malnourished pairs on the same gel. Gel was used as a independent variable in the statistical analysis in order to control for differences in exposures.

Glycogen phosphorylase a *assay*

Glycogen phosphorylase *a* activity was assessed by the reverse reaction.[26] Hepatocyte samples were thawed on ice and centrifuged at 16 000 × g for 20 min. Thirty μL of the supernatant was then incubated with 60 μL of assay solution (200 nM KF, 100 mM [^{14}C]glucose-1-phosphate, 1% glycogen; pH 6.1). Total glycogen phosphorylase activity was measured in the presence of 3 mM 5′-AMP. Incubations were carried out at 37°C for 20 min and terminated by spotting 30 μL of the assay mixture on Whatman 1 filter paper. Filters were incubated in 66% ethanol to precipitate glycogen onto the filter and then washed twice in 66% ethanol for 20 min and once in acetone for 3 min Filters were air-dried and [^{14}C] glycogen bound to filter was determined by liquid scintillation counting.

cAMP-dependent protein kinase activity in liver

Three rats per dietary group were anaesthetized with Na-pentobarbital at day 14 of feeding and livers perfused with saline, removed, and immediately frozen in liquid nitrogen. Liver homogenates were incubated with RI-specific or RII-specific agonists as indicated in the figure legend and PKA activity was measured using the Kemptide assay,[27] as previously described.[19,20] Nonspecific activity was measured by addition of 4 μmol/L protein kinase inhibitor peptide.

In vivo concentrations of pCREB and cAMP in liver

Five rats per dietary group were anaesthetized with Na-pentobarbital at day 3 and day 14. Freeze-clamped livers were homogenized in buffer B, nuclei were isolated and quantity of immunoreactive pCREB was measured by Western blotting, as described. Total cAMP in homogenates was measured by acetylated-radioimmunoassay.[28]

Statistical analysis

Data were analyzed by 2-way analysis of variance (ANOVA) using the general linear models procedure using the SAS statistical software for personal computer (SAS Institute, Carey, NC USA).

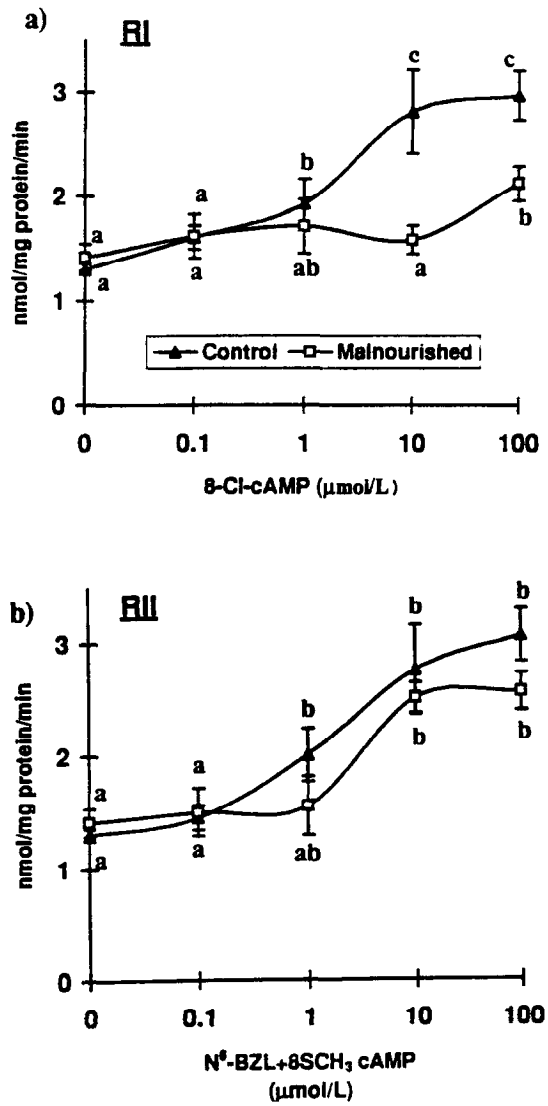

Figure 1 PKA activity in response to increasing concentrations of RI agonist (*a*) or RII agonist (*b*) in liver homogenates of day 14 control and malnourished rats. Activity of PKA was measured over 5 min using the Kemptide assay. Values are means (nmol/mg protein/min) ± SEM, (n = 4). Values with different letters differ significantly ($P < 0.01$).

Differences between groups were then determined using the least significant difference test $P < 0.05$). Student's t test was used to analyze differences on assays performed at one agonist dose. Values are reported as means ±SEM.

Results

PKA activity in liver homogenates

Basal activity of PKA activity did not differ between control and malnourished rats (*Figures 1a* and *1b*). In contrast, total PKA activity, assessed by treatment with a maximally stimulating concentration of db-cAMP (40 μM), was lower in the malnourished rats compared to controls at 1.92 ± 0.85 and 3.75 ± 0.34 nmol/mg protein (n = 4, $P < 0.01$), respectively. Treatment of d 14 liver homogenates with 8-Cl-cAMP (RI agonist) at concentrations of 1 μM or higher stimulated PKA activity in control rats; however,

activity was only increased at 100 μM 8-Cl-cAMP in the malnourished rats (*Figure 1a*). PKA activity was stimulated by 1 μM N^6-BZL-cAMP and 8-SCH_3-cAMP (RII agonist) in control rats and 10 μM in malnourished rats. Maximal activation of PKA in response to treatment with RI agonist was lower in malnourished rats relative to controls (*Figures 1a*). In contrast, there was no difference between dietary groups in response to any concentration of the RII agonist pair (*Figure 1b*).

Glycogen phosphorylase a activity in hepatocytes

Increased cAMP in hepatocytes leads to the activation of glycogen phosphorylase. We tested whether decreases in the RI activity of malnourished rat liver would impair the activation of glycogen phosphorylase *a* by cAMP analogs. Basal activity, measured in the absence of added agonist, (*Figures 2a, b* and *3a, b*) and total activity of glycogen phosphorylase, measured in the presence of 3 mM 5′-AMP, were not affected by diet at day 3 or at day 14. Total activity at day 3 was 390.3 ± 92.5 vs 295.8 ± 46.1 nmol/min/mg protein in control and malnourished rats, respectively, (n = 5) and at day 14 was 323.6 ± 80.7 vs 311.8 ± 95.3 nmol/min/mg protein in control and malnourished rat hepatocytes, respectively (n = 5).

Because the very low protein diet did not affect glycogen phosphorylase activity *per se*, we were able to test whether very low protein diets reduced the ability of cAMP to activate glycogen phosphorylase in hepatocytes. Glycogen phosphorylase activity was increased over basal in response to 0.1 μM RI or to 0.1 μM RII agonists in both control and malnourished rat hepatocytes after 14 days of feeding (*Figures 2a, 2b*). Activity was further stimulated by 1 μM RI agonist in hepatocytes of control rats only. RI-stimulated activity was lower in hepatocytes of malnourished rats compared to controls at all concentrations of agonist (*Figure 2a*), whereas RII-stimulated activity did not differ between dietary groups (*Figure 2b*).

To determine if the increase in glucagon-stimulated cAMP production that develops in hepatocytes after day 14 of feeding very low protein diets[20] could compensate for the reduction in cytosolic RI, activation of glycogen phosphorylase by glucagon was compared in hepatocytes from rats fed 0.5% protein diets for 3 or 14 days. RI agonist-stimulated activity was lower in the malnourished rat hepatocytes relative to the control group at both d 3 and d 14 (*Figures 3a, b*). Similarly, activity was also lower in the malnourished rat when hepatocytes were stimulated with 200 μM db-cAMP (*Figure 3a, b*). No difference was observed between dietary groups when hepatocytes were stimulated with RII agonist at either day 3 or day 14. Glycogen phosphorylase was activated by 2 μM glucagon in both control and malnourished rat hepatocytes at both day 3 and day 14 (*Figure 3a, b*). As with the RI-specific agonist, glucagon-stimulated glycogen phosphorylase activity was lower in hepatocytes from malnourished rats at d 3 (P < 0.01 *Figure 3a*). In contrast, glucagon-stimulated activity was not different between dietary groups at d 14 (*Figure 3b*), when cAMP production in response to glucagon is increased.[20]

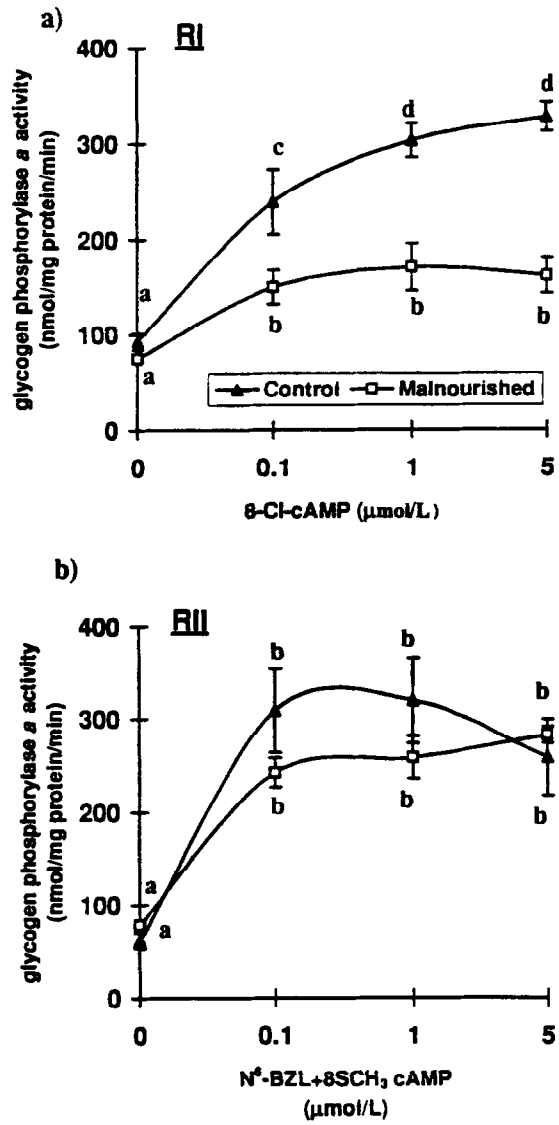

Figure 2 Glycogen phosphorylase *a* activity in response to increasing concentrations of RI agonist (*a*) or RII agonist (*b*) in hepatocytes of day 14 control and malnourished rats. Activity was measured 1 min after the addition of agonist. Values are means (nmol/mg protein/min) ± SEM, (n = 4). Values with different letters differ significantly (P < 0.01).

Quantitation of pCREB in hepatocytes

A second important cellular response to cAMP is the activation of transcription of cAMP responsive genes in the nucleus. As a first step toward understanding whether diet-induced decrease in RI impairs transcriptional activation in response to cAMP, we measured the increase in quantity of pCREB in nuclei from control and malnourished rat hepatocytes treated with cAMP agonists. Treatment of control rat hepatocytes with 1 μM RI or 1 μM RII agonist increased the quantity of immunoreactive PKA-C in the nuclear fraction (*Figure 4a*). This increase was paralleled by an increase in quantity of nuclear pCREB after incubation with 1–10 μM RI or RII agonist (*Figure 4b*), demonstrating that both RI and RII agonists can increase the quantity of pCREB.

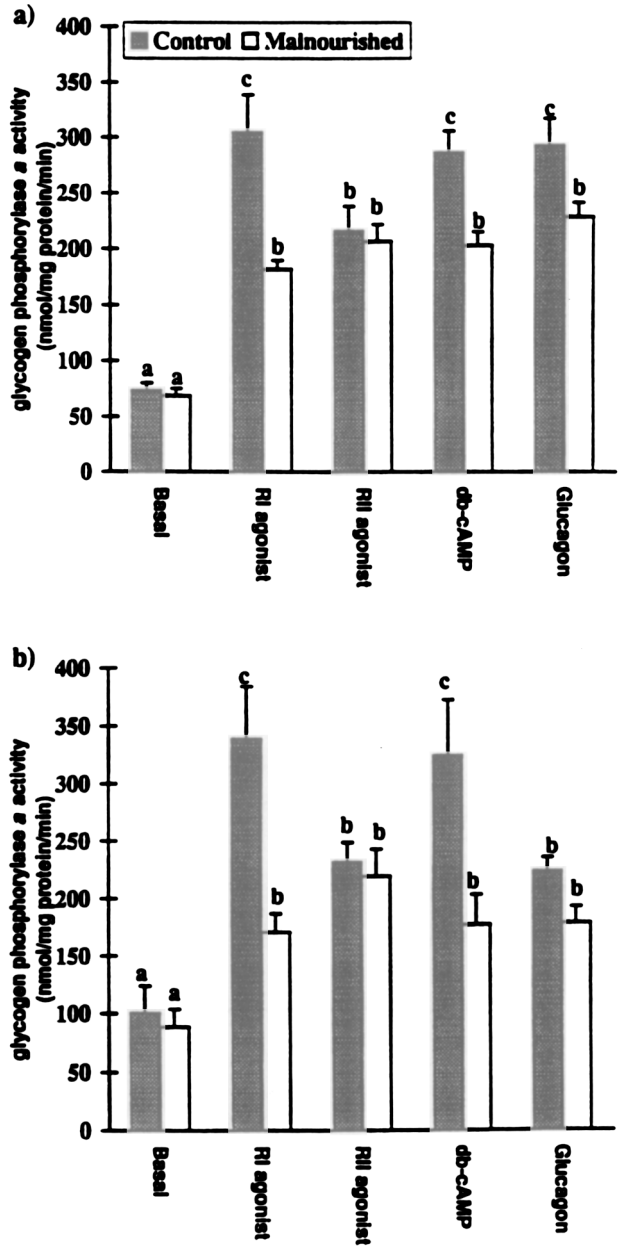

Figure 3 Glycogen phosphorylase *a* activity in response to agonists in hepatocytes from control and malnourished rats at day 3 (*a*) and day 14 (*b*) of dietary treatment. Activity was measured 1 min after the addition of the following agonists: media (basal), 1 μM 8-Cl-cAMP (RI agonist), 1 μM of both N^6-BZL-cAMP and 8-SCH_3-cAMP (RII agonist), 200 μM db-cAMP and 2 μM glucagon. Values represent means (nmol/mg protein/min) ± SEM, (n = 4). Values with different letters differ significantly ($P < 0.05$).

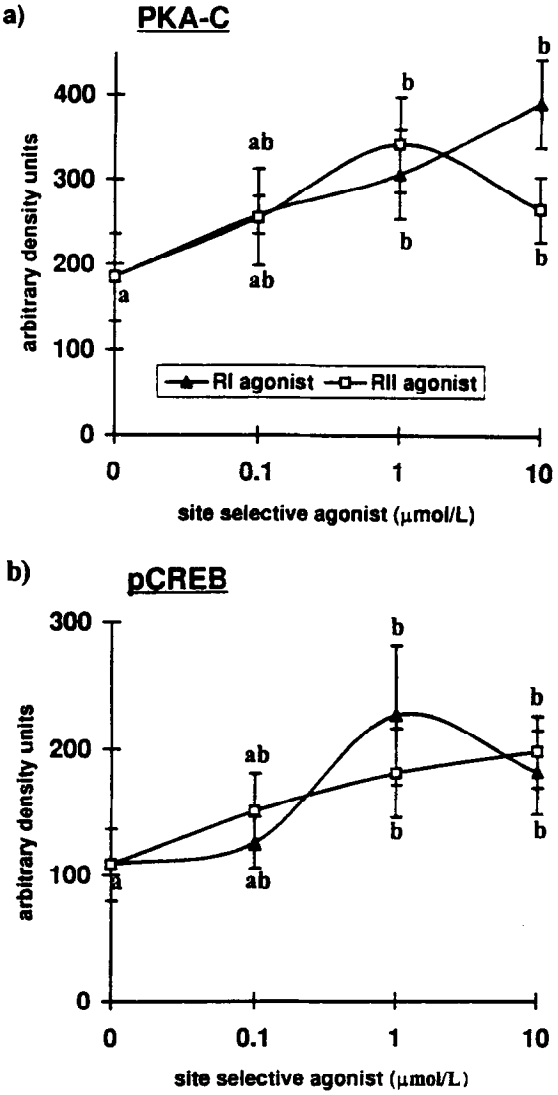

Figure 4 Quantity of PKA-C (*a*) and pCREB (*b*) protein in the nuclear fraction of control rat hepatocytes after treatment with increasing concentrations of 8-Cl-cAMP (RI agonist) or N^6-BZL-cAMP and 8-SCH_3-cAMP (RII agonist). Nuclear protein was separated on SDS-PAGE and transferred to PVDF membranes. Membranes were probed for pCREB, stripped and reprobed for PKA-C, as described in Materials and Methods. Values represent means (arbitrary density units) ± SEM (n = 3). Values with different letters differ significantly.

There was no difference in the quantity of total immunoreactive CREB in the nuclear fraction between dietary groups at day 14 (*Figure 5a*). Although there was a trend for lower pCREB in malnourished hepatocytes, basal pCREB was not different between dietary groups at d 14 (*Figures 5c* and *6b*; P = 0.06). However, quantity of PKA-C and pCREB in the nuclear fraction after treatment with 1 μM RI agonist was lower in the malnourished group (*Figure 5b c*). There was no difference in nuclear PKA-C or pCREB quantity between dietary groups after stimulation with 1 μM RII agonist (*Figure 5b, c*). Similar to the effects of RI-agonist, quantity of pCREB was lower in hepatocytes of malnourished rats after treatment with 200 μM db-cAMP compared with control rat hepatocytes (*Figure 6b*).

To assess whether there were differences in response to glucagon over time of feeding the very low protein diet, as we had observed with the activation of glycogen phosphorylase *a*, pCREB quantity was compared at day 3 and day 14 of feeding. Quantity of nuclear CREB was not different between groups at day 3 (109.6 ± 5.4 vs 128.6 ± 14.0 arbitrary density units in control and malnourished rat hepatocytes, respectively). However, basal pCREB was 55% lower in hepatocytes from rats fed 0.5% protein diet for 3 days compared with rats fed control diets for 3 days

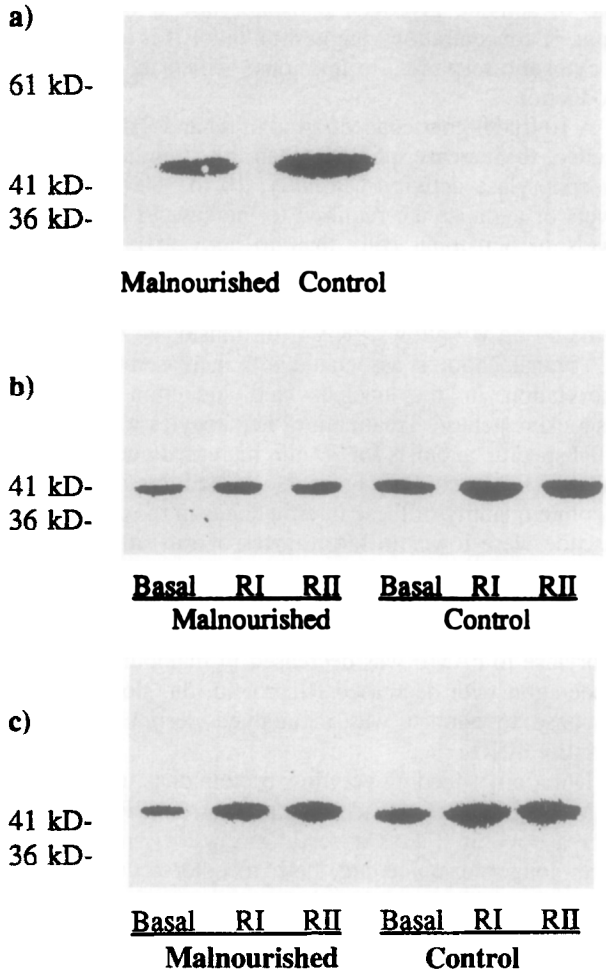

Figure 5 Quantity of immunoreactive total CREB (*a*) PKA-C (*b*) and pCREB (c) protein in the nuclear fraction of hepatocytes from day 14 control and malnourished rats. Western blots are representative of the following means (arbitrary density units) ± SEM; (*a*) total CREB in control and malnourished rat hepatocytes: 152.9 ± 19.8 vs 116.2 ± 20.3 (n = 6); (*b*) PKA-C concentrations in control and malnourished rat hepatocytes after treatment with media (basal): 1153.8 ± 155.5 vs 834.2 ± 296.1, 8-Cl-cAMP (RI agonist): 3250.0 ± 150.1 vs 1948.2 ± 299.8** or N^6-BZL-cAMP and 8-SCH_3-cAMP (RII agonist): 1686.5 ± 284.3 vs 1593.5 ± 135.5, (n = 3); (c) pCREB concentrations in control and malnourished rat hepatocytes after treatment with media (basal): 287.3 ± 41.0 vs 175.1 ± 39.1, RI agonist: 589.9 ± 48.2 vs 314.1 ± 50.2** or RII agonist 499.0 ± 61.2 vs 438.9 ± 50.3 (n = 5). Nuclear protein was separated on SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed for CREB or for pCREB, stripped and reprobed for PKA-C, as described in methods and materials. **$P < 0.05$ compared with control diet, as analyzed by Student's t test.

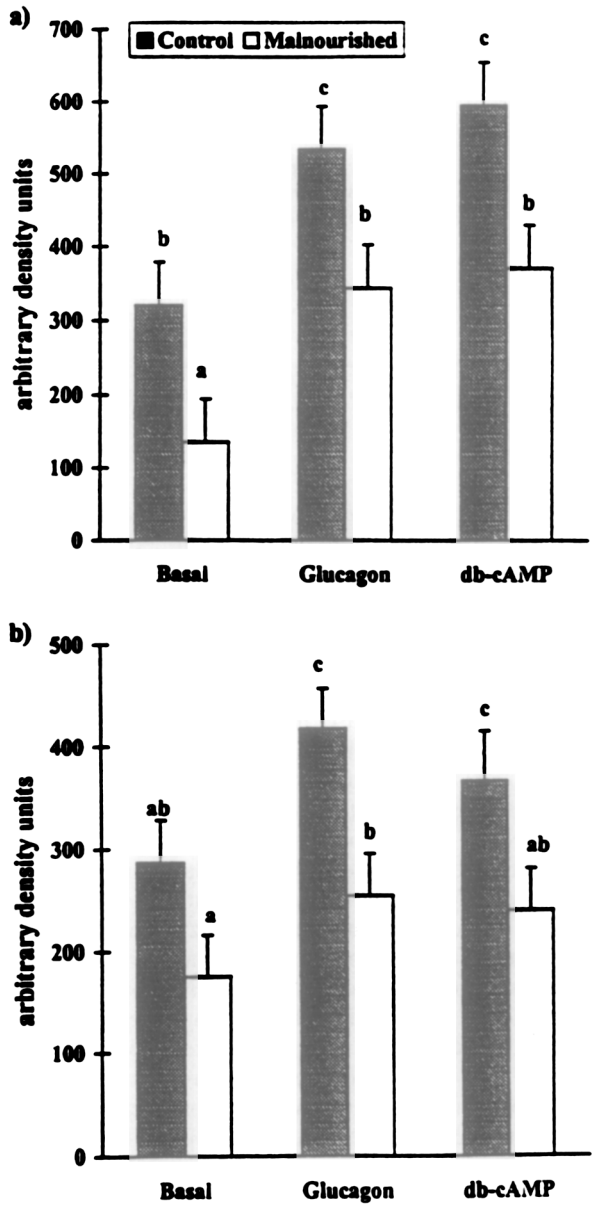

Figure 6 pCREB quantity in response to the addition of media (basal), 2 μM glucagon or 200 μM db-cAMP in hepatocytes from control and malnourished rats at day 3 (*a*) and day 14 (*b*) of dietary treatment. Values represent means (arbitrary density units) ± SEM, n = 4 rats at day 3 and n = 10 rats at day 14. Values with different letters differ significantly ($P < 0.05$).

(*Figure 6a*; $P < 0.01$). After treatment with 2 μM glucagon for 30 min, quantity of pCREB was significantly lower in the malnourished group compared to controls at both day 3 and day 14 (*Figures 6a, b*). Although the quantity of pCREB was lower in hepatocytes from malnourished rats, the relative increase after glucagon treatment with respect to basal pCREB was similar between dietary groups (1.79 ± 0.16 and 1.31 ± 0.30 on day 3 and 1.64 ± 0.09 fold and 1.62 ± 0.19 fold on day 14 for control and malnourished, respectively).

Quantity of pCREB was also measured in freeze-clamped livers from control and malnourished rats. pCREB levels were similar in control and malnourished rat livers at day 3 (*Table 1*); however, pCREB concentrations were increased in malnourished rat livers relative to controls at d 14. This increase in pCREB at d 14 was associated with higher in vivo concentrations of cAMP in malnourished rat liver relative to controls (*Table 1*).

Discussion

Here we report that cellular responses to PKA activation in rat hepatocytes are diminished after feeding very low protein diets for 3 or 14 days. Increases in the activity of

Table 1 cAMP and pCREB concentration in freeze-clamped livers of control and malnourished rats

	Control	Malnourished
cAMP		
	(pmol cAMP/mg protein)	
Day 3	26.3 ± 11.2[a]	32.7 ± 12.6[a]
Day 14	39.5 ± 11.3[a]	69.0 ± 4.6[b]
pCREB		
	(arbitrary density units)	
Day 3	397.2 ± 87.6[ab]	274.8 ± 28.1[a]
Day 14	521.5 ± 77.6[b]	725.6 ± 52.4[c]

Values are means ± SEM (n = 5). Values with different letters differ $P < 0.01$.

glycogen phosphorylase *a* in response to activation of PKA by RI-specific agonist were decreased in hepatocytes from malnourished rats compared to controls. This loss of responsiveness to RI agonist parallels the 56% decrease in RI protein in malnourished rat liver.[19,20] In contrast, responses to RII agonist were unaffected by diet, consistent with the maintenance of hepatic RII protein in the liver of malnourished rats.[19,20] A decrease in the ability of glucagon to regulate glutathione synthesis in the malnourished rat liver has previously been reported,[22] suggesting that the loss of RI-mediated responses after very low protein diets impacts on a variety of cAMP controlled pathways in the liver.

Activation of glycogen phosphorylase in response to increased cAMP results in the degradation of liver glycogen.[4] The reduced activation of this enzyme in response to cAMP in rats fed 0.5% protein diets may partially account for the disruption of glucose homeostasis observed during protein-energy malnutrition. For example, glycogen concentration is increased in livers of rats fed 0.5% protein for 14 days[29] and in children suffering from kwashiorkor.[30] Furthermore, protein-energy malnutrition decreases fasting plasma glucose concentrations and impairs the ability of glucagon to increase plasma glucose in children[31] and rats.[32] Taken together, these data suggest that mobilization of glycogen in response to activation by glucagon may be impaired in vivo during protein-energy malnutrition. Impaired responses to glucagon during protein-energy malnutrition may be mediated by the decrease in the RI-mediated activation of glycogen phosphorylase reported here.

Hepatocytes express both the RI and RII isoforms of PKA, with RI present in a slightly higher abundance (60 to 75%), suggesting that both isoforms are important in maintenance of hepatic function.[33] However, it is not clear if hormones can differentially activate RI and RII. Low concentrations of glucagon have been reported to preferentially activate RI in hepatocytes, whereas higher glucagon concentrations activate both RI and RII.[14] In contrast, in another study, the fractional saturation of RI and RII in hepatocytes was similar over a broad range of glucagon concentrations.[33] Our data suggest that glucagon-activation of glycogen phosphorylase *a* is primarily mediated by RI since glucagon stimulation of glycogen phosphorylase was decreased in parallel with RI specific responses in hepatocytes from malnourished rats. However, RII agonists were able to activate glycogen phosphorylase *a* over a broad range of concentrations, suggesting that RII is also involved in cytosolic responses to hormones which increase cAMP production.

A 10 fold higher concentration of RI and RII agonist was needed to increase pCREB than to stimulate glycogen phosphorylase activity. Similarly, 10 to 1000 fold higher levels of agonists are required to increase PEPCK mRNA levels in hepatoma cells than to activate phosphorylase activity,[23] suggesting that higher cAMP levels are needed to stimulate transcription. Dissociation of the PKA holoenzyme by cAMP allows PKA-C to translocate to the nucleus.[3] Translocation is associated with increased CREB phosphorylation in the nucleus and induction of cAMP-responsive genes.[9] Treatment of hepatocytes with glucagon or RI specific agonists for 30 min increased nuclear PKA-C and pCREB in control and malnourished rats. Whereas the absolute quantity of these intermediates in the cAMP signal cascade were lower in hepatocytes of rats after 3 and 14 days of feeding very low protein diets, stimulation by agonist relative to basal was maintained. Thus, while the rapid, cytosolic response of activation of glycogen phosphorylase to cAMP was decreased in malnourished rats in association with decreased RI protein, the slower, nuclear response to agonist was maintained despite lower total quantity of RI.

Duration of feeding very low protein diets influences the cAMP signalling cascade. RI protein and PKA activity decrease within 3 days of feeding very low protein diets.[20] After longer-term feeding, both receptor-activated cAMP production and adenylyl cyclase activity are increased.[20] In this investigation, hepatocytes from malnourished rats had lower basal pCREB concentrations at day 3 compared with controls. However, by day 14, there was no longer any difference between diet groups. Activation of glycogen phosphorylase *a* by glucagon also recovered by day 14 of malnutrition. It is likely that the increased capacity for cAMP production in response to glucagon at day 14 contributes to the increase in basal pCREB in hepatocytes and the recovery of glucagon-stimulated glycogen phosphorylase activity at day 14. Similarly, pCREB concentrations in freeze-clamped livers were increased at day 14 compared to day 3. This increase was associated with an increase in cAMP in liver at day 14, but not at day 3. Thus, increased receptor-stimulated cAMP production and adenylyl cyclase activity[20] may be a mechanism for the hepatocyte to recover glucagon-mediated responses despite a decrease in PKA activity.

cAMP regulates transcription of many genes, such as phosphoenolpyruvate carboxykinase (PEPCK)[5] and tyrosine amino transferase (TAT).[34] Both basal and stimulated levels of pCREB may influence cAMP-responsive gene transcription. In hepatoma cells, CREB binding to the PEPCK-CRE, a high affinity binding site for CREB, is not strongly stimulated by PKA, whereas CREB binding to the TAT-CRE, a weak site, was increased by PKA.[35] The TAT gene was reported to show lower basal levels of transcription and a higher fold induction by PKA that the PEPCK gene in newborn mouse livers.[36] This suggests that changes in basal levels of pCREB may increase CREB binding to weak affinity sites, such as the CRE for PEPCK. Consistent

with the increase in pCREB in freeze clamped liver at d 14 (*Table 1*), PEPCK activity is increased in rats fed low-protein diets.[37] However, it is not known if this is due to an increase in PEPCK mRNA.

Since the cloning of CREB, a number of CRE-binding proteins have been identified, including ATF-1[38] and CREM.[39] These proteins share sequence homology with CREB and can heterodimerize in vitro.[40] Mice with a disruption of the CREB gene have increased levels of CREM and appear healthy,[41] suggesting that compensation within the CREB family of transcription factors can occur. Further investigations are required to determine whether protein malnutrition induces compensatory changes in the CREB family of transcription factors and affects transcription rates of cAMP-responsive genes.

In summary, we have demonstrated that diet-induced loss of the RI subunit of PKA impairs down-stream responses to RI activation in both the cytosol and nucleus of rat hepatocytes. In the nucleus, relative increases in the quantity of pCREB are maintained in response to stimulation by glucagon or RI-agonist in rats fed very low protein diets; however, the absolute quantity of pCREB is lower in malnourished rat hepatocytes compared to control hepatocytes. In contrast, activation of glycogen phosphorylase by glucagon and RI-specific agonist is blunted after 3 days of feeding a very low protein diet. By day 14 of feeding, increased receptor-stimulated cAMP production in the liver of malnourished rats (*Table 1*)[20] appears to partially compensate for the reduction in PKA-mediated responses because activation of glycogen phosphorylase by glucagon was not decreased at d 14 (*Figure 3b*). These data suggest that cAMP-mediated regulation of glycogen metabolism in the cytosol is more sensitive to diet-induced decreases in the RI subunit of PKA than activation of CREB in the nucleus. This disruption of cAMP-mediated control of hepatic glycogen metabolism may contribute to the abnormal regulation of glucose homeostasis observed during protein-energy malnutrition.

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