Down-regulation of negative acute-phase response genes by hypotonic stress in HepG2 hepatoma cells

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Abstract An increased hepatocellular hydration state (HS) that can be induced by hypotonic stress or a high glutamine uptake modulates the transcription of given genes in liver. This could be important in the acute phase (AP) of a systemic inflammation where both HS and glutamine uptake transiently increase in liver. In HepG2 hepatoma cells cultured in conditions of hypotonic stress or a high extracellular glutamine availability, a specifically decreased expression of two human mRNAs, namely those of α1-microglobulin/bikunin precursor (AMBP) and α2-HS-glycoprotein, that are also down-regulated in liver by AP, could be seen. A functional analysis of the *AMBP* promoter indicated that this hypotonic stress-induced down-regulation takes place at a transcriptional level. In these experiments, the mRNA level and transcription of the *glyceraldehyde-3-phosphate dehydrogenase* gene that are known to be unmodified in AP did not exhibit any change. Given that hypotonic stress also up-regulates the transcription of a liver gene that is also up-regulated in AP [Meisse et al. (1998) FEBS Lett. 422, 346–348], the AP-associated increase in hepatocellular HS now appears to participate in the transcriptional control of both sets of genes that are up- or down-regulated in AP.

Key words: Acute-phase gene; Glutamine; Hepatoma cell; Hydration state; Transcriptional regulation

1. Introduction

In recent years, evidence has accumulated that an increased hydration state (HS) can promote anabolic effects on glucidic, lipidic and protein metabolism in hepatocytes [1,2]. The hepatocellular HS is modulated by hormones, aniso-osmolarity, amino acids and, notably, glutamine uptake. This HS provides a signal for control of the intracellular level of specific mRNAs [3]. For instance, an increased hepatocellular HS experimentally induced in vitro has been associated with an increased level of the mRNA for a transcription factor such as c-*jun* [3], cytoskeleton proteins such as β-actin and tubulin [3], and the enzymes argininosuccinate synthetase [4] and ornithine decarboxylase [3] as well as with a decreased mRNA level for the enzymes phosphoenolpyruvate carboxykinase (PEPCK) and tyrosine aminotransferase [3]. However, quite a limited number of studies, namely those for an intracellular protein, i.e. β-actin, and an excreted protein, i.e. α2-μacroglobulin (α2-M), have as yet correlated such an up-regulated, hepatic mRNA level with an increased transcription [5,6]. Whether the opposite situation, i.e. the down-regulation of an mRNA level by the hepatocellular HS, also involves a transcriptional step and which regulatory region(s) in the affected gene is involved at this stage are currently unknown. Most importantly, the relevance of such hepatocellular HS-controlled, up- or down-regulation to given pathological situations largely remains to be explored.

The acute phase (AP) of a systemic inflammation is a unique condition to clarify the above issues, for three major reasons. Firstly, the HS of liver cells is known to be increased in vivo by an endotoxin-induced AP in rat. In such endotoxin-challenged animals the hepatocytes exhibit increases in intracellular water content and volume [7]. Secondly, a 8–10-fold increase in hepatic uptake of blood-borne glutamine has been reported in patients with sepsis [8] and in endotoxin-treated rats [8–11]. It is likely that this increased uptake accounts, at least partly, for the increased hepatocellular HS observed in vivo in AP. Thirdly, the hepatic response to AP includes transient changes in the production of so-called acute-phase proteins (APP) that are comprised of up- and down-regulated plasma proteins designated positive and negative APPs, respectively [12,13]. Although the transcriptional control of APP genes by pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1) and IL-6 is largely proven [12–14], the question now arises as to whether the hepatocellular HS also participates in this control. Indeed, some of us have recently shown that a hypotonic stress-induced increase in this hepatocellular HS is sufficient to up-regulate the mRNA level for a positive APP, namely rat α2-M, via a transcriptional step [6]. We now report for the first time that hypotonic stress, e.g. induced by a hypotonic or a glutamine-enriched culture medium, is sufficient to specifically down-regulate the mRNA level and transcription of negative APP genes, namely the α1-microglobulin/bikunin precursor (AMBP) and the α2-HS-glycoprotein (AHSG) genes. Therefore, the hepatocellular HS is now added to the set of inflammation-driven regulatory pathways that are able to up- or down-regulate the hepatic transcription of positive or negative APP genes, respectively.

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Abbreviations: α2-M, α2-macroglobulin; AHSG, α2-HS-glycoprotein; AMBP, α1-microglobulin/bikunin precursor; AP, acute phase; APP, acute-phase protein; AR, aldose reductase; β-gal, β-galactosidase; BT, betaine transporter; Cat, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HS, hydration state; hypo, hypotonic medium; iso, isotonic medium; isoraff, raffinose-supplemented, isotonic medium; PEPCK, phosphoenolpyruvate carboxykinase; tk, thymidine kinase

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2. Materials and methods

2.1. Cell cultures

The human HepG2 hepatoma cell line was plated and maintained at 37°C in RPMI 1640 (Sigma) medium containing 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 10% fetal calf serum (FCS). However, in all media listed below FCS was reduced to 2%. Tonicity experiments were carried out with an isotonic medium (iso) (NaCl 145 mM; 300 mosmol/kg of H_2O) or a hypotonic medium (hypo) (NaCl 95 mM; 200 mosmol/kg of H_2O) made by adjusting the NaCl concentration of the RPMI medium. A further, raffinose-supplemented, isotonic medium (isoraff) was made by supplementing the hypo medium with 100 mM raffinose. Cell cultures with different amounts of glutamine were carried out in RPMI 1640 medium supplemented (glutamine-enriched medium) or not (control medium) with 10 mM L-glutamine. Cell viability, as evaluated by measuring the intracellular ATP content, was unchanged at the end of incubation periods in the cells incubated in either medium (data not shown).

2.2. RNA isolation and Northern blot quantitation with cDNA probes

Total RNAs were extracted by an acidic guanidinium thiocyanate/phenol/chloroform procedure, electrophoresed (20 μg per lane), blotted and hybridized with [α^{32}P]dCTP-labeled cDNA as in [15]. Full-length cDNA probes included those for the human AMBP [15], the human AHSG (courtesy of Prof. P. Arnaud, Charleston, SC, USA), the human GAPDH (a kind gift from Dr. J.P. Borel, Reims, France) and the rat 18S (kindly provided by Dr. R. Vrancks, Paris, France). The autoradiographic signals obtained with a given cDNA probe were quantitated by densitometric scanning with an image analyzer (Biocom 500; Biocom, Les Ulis, France). In all experiments we verified that the signal-to-amount ratio was linear in the range of mRNA amounts studied with each probe (not detailed). All densitometric values for a given mRNA were normalized with the 18S signals obtained on the same blot.

2.3. Reporter plasmids, HepG2 cell transfections and Cat/β-gal assays

p47 is a pCMV-β-galactosidase (β-gal) plasmid with the bacterial β-*gal* gene under the control of the cytomegalovirus immediate-early enhancer/promoter region. pBL*cat*5 with the chloramphenicol acetyltransferase (*cat*) gene under the control of Herpes simplex thymidine kinase (tk) promoter (−109/+51) and the promoter-free pBL*cat*6 plasmid have been described elsewhere [16]. pGAPDH/*cat* with the human *GAPDH* promoter (−488/+21) fused to *cat* in pUC/*cat* and the promoter-free pUC/*cat* plasmid [17] were a courtesy from Dr. M. Alexander (Harvard). pSV2/*cat* with the early enhancer/promoter of the simian virus 40 fused to *cat*, p-4964AMBP/*cat* and p-2929AMBP/*cat* with the human *AMBP* enhancer/promoter (from −4964 to +57 and from −2929 to +57, respectively) fused to *cat* in pUMSV0*cat* and the promoter-free pUMSV0*cat* plasmid have been detailed previously [18]. The HepG2 cells were plated in 6 cm plastic Petri dishes and first cultured in the standard plating medium described above. When the cells reached 70% confluence, the standard medium was replaced with minimum essential medium and 8 μg of Cat plasmid and 1 μg p47 were co-transfected by a $Ca_3(PO_4)_2$ precipitation method [18]. After 12 h exposure to the precipitate, the cells were shifted to the iso, hypo or isoraff medium as listed above, further cultured for 24 h and finally harvested by scraping. A cell extract was assayed for β-gal activity and for Cat activity and the final values of Cat activities are expressed as cpm [^{3}H]acetyl-chloramphenicol/β-gal unit as previously detailed [19]. The low activity provided by each control, promoterless plasmid was subtracted from that obtained with the paired promoter/*cat* construct(s). In every experiment the transfection of each plasmid was done in triplicate.

2.4. Statistics

Statistical significance of differences was analyzed using the Mann-Whitney non-parametric test.

3. Results

We first evaluated whether a hypotonic stress down-regulates in a gene-specific fashion the level of endogenous mRNAs in the human HepG2 hepatoma cells. The hepatic mRNAs under study comprised the GAPDH mRNA that is not regulated by AP [20,21] and the AMBP and AHSG mRNAs that both encode well-known negative APPs [13,15]. As judged from Northern blots (Fig. 1), the abundance of the endogenous GAPDH mRNA was not modified by a cell culture carried out for 24 h in a low-NaCl, hypo medium (as defined in Section 2) as compared to the control, iso medium. In contrast, the AMBP and AHSG mRNA levels were significantly decreased by this incubation in the hypo medium. In order to discriminate between a possible NaCl-specific effect and a genuine, hypotonicity-driven one, an isoraff medium that was simultaneously isotonic and low in NaCl was also tested. In the isoraff medium the abundance of AMBP and AHSG mRNAs returned to control values while the GAPDH mRNA level remained unchanged (Fig. 1). Therefore hypotonic stress per se down-regulates the expression of mRNAs that encode negative APPs, namely AMBP and AHSG, while it does not affect the expression of an mRNA whose level is known to be AP-independent, namely GAPDH.

Given that glutamine uptake by liver cells increases during AP [8–11] we evaluated whether glutamine specifically down-regulates the level of mRNAs for negative APPs. Glutamine is known to be transported across liver cell membranes by a Na-dependent transporter whose activity in HepG2 cells overcomes that in hepatocytes [22,23]. Therefore our experiments were carried out in HepG2 cells incubated in the presence of large amounts of extracellular glutamine. When these cells were cultured in a glutamine-enriched medium (see Section 2) for 24 h the abundance of both AMBP and AHSG mRNAs was significantly decreased whereas that of GAPDH mRNA remained unchanged (Fig. 2). The effect of a glutamine addition upon AMBP and AHSG mRNA levels was a relatively slow and persistent event as it was not observed after a 12 h incubation period but it was still visible at 48 h (data not shown). These results indicate that glutamine down-regulates the level of two negative APP mRNAs, i.e. AMBP and AHSG, whereas it does not affect the GAPDH mRNA level in HepG2 cells. Overall, both hypotonic stress and extracel-

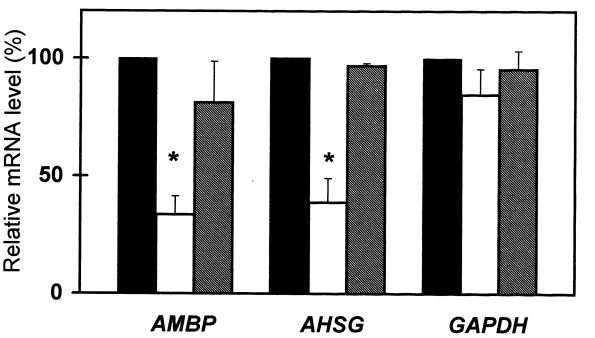

Fig. 1. Influence of hypotonic stress upon AMBP, AHSG and GAPDH mRNA levels in HepG2 cells. The HepG2 cells were maintained for 24 h in an isotonic (iso; black bars), low-NaCl, hypotonic (hypo; open bars) or low-NaCl, raffinose-supplemented, isotonic medium (isoraff; hatched bars). Total cellular RNAs were subjected to Northern blot analysis and successively probed with human AMBP, AHSG, GAPDH and 18S cDNAs. For each mRNA population, the autoradiographic signals were normalized with the 18S signals on the same blot. Values (mean ± S.E.M.) for n independent experiments ($n \geq 3$) are expressed as percent of the control value (iso). *Significant difference ($P < 0.05$) between hypo and iso and hypo and isoraff.

lular glutamine enrichment promote an identical end result, i.e. a specific down-regulation of the mRNA level for negative APPs in HepG2 cells.

We next tested whether the effect of hypotonic stress as observed above on AMBP mRNA takes place at the transcriptional level and in a gene-specific fashion. Given that the endogenous expression of AMBP is low in HepG2 cells, nuclear run-on experiments turned out to be poorly informative (data not shown). Therefore, we performed experiments where the promoter of AMBP or control gene was fused to the bacterial *cat* reporter gene. The transcriptional activities of these promoter/*cat* constructs transiently transfected (along with a β-gal plasmid for normalization) in HepG2 cells that underwent or not a hypotonic stress are shown in Fig. 3. First, the transcriptional activity of the *GAPDH* promoter was not affected by a hypo medium nor was it modified by an isoraff medium. Likewise, the transcriptions driven by promoters of viral origin, namely the *SV40* early enhancer/promoter and the HSV *tk* promoter, were not affected by either culture condition. In contrast, the transcriptional activity of the *AMBP* gene encoding a negative APP was significantly decreased in HepG2 cells incubated in the hypo medium and returned to control values when the cells were cultured in an isoraff medium. This result was observed with two AMBP/*cat* constructs covering the *AMBP* gene from −4964 to +57 or from −2929 to +57. Similar results were obtained in experiments where HepG2 cells were transfected by electroporation instead of $Ca_3(PO_4)_2$ precipitation (data not shown). Elsewhere, we ruled out that the specific activities of the Cat or β-gal enzymes might be influenced by the ionic content in cells incubated in hypo medium. Indeed, the activities of purified Cat and β-gal enzymes remained unchanged when tested in the presence of variable amounts of cell extracts from mock-transfected HepG2 cells incubated in hypo or iso media (data not shown). Therefore, our results demonstrate that hypotonic stress per se is sufficient to decrease the transcriptional activity of a human gene (*AMBP*) encoding a negative APP in liver cells and that this effect is mediated by the 5′ control region of this tonicity-responsive gene.

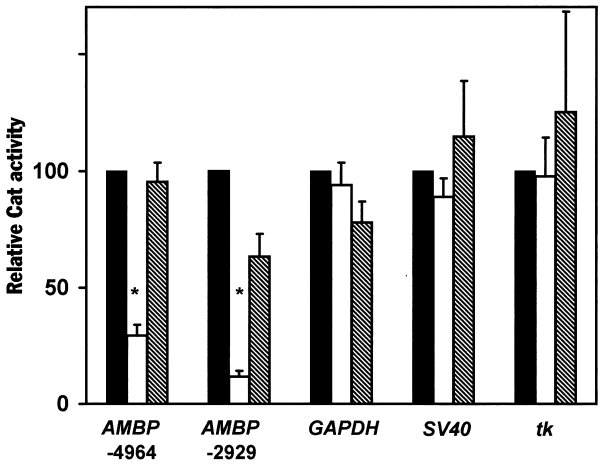

Fig. 3. Influence of hypotonic stress upon various promoter/*cat* constructs transiently transfected in HepG2 cells. The transcriptional activities of two control promoters of viral origin (*SV40*, simian virus 40 early promoter/enhancer; *tk*, Herpes simplex thymidine kinase promoter), a control promoter of human origin (*GAPDH*) and two promoters for human, a negative APP gene (*AMBP*) are shown. The extent of the 5′ flanking region for the AMBP promoter in various constructs is summarized as follows: *AMBP* (−4964) and *AMBP* (−2929) cover the *AMBP* promoter from −4964 to +57 and −2929 to +57, respectively. The constructs were tested in HepG2 cells cultured for 24 h in an isotonic (iso; black bars), low-NaCl, hypotonic (hypo; open bars) or low-NaCl, raffinose-supplemented, isotonic medium (isoraff; hatched bars). For each promoter, the values (mean ± S.E.M.) for *n* independent experiments ($n \geq 3$) are expressed as percent of the control value (iso). *Significant difference ($P < 0.05$) between hypo and iso and hypo and isoraff.

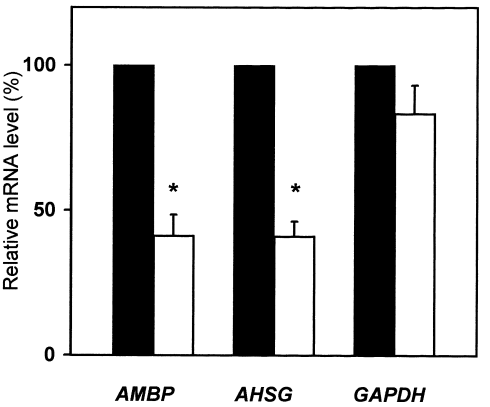

Fig. 2. Influence of a glutamine-enriched culture medium upon AMBP, AHSG and GAPDH mRNA levels in HepG2 cells. The HepG2 cells were maintained for 24 h in the absence (control medium; black bars) or in the presence (glutamine-enriched medium; open bars) of 10 mM glutamine. Values (mean ± S.E.M.) for *n* independent experiments ($n \geq 4$) are expressed as percent of the control value (control medium). *Significant difference ($P < 0.05$).

4. Discussion

Although the pro-inflammatory cytokines TNF-α, IL-1 and IL-6 and related intracellular signalling pathways and nuclear factors that trigger the transcription of APP genes in liver have been largely deciphered (reviewed in [12,14]) it cannot be excluded that other AP mediators contribute to the changes in APP levels observed in vivo. Since in AP both an increased hepatocellular HS and an increased glutamine uptake in liver take place [7,8], we investigated whether either event participates in a specific regulation of APP mRNA levels in vitro. We have now demonstrated that hypotonic stress and an increase in extracellular glutamine availability are both able to specifically decrease the level of hepatic mRNAs that encode well-known negative APPs, i.e. AMBP and AHSG, without modifying that of another mRNA whose product is not an APP, namely GAPDH. The observed similarity in the final effects produced by hypotonic stress and glutamine was not unexpected. Indeed, up-regulated glutamine uptake in hepatocyte has been shown to increase the hepatocellular HS, an event that in turn results in cell swelling [24,25]. Therefore, our study now suggests that during AP glutamine participates in the regulation of AMBP and AHSG mRNA levels in the liver via an increase in hepatocellular HS and cell swelling. Accordingly, we have selected hypotonic stress to further study the transcription of a negative APP gene under conditions of increased hepatocellular HS.

Our data demonstrate that the down-regulation of the AMBP mRNA level promoted by hypotonic stress is mediated, at least partly, by the 5′ flanking region of the cognate

gene even though a role for hypotonic stress on AMBP mRNA stability cannot be excluded. A tonicity-responsive element (TonE: TGGAAAATC) has been previously characterized in two genes, namely the *aldose reductase* (*AR*) [26,27] and *betaine transporter* (*BT*) [28,29] genes that are both up-regulated by hypertonic stress in renal medulla. The *AR* gene is also up-regulated by hypertonic stress in liver and a short enhancer (AEE; core: GAAGAGTG) has been identified in the rat gene where this element controls the hypertonicity-driven transcription in a fashion that does not depend on the neighboring TonE [30].

Whether these hypertonicity-responsive elements are also able to participate in a response to hypotonic stress had not been studied. A search in the *AMBP* 5′ flanking region has now revealed that a perfect TonE sequence is present at position −3744. However, this sequence alone is unlikely to account for our present results as an AMBP/*cat* construct lacking this TonE sequence (p-2929AMBP/*cat*) fully retains the negative response to hypotonic stress. Elsewhere, another element (oligo B: TGAGACNNNGTCTCA, which is palindromic in nature and harbors a A/T-rich cluster ahead of it) mediates in various cell lines the hypertonicity-driven up-regulation of several genes [31]. Thus, the set of hypertonicity-responsive elements TonE and oligo B that are up-regulated by hypertonic stress appear not to be involved or, at least, to be dispensable in the response of the AMBP gene to hypotonic stress in liver. Therefore, the 5′ flanking sequence of the *AMBP* gene now provides a new tool to identify the sequence(s) and transcription factor(s) involved in the hepatic response to hypotonic stress.

This is the first report that hypotonic stress per se is sufficient to down-regulate the transcription of a negative APP-encoding gene. Strikingly, this down-regulation is opposed to the hypotonicity-driven, transcriptional up-regulation of a gene encoding a well-known positive APP, as recently shown with the rat *a2-M* gene [6], or that of another gene, namely the rat β-*actin* gene [5] whose up-regulation in endotoxin-challenged hepatocytes has been observed [32]. Also of great interest is the fact that c-*jun* is another gene whose hepatic mRNA level is up-regulated by hypotonicity and AP [3,33]. All together, these past results and our present findings indicate that the transient (and possibly glutamine-driven) hypotonic stress of liver cells that takes place in AP [7] simultaneously participates in the up- and down-regulated synthesis of both sets of positive and negative APPs, respectively, in liver.

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