Osmotic Regulation of Bile Acid Transport, Apoptosis and Proliferation in Rat Liver

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Key Words

Integrins • Src • Fyn • EGFR • CD95

Abstract

Changes in mammalian cell volume as induced by either anisoosmolarity, hormones, nutrients or oxidative stress critically contribute to the regulation of metabolism, membrane transport, gene expression and the susceptibility to cellular stress. Osmosensing, *i.e.* the registration of cell volume changes, triggers signal transduction pathways towards effector pathways (osmosignaling) which link alterations of cell volume to changes in cell function. This review summarizes our own work on the understanding of how osmosensing and osmosignaling integrate into the overall context of bile acid transport, growth factor signaling and the execution of apoptotic programs.

Osmotic regulation of bile acid transport

Hepatic bile formation is due to the vectorial trans-hepatocellular transport of solutes and involves the coordinated action of transport proteins at the basolateral (sinusoidal) and the apical (canalicular) membrane of the liver parenchymal cell [1-4]. The hepatocellular hydration state, *i.e.* hepatocyte volume, exerts powerful control on the transcellular transport of solutes, such as conjugated bile acids, glucuronide and glutathione conjugates [5-9]. Since liver cell hydration is a dynamic parameter, which changes within minutes under the influence of hormones, nutrients and oxidative stress, the functional relevance of the liver hydration state for hepatic bile formation is evident and of physiological and pathophysiological interest [10, 11].

One major mechanism of short-term regulation of bile formation is regulated transporter insertion into and retrieval from the canalicular membrane [8, 12]. By this mechanism, the number of transporter molecules in the canalicular and sinusoidal membrane can change

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within minutes and thereby affect transport capacity ($V_{\max}$). One major regulator of this process are changes of liver cell volume, which can occur in response to hormones, nutrients, oxidative stress or changes in ambient osmolarity (for review see [7, 13]). In perfused rat liver, cell shrinkage inhibits, whereas cell swelling stimulates taurocholate (TC) excretion into bile, regardless of whether cell volume is modified by anisotonic exposure, insulin, cumulative amino acid uptake or ethanol [5, 8]. In perfused rat liver, a 10% increase of hepatocyte water content doubles within minutes the $V_{\max}$ of TC excretion into bile, whereas hyperosmotic cell shrinkage decreases $V_{\max}$ [5].

The swelling-induced increase in transport capacity is abolished in presence of colchicine, indicating the requirement of intact microtubules [6]. Hyperosmotic hepatocyte shrinkage triggers a rapid retrieval of Bsep from the canalicular membrane and its transfer into an intracellular vesicular compartment as shown by immunohistochemistry, whereas cell swelling triggers the insertion of intracellularly stored Bsep into the canalicular membrane [14]. Likewise, hyperosmotic hepatocyte shrinkage triggers retrieval of the conjugate export pump Mrp2 from the canalicular membrane, whereas hypoosmotic hepatocyte swelling induces a rapid insertion of Mrp2 into the canalicular membrane, as shown by immunohistochemistry and immunogold electron microscopy [12, 15]. These osmodependent effects on transporter localization are also found at the functional level, as reflected by corresponding changes in the canalicular secretion of the glutathione-conjugates in perfused rat liver [15, 16]. Also the expression of Bsep and Mrp2 is osmoregulated. Hypoosmotic hepatocyte swelling increases Bsep and Mrp2 mRNA and protein expression, whereas hyperosmotic cell shrinkage decreases [17, 18].

Osmosensing and osmosignaling pathways towards canalicular secretion

Regulation of canalicular secretion by changes of cell hydration requires structures which pick up changes in liver cell volume (osmosensing) and which further signal this information towards effector sites (osmosignaling). Recent studies identified the integrin system as one major osmosensor in hepatocytes [19-21]. Integrins are a family of extracellular matrix adhesion molecules involved in “mechanotransduction” and growth factor signaling [22-25]. In liver, $\alpha_1\beta_1$, $\alpha_3\beta_1$ and

$\alpha_9\beta_1$ integrins are the most important ones [26-28]. As shown recently by means of immunohistochemistry, hypoosmotic exposure of hepatocytes induces the rapid appearance of the active conformation of the β_1 subunit in the plasma membrane, indicating integrin activation in response to hepatocyte swelling [19-21]. Downstream consequences of hypoosmotic integrin activation are the activation of focal adhesion kinase (FAK), c-Src-kinase, the epidermal growth factor receptor (EGFR) and of mitogen activated protein (MAP) kinases Erk-1/2 and p38^{MAPK} [19, 20]. Dual activation of both, Erks and p38^{MAPK} is required for the swelling-induced choleretic effect and choleresis induced by hypoosmotic hepatocyte swelling is abolished in presence of inhibitors of either the integrin system, c-Src or one of the two MAP-kinases [14, 19, 29, 30]. The role of integrins as osmosensors is underlined by the fact that integrin-inhibitory peptides containing an RGD motif abolish osmosignaling towards c-Src and MAP kinases as well as the stimulation of bile formation, which is otherwise triggered by hypoosmotically induced cell swelling [19]. Inhibitory RGD peptides prevent integrin binding to the RGD attachment sites of extracellular matrix proteins, such as fibronectin, and thereby impair the dynamics of integrin/matrix interactions, which are critical for an effective mechanotransduction [31]. It is important to note that tauroursodesoxycholate (TUDC) also activates integrin-dependent osmosensing and osmosignaling pathways in a cell volume-independent way by a direct interaction of the bile acid with the binding pocket of the $\alpha_3\beta_1$ integrin (Gohlke, Reinehr & Häussinger, unpublished data), which may explain the choleretic action of this bile acid [19, 30]. Likewise, urea and some of its derivatives can activate β_1 -integrin in a volume-independent way and thereby partially mimic a “swollen hepatocyte response” [32, 33].

Early endosomes have been identified as an osmosensing compartment, which is activated in response to hyperosmotic hepatocyte shrinkage [34]. Evidence was presented that a hyperosmolarity-induced and chloride-driven endosomal acidification triggers within seconds ceramide formation, which in turn activates protein kinase C (PKC) ζ , which further results in an activation of NADPH oxidase isoforms due to an activating phosphorylation of the regulatory subunit p47^{phox} [34]. As a consequence, hyperosmotic hepatocyte shrinkage produces oxidative stress [35-37], which is involved in the hyperosmotic retrieval of Mrp2 and Bsep from the canalicular membrane [38].

Moreover, exogenously added hydroperoxides were shown to induce the retrieval of Mrp2 from the canalicular membrane [38]. In line with findings that oxidative stress contributes to the cholestatic state after hyperosmotic hepatocyte shrinkage is also the observation that hydrophobic bile acids not only induce cholestasis, but also oxidative stress via NADPH oxidase activation [39, 40].

This hyperosmolarity-induced and NADPH oxidase-mediated ROS formation leads to an activation of the Src-kinases Yes and Fyn [41] and an activation of c-Jun-N-terminal kinase (JNK) [37]. It was shown in the perfused rat liver that the retrieval of Bsep and Mrp2 is sensitive to inhibition of Fyn, whereas Yes and JNK are not involved in hyperosmolarity-induced changes in bile acid transported localization [42]. In line with this, Fyn knockdown in rat hepatocyte couplets using an siRNA-approach, as well as pharmacological inhibition of Fyn, largely abolishes the hyperosmolarity-induced cholestasis [42]. Also the antioxidant N-acetylcysteine (NAC) prevents cell shrinkage-induced cholestasis in both, rat hepatocyte couplets and the perfused rat liver [42]. Evidence was presented that cortactin may act as a target of hyperosmolarity-induced and Fyn-mediated phosphorylation in rat hepatocytes [42], which was also described in fibroblasts [43]. Phosphorylation of cortactin was shown to favour its dissociation from the actin cytoskeleton [44] and this was also found in rat hepatocyte couplets in a hyperosmolarity-induced and Fyn-mediated way [42]. Thus, retrieval of Bsep and Mrp2 from the canalicular membrane in response to cell shrinkage is induced by a NADPH oxidase-driven ROS formation, which leads to a Fyn-mediated cortactin phosphorylation and its subsequent dissociation from the actin cytoskeleton thereby augmenting endocytosis [42, 45].

Hepatocyte shrinkage and apoptosis induction

Apoptosis is characterized by cell shrinkage, nuclear condensation, DNA fragmentation and apoptotic body formation. These features distinguish apoptosis from other types of cell death, such as necrosis [46]. Whereas some signs of apoptosis, such as externalization of phosphatidylserine, altered mitochondrial function or activation of caspases are cell type- and death signal-dependent, apoptotic cell volume decrease (AVD) is an early and ubiquitous event [47-50]. For example, in

Jurkat cells CD95 activation inhibits Na^+/H^+ -antiport [47], the Na^+/K^+ -ATPase [51], activates an outwardly rectifying chloride channel [52] and stimulates efflux of taurine [53], resulting in AVD. In a variety of non-hepatic cell lines AVD in response to $\text{TNF}\alpha$, cycloheximide or staurosporine is achieved by cellular losses of K^+ and Cl^- , followed by cytochrome c release, caspase-3 activation, DNA fragmentation and ultrastructural alterations [54, 55]. Inhibition of AVD by blocking volume-sensitive K^+ - or Cl^- -channels [56] or by elevation of extracellular K^+ [57] counteracts apoptosis, whereas inhibition of the Na^+/H^+ -exchange accelerates apoptotic cell death [47]. The contribution of AVD to apoptotic signal transduction is less clear, but hyperosmolarity can mimic AVD and thereby stimulate apoptosis (see below). This, however, is cell type-specific and depends on the efficacy of regulatory volume increase mechanisms [58-60]. In rat hepatocytes, hyperosmotic cell shrinkage triggers ligand-independent CD95-activation and subsequent CD95 trafficking to the plasma membrane, thereby sensitizing hepatocytes towards CD95 ligand (CD95L)-induced apoptosis or even inducing apoptosis when the hyperosmotic challenge is strong enough [60, 61].

Whereas under normoosmotic resting conditions CD95 is localized inside the hepatocyte and exhibits no detectable membrane localization, this death receptor is targeted within 1-2 hours to the plasma membrane in response to pro-apoptotic stimuli, such as CD95L, hyperosmolarity or hydrophobic bile acids [60-64]. Hyperosmotic CD95-activation and membrane trafficking are complex processes and have been studied in detail. An almost instantaneous production of reactive oxygen species (ROS) [60] following hyperosmotic exposure was identified as an early signaling event, which triggers CD95-activation and membrane trafficking. The underlying mechanisms were recently identified [34] and resemble those triggered by CD95L [65] or hydrophobic bile acids [39, 66]. Although mitochondria or the endoplasmic reticulum (ER) were discussed as potential sources of oxygen radical formation [67], it is now clear that the rapid ROS generation in response to hyperosmotic hepatocyte shrinkage is largely mediated by NADPH oxidase isoforms [68]. Hyperosmolarity rapidly induces an activating p47^{phox} -serine phosphorylation [34], which triggers self-assembly of the NADPH-oxidase subunits and subsequent ROS-generation. Hyperosmotic p47^{phox} -serine phosphorylation is sensitive to inhibitors

of PKC ζ , and as a surrogate marker of its hyperosmotic activation, this PKC isoform is translocated to plasma membrane after hyperosmotic exposure [34]. In addition, hyperosmotic exposure leads to an almost instantaneous increase of ceramide levels, which is sensitive to inhibitors or knockdown of acidic sphingomyelinase (ASM) [34]. PKC ζ is known to be activated by ceramide [69]. Thus it is likely that hyperosmolarity induces a rapid activation of ASM and an increase of intracellular ceramide levels, which triggers PKC ζ -activation and subsequent p47^{phox}-serine phosphorylation, which leads to NADPH-oxidase activation and ROS generation. Inhibitor studies support this notion [34].

Hyperosmotic hepatocyte shrinkage rapidly increases the cytosolic Cl⁻-concentration [70-72] and evidence has been presented for a direct activation of the vesicular proton pump by Cl⁻ [73-75], independent of a role of chloride for compensation of the electrogenicity of the vacuolar proton ATPase. In hepatocytes ASM is not only located at the plasma membrane or in the lysosomes [76, 77], but also in an early endosomal compartment with an apparent pH_{ves} of about 6.0 [34]. Here, hyperosmolarity induces an almost instantaneous endosomal acidification by shifting apparent vesicular pH from 6.0 to about 5.6. This hyperosmolarity-induced endosomal acidification was sensitive to bafilomycin, an inhibitor of vacuolar-type H⁺-ATPase, and the anion channel blocker 4,4'-diisothiocyano-stilbene-2,2'-disulfonic acid disodium salt (DIDS). ASM has a pH optimum around 5 [78, 79] and acidity is thought to increase the affinity of ASM to its substrate [80]. Thus hyperosmolarity-induced endosomal acidification by about 0.4 pH-units may induce ASM-activation and ceramide formation [34]. In line with this, the hyperosmolarity-induced increase of hepatocellular ceramide levels is abolished in presence of bafilomycin and DIDS.

Hyperosmolarity-induced ROS generation was identified as an important upstream event for activation of both, the Src-family kinase Yes and of c-Jun-N-terminal kinases (JNK) [34, 60]. Activated Yes associates with the epidermal growth factor-receptor (EGFR) and the latter becomes tyrosine-phosphorylated on position Y⁸⁴⁵ within the tyrosine kinase domain, followed by autophosphorylation at Y¹¹⁷³ and EGFR activation. Whereas Yes activates the EGFR, the JNK signal mediates an association of the EGFR with CD95, as shown by co-immunoprecipitation and fluorescence

resonance energy transfer (FRET) studies [60-64]. CD95 then becomes tyrosine-phosphorylated by the EGFR tyrosine kinase activity, which is a signal for a microtubule-dependent targeting of the EGFR/CD95 complex to the plasma membrane [60], where formation of the death-inducing signaling complex (DISC) occurs with activation of caspases 8. Human CD95 contains 3 tyrosine residues: one in the extracellular domain (Y⁹¹) and two within the death domain (Y²³² and Y²⁹¹) [81]. It has been demonstrated by single-, double- and triple-mutation (Y→F) of YFP-coupled CD95-constructs, that phosphorylation of both tyrosine residues within the death domain (Y²³² and Y²⁹¹) is critical for hyperosmolarity-induced CD95-membrane trafficking and DISC formation [64].

Although hyperosmotic shrinkage of hepatocytes targets CD95 to the membrane followed by DISC formation and activation of the caspases, this does not necessarily lead to hepatocyte apoptosis, suggesting that death-inducing signals can be counterbalanced by yet unidentified survival signals. However, in any case mild hyperosmolarity sensitizes hepatocytes to apoptosis induced by CD95 ligand [60, 61, 82] by triggering CD95 translocation to the plasma membrane. This may be important, because Kupffer cells were shown to express the membrane-bound CD95L in response to endotoxin [83, 84], which can bind to its receptor only when presented at the hepatocyte surface [61]. This may explain why shrunken hepatocytes are more susceptible towards stress-induced cell death. This may also explain the clinical observation that transplantation of livers from hypernatremic donors exhibit high rates of primary graft dysfunction.

Hyperosmotic CD95 tyrosine phosphorylation is an essential step for CD95-activation, subsequent CD95 targeting to the plasma membrane and apoptosis induction. In line with this, pharmacological maneuvers which prevent hyperosmotic CD95 tyrosine phosphorylation, such as inhibitors of sphingomyelinases, PKC ζ , p47^{phox}, Yes, JNK, EGFR or of EGFR/CD95 association, all abolish CD95 trafficking to the plasma membrane and DISC formation. Also taurine and glutamine can antagonize hyperosmotic CD95 targeting to the plasma membrane, probably due to an attenuation of hyperosmotic shrinkage [60, 61].

Inhibition of hyperosmotic CD95 activation and sensitization towards apoptosis is also found following other covalent modifications of the CD95. For example, cyclic AMP, which exerts anti-apoptotic

Fig. 1. Osmosensing and osmosignaling in response to hypoosmotic and hyperosmotic hepatocyte swelling and shrinkage, respectively. Cell swelling is sensed by integrins and triggers an activation of focal adhesion kinase, c-Src, EGFR and MAP-kinases, which mediate cholestasis, cell proliferation and inhibition of proteolysis. Cell shrinkage is sensed by a chloride-dependent acidification of endosomes, which activates acidic sphingomyelinase and triggers ceramide formation. Ceramide activates protein kinase ζ , which phosphorylates p47^{phox}, which activates NADPH oxidase(s). The resulting generation of reactive oxygen species activates c-Jun-N-terminal kinase (JNK) and the Src kinase family members Yes and Fyn. Fyn mediates retrieval of Bsep and Mrp2 from the canalicular membrane and triggers cholestasis. Yes activates the EGFR, whereas JNK mediates EGFR/CD95 association, followed by EGFR-catalyzed CD95 tyrosine phosphorylation. The latter allows for CD95 oligomerization, translocation to the plasma membrane and formation of the death-inducing signalling complex.

effects in a variety of cell types including hepatocytes [85], also inhibits hyperosmotic CD95 activation in hepatocytes. This is on the one hand explained by a protein kinase A (PKA)-dependent inhibition of the hyperosmotic EGFR activation, which prevents CD95 tyrosine phosphorylation and on the other hand by a PKA-dependent serine/threonine phosphorylation of the CD95 [63]. Serine/threonine phosphorylation of CD95 acts as an internalization signal, whereas CD95 tyrosine phosphorylation is a signal for CD95 targeting to the plasma membrane. Another covalent modification

of the CD95 is CD95-tyrosine nitration [86], which occurs in response to endotoxin or peroxynitrite (ONOO⁻). ONOO⁻-induced CD95-tyrosine nitration prevents CD95-tyrosine phosphorylation in response to hyperosmolarity [86]. Conversely, when CD95-tyrosine phosphorylation is induced prior to ONOO⁻-addition, no CD95-tyrosine nitration is inducible, indicating that CD95-tyrosine phosphorylation and nitration are mutually exclusive [86]. CD95-tyrosine nitration is also induced *in vivo* following endotoxin administration, probably due to the induction of NO synthase and

myeloperoxidase [87]. Under these conditions CD95-tyrosine nitration may serve to limit the apoptotic cell death in response to inflammatory stimuli.

Hepatocyte swelling and hepatocyte proliferation

In contrast to the hyperosmotically induced activation of apoptotic cell death cascades, cell swelling is a prerequisite for cell proliferation. Hyperosmolarity-induced hepatocyte swelling involves an $\alpha_5\beta_1$ -integrin-mediated c-Src-activation, which triggers EGFR phosphorylation, subsequent Erk-1/-2 activation and proliferation [19, 88]. Also cell swelling induced by insulin induces an $\alpha_5\beta_1$ -integrin-dependent activation of FAK, c-Src and of Erks and p38^{MAPK}, the latter of which mediates the anti-proteolytic effect of insulin [21]. Apart from its metabolic effects, insulin stimulates proliferation in the liver and other organs [89-93]. Much effort has been devoted to the understanding of insulin signaling and its complexity [94-98], which involves tyrosine phosphorylation of the insulin receptor substrate-1 and activation of a variety of protein kinases such as mitogen-activated protein (MAP) kinases Erk-1/-2 and p38^{MAPK} [91, 99]. In rat liver, insulin stimulates Na⁺/H⁺ antiport and K⁺/Na⁺/2Cl⁻ co-transport, thereby inducing hepatocyte swelling [100]. Recent data [88] point to an important contribution of insulin-induced cell swelling to the known proliferative effects exerted by the hormone [89-93]. Both, insulin and EGF increase DNA synthesis in hepatocytes kept under serum-free conditions [101-103], but it remained unclear whether there is convergence of insulin- and EGF-derived signals. As shown in both, perfused rat liver and primary rat hepatocytes, insulin leads to an activation of the EGFR, which is triggered by insulin-induced hepatocyte swelling [88]. EGFR activation by insulin was sensitive to bumetanide (which prevents insulin-induced hepatocyte swelling) and to inhibition by an integrin-antagonistic RGD peptide, an integrin β_1 subtype-blocking antibody and the c-Src inhibitor PP-2. These findings point to an involvement of the recently described swelling induced and integrin-dependent osmosensing/signaling pathway [19, 21]. Both, insulin and hypoosmolarity induced a significant increase in BrdU-uptake in primary rat hepatocytes suggestive for hepatocyte proliferation, which

was again sensitive to RGD peptide-, integrin β_1 -blocking antibody, PP-2, AG1478 and PD098059 suggestive for an involvement of $\alpha_5\beta_1$ -integrin, c-Src, EGFR tyrosine kinase and Erk-1/-2 [88].

Since EGFR activation participates in both, cell swelling-dependent proliferation and cell shrinkage-induced apoptosis, the question arises on the mechanisms which provide the switch between EGFR-based proliferation and EGFR-dependent apoptosis. JNKs are a likely candidate, because JNKs provide the signal for CD95/EGFR association and subsequent EGFR-mediated CD95 tyrosine phosphorylation and activation of the CD95 death machinery [60]. Recent data suggest that insulin-induced and swelling-dependent EGFR-activation can be converted into a CD95-mediated death signal upon addition of free fatty acids leading to a sustained JNK-activation (Reinehr & Häussinger, unpublished data). Further work is required to strengthen this hypothesis.

Conclusions

The $\alpha_5\beta_1$ -integrin systems represents an osmosensor registering liver parenchymal cell swelling, which triggers cholestasis, proteolysis inhibition and proliferation via osmosignaling pathways involving FAK, Src, EGFR and MAP kinases Erks and p38^{MAPK}. On the other hand, hepatocyte shrinkage leads to an acidification of an early endosomal compartment triggering an ASM-, ceramide- and PKC ζ -dependent ROS generation which leads to Yes, Fyn and JNK activation. Fyn is involved in triggering cholestasis due to retrieval of Bsep and Mrp2 from the apical membrane, whereas Yes transactivates EGFR and a JNK signal couple EGFR to CD95 activation and apoptosis induction. Fig. 1 schematically depicts our current view on hypoosmotic and hyperosmotic sensing and signaling pathways in hepatocytes.

Abbreviations

ASM (acidic sphingomyelinase); AVD (apoptotic cell volume decrease); Bsep (bile salt export pump); CD95 (Fas receptor, APO-1); DISC (death-inducing signaling complex); EGFR (epidermal growth factor receptor); ER (endoplasmic reticulum); FAK (focal

adhesion kinase); FRET (fluorescence resonance energy transfer); JNK (c-Jun-N-terminal kinase); MAP kinase (mitogen activated protein kinase); Mrp2 (multidrug resistance protein 2); NAC (N-acetylcysteine); ONOO⁻ (peroxynitrite); PKA (protein kinase A); PKC ζ (protein kinase C ζ); ROS (reactive oxygen species); TC (taurocholate); TUDC (tauroursodesoxycholate); V_{max} (transport capacity).

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