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PROSPECT

Impact of Cell Swelling on Proliferative Signal Transduction in the Liver

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Abstract Cellular swelling has emerged as an important initiator of metabolic and proliferative changes in various cells. Because of the unique regenerative capacity of the adult liver, researchers have delineated key intracellular signals that are activated following mitogens, injury, and partial hepatectomy. Although hepatocellular swelling is commonly observed following these regenerative stimuli, only recently has the relationship between cell volume increase and proliferative activity been investigated; to date, the data implicating cell volume increase with hepatocyte regeneration has been mostly indirect. Hepatocyte swelling has been demonstrated in various clinical scenarios from sepsis, hepatic resection, ischemia-reperfusion injury, glucocorticoid excess, and hyperinsulinemia. Using various in vivo and in vitro models of hepatocyte swelling, particularly hypo-osmotic stress, investigators have demonstrated changes in cellular structure: (1) cell membrane stretch, (2) cytoskeletal microtubule and microfilament reorganization, and (3) alterations in cytoskeletal-membrane complexes. Similar studies have demonstrated a causal relationship between cell volume increase and intracellular signals: (1) activation of cytoplasmic signaling cascades such as MAPKs, PI-3-K, and PKC, (2) activation of proliferative transcription factors NF-κB, AP-1, STATs, C/EBPs, and (3) transcription of metabolic and immediate early genes of regeneration. Through mechanotransduction, or the translation of physical changes to chemical signals, cell volume is a potent effector of these signaling events. Growing evidence demonstrates a link between these physical and chemical changes in the swelling-mediated growth in the liver. J. Cell. Biochem. 83: 56–69, 2001.

Key words: cell volume; cell swelling; cytoskeleton; tensegrity; mechanotransduction; ion channels; MAP Kinase; PI-3-Kinase; PKC; transcription factors; immediate early genes; proliferation

Because of the unique ability of the adult liver to regenerate following injury, investigations into its proliferative machinery are both scientifically and clinically needed. Understanding the mechanisms involved in hepatocyte proliferation would not only elucidate the physiology of cell growth and oncogenesis, but it may help patients in need of liver transplantation. As the dearth of liver donors remains at crisis, the ability to support marginal hepatic function by either expediting liver healing, by facilitating living related donor regeneration to expand the donor pool, or by engineering autologous hepatocytes would alleviate a growing health problem.

In this review of the literature, the effects of hepatocellular swelling due to various physiologic and pathophysiologic conditions are discussed. Although a number of in vivo and in vitro models have been used, the results consistently demonstrate that cell volume increases metabolism and gene expression. In addition, a growing body of data suggests that hepatocyte swelling induces a number of intracellular pathways implicated in cell cycle progression, proliferation, and even oncogenesis. Collectively, these data demonstrate that swelling is a mitogenic signal promoting growth for liver cells.

Liver Swelling in Health and Disease

Physiologic hepatocyte swelling. The liver acts as a filter to absorb and process nutrients, toxins, and hormones from both the portal and

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systemic circulation. Changes in the levels of these factors have been shown to alter hepatocyte cell volume. To add to the complexity of these volume changes, investigators have shown through in vivo studies that the degree to which various factors alter hepatocyte hydration is influenced by the host's nutritional status [Vom and Haussinger, 1996].

Rat liver perfusion studies have demonstrated hepatocyte cell volume increase with exposure to insulin and IGF-1, and that this effect was diminished in starved rats [Vom and Haussinger, 1996]. In vivo studies in rats have shown that the amino acids glycine, glutamine, and alanine cause hepatocyte volume increase, but unlike insulin, this response increased in starved rats [Vom and Haussinger, 1996]. When perfused with physiologic concentrations of various amino acids, isolated rat liver increased their mass by 4 to 6% [Wettstein et al., 1990]. In isolated perfused rat liver studies, the bile acids taurocholate and tauroursodeoxycholate induced hepatocyte volume increase which then resulted in increased taurocholate excretion and bile flow [Haussinger et al., 1992a]. These observations suggest an important feedback mechanism in which nutritional status influences liver volume, which in turn modulates hepatocyte metabolic function. Clinically, these findings are seen in nutritionally deficient patients whose regenerating livers following resection are retarded.

Swelling in liver injury. As the largest solid organ in the body, the liver is susceptible to injury both directly and indirectly. In addition, as the filter for both portal and systemic blood, the liver is constantly exposed to various bloodborne insults from various disease processes.

The liver is exposed to toxins and cytokines due to infections, and various studies demonstrate subsequent liver volume changes. In rats suffering from septic peritonitis for 24 h, both liver weight and protein synthesis increased [Pedersen et al., 1986]. Similarly, the infusion of tumor necrosis factor-alpha (TNF-α) resulted in a marked increase in liver mass [Feingold and Grunfeld, 1987]. This observation may explain why endotoxin increased both rat liver mass and hepatocyte volume [Qian and Brosnan, 1996], and demonstrates that the liver size increases because of the cell size. Although these studies suggest that the signaling events induced by TNF-α are cell volume mediated, clearly swelling is only part of the picture as compelling evidence supports a ligand-receptor mechanism as being responsible for TNF-α stimulated activity [Yamada et al., 1998]. Finally, many exogenous compounds have been shown to be osmotically active solutes across the cell membrane. Among its many effects, ethanol inhibits of hepatic proteolysis by increasing the activity of Na^+-K^+-$2Cl^-$ cotransport system [Vom and Haussinger, 1998].

Surgery also subjects the liver to significant injury. The remnant liver following partial hepatectomy represents one model of liver injury, and the hemodynamic changes seen here are striking. In the rat, an early increase in portal venous pressure within the remnant liver results in increased endothelial fenestrations and sinusoidal wall porosity [Morsiani et al., 1995; Rice et al., 1977]. This sinusoidal dilatation allows the direct exposure of portal blood to parenchymal cells [Morsiani et al., 1995], thus allowing greater contact between volume altering factors in the blood and hepatocytes. In orthotopic liver transplantation, the osmolytes within the University of Wisconsin preservation solution induce hepatocyte cell volume changes [Sadoshima et al., 1996]. The modulation of hormone levels such as insulin and glucagon following surgical stress also causes hepatocyte volume increase [Haussinger and Lang, 1992]. Although the exact timing of initial cell volume change is not yet known, it occurs early and precedes the intracellular growth response to injury [Kutz and Burg, 1998]. In glucocorticoid-treated dogs, electron microscopy demonstrated increased hepatocyte cell volume [Kuhlenschmidt et al., 1991]. In addition, dexamethasone has been shown to upregulate multidrug-resistance protein 2 (MRP2) [Kubitz et al., 1999] and canalicular bile salt export pump (Bsep) mRNA [Warskulat et al., 1999] in rat hepatocytes exposed to hypoosmotic stress. In rat orthotopic liver transplantation experiments, the upregulation of TNF-α, c-Jun N-terminal kinase (JNK), activator protein-1 (AP-1), and nuclear factor-κB (NF-κB) following reperfusion were independent of Kupffer cells, suggesting that events within hepatocytes themselves induce this cascade of signals [Bradham et al., 1999]. Since TNF-α causes hepatocellular swelling [Feingold and Grunfeld, 1987], reperfusion injury in transplantation may initiate proliferative signaling by cytokine-mediated hepatocyte volume increase.

Liver swelling in growth. Studies demonstrating a relationship between hepatocyte volume increase and liver growth in vivo show convincingly that cell swelling is a physiologic proliferative mechanism. Recently, investigators have demonstrated that hepatocyte volume in the regenerating rat livers following partial hepatectomy increased 25% at 6 h then returned to control values by 12 h [Freeman et al., 1999]. Further studies in regenerating livers correlated DNA synthesis with amino acid uptake-induced swelling [Freeman et al., 1999] (see below).

Effects of Swelling on Hepatocellular Function

Although isolated liver perfusion and in vitro hepatocyte studies have demonstrated that volume increases due to hypo-osmotic stress or hormones are rapid and transient, with subsequent regulatory volume decrease (RVD) occurring within minutes, the alterations in metabolism and gene expression are prolonged [Yano et al., 1996]. These effects of cell volume increase on hepatocyte metabolism are diverse.

Metabolism. Hepatocellular volume increase induced by hypo-osmotic stress, insulin, and quinine stimulated glycogenesis, in part due to increased cation uptake [al-Habori et al., 1992]. In vitro studies suggest that cell swelling-induced glycogenesis occurs through synthase phosphatase activation following Cl^- extrusion [Meijer et al., 1992]. It is likely that the movement of both ions following hepatocyte swelling can be explained by the activation of the Na^+-K^+-$2Cl^-$ cotransport system.

Hepatocyte swelling caused by changes in hormone levels affects protein metabolism. In starved rats, diminished hepatocyte proteolysis reflects diminished insulin-induced cell swelling. In these same animals, various amino acids induced both enhanced swelling and proteolysis in hepatocytes [Vom and Haussinger, 1996]. These two studies demonstrate that cell volume increase due to endogenous compounds impact metabolism. In primary rat hepatocytes, this anti-proteolytic effect was inhibited by colchicine, thus suggesting a microtubule dependent mechanism [Haussinger et al., 1994]. Because hepatic proteolysis involves pH-sensitive proteinases housed normally in acidic lysosomal compartments, the swelling-induced alkalinization of these vesicles may contribute to proteolytic inhibition. These changes delineate a possible mechanism linking cell volume to function via changes in the intracellular milieu [Busch et al., 1994]. More recently, hypo-osmotic stress induced hepatocyte swelling has been shown to regulate proteolysis by the sequestration of autophagic vacuoles in a p38 dependent fashion [Vom et al., 2001].

Fat metabolism is also affected by liver hydration. In isolated perfused rat liver, ketogenesis and CO_2 production from alpha-ketoisocaproate were increased with hypo-osmotic stress and to a lesser extent from glutamine, indicating that volume changes may influence cellular respiration and mitochondrial function. [Haussinger et al., 1992].

Hypo-osmotic medium perfused into a rat liver has been found to increase the rate of taurocholate excretion, and this effect is both G-protein and tyrosine kinase-dependent [Haussinger et al., 1992]. In this model, cell swelling was protective against taurocholate-induced cholestasis as was seen in hyper-osmotic cell shrinkage. More recently, hypoosmotic stress has been shown to stimulate taurocholate uptake by increasing Na-taurocholate cotransporter translocation in a PI-3-K-dependent fashion [Webster et al., 2000]. Therefore, hepatocyte volume may have a housekeeping role to maintain biliary function.

Metabolic gene transcription. The earliest evidence implicating hepatocyte volume to gene expression — a relationship which later led to the idea of cell volume-induced growth and proliferation — was acquired in studies linking hypo-osmotic stress to metabolic gene transcription. In both perfused rat livers and H4IIE rat hepatoma cells, hypo-osmotic stress decreased both tyrosine aminotransferase (TAT) and phosphoenolpyruvate carboxykinase (PEPCK) mRNA levels [Newsome et al., 1994]. This abrogation of metabolic gene transcription suggests that upon swelling, hepatocytes decrease carbohydrate metabolic machinery in order to undergo cell cycle progression.

HEPATOCYTE SWELLING AND PHYSIOLOGIC RESPONSE

Hypo-Osmotic Stress and Cellular Hydration In Vitro

Hypo-osmotic stress as a valid model of hepatocyte swelling. In order to study the effects of hepatocellular volume increase, investigators

TABLE I. Effects of Hypo-Osmotic Stress on Liver Cell Metabolism

Cell type	Stimulus	Effect	Reference
Primary rat hepatocytes	Hypo-osmotic	⇑Biliary taurocholate excretion	Noe et al. [1996]
		pH ⇑ in vesicles, ⇓ in cytosol	Schreiber et al. [1994]
		⇑ H^+ retention	Gleeson et al. [1990]
		⇓ G-actin/total actin ratio	Theodoropoulos et al. [1992]
		⇑ Ketogenesis	Haussinger and Lang [1992]
		⇑ Glycogenesis	Vom and Haussinger [1998]
		⇓ Cl^-	Meijer et al., [1992]
	Quinine	⇑ Glycogenesis	Vom and Haussinger [1998]
Perfused rat liver	Insulin	⇑ K^+	Vom and Haussinger [1996]
	IGF-1	⇓ Proteolysis	Vom and Haussinger [1996]
	Taurocholic acid	⇓ Proteolysis	Vom and Haussinger [1996]
	Gly, glut, ala	⇓ Proteolysis	Vom and Haussinger [1996]
	Hypo-osmotic	⇓ TAT and PEPCK mRNA	Warskulat et al. [1999]
	Glut, gly, ala, phe, ser, pro	Biphasic K^+ ⇑ then ⇓	Wettstein et al. [1990]
	Ethanol/acetaldehyde	⇓ Proteolysis	Vom and Haussinger [1998]
H4IIE hepatoma cells	Hypo-osmotic	⇓ TAT and PEPCK mRNA	Warskulat et al. [1999]
		⇑ ERK-2, JNK-2, p38 activation	Wiese et al. [1998]
		⇑ c-jun and MKP-1 mRNA	
Mouse liver slices	Hypo-osmotic	⇑ Ca^{2+} uptake	Khalbuss and Wondergem [1991]

exposed various stimuli to perfused livers, primary hepatocytes, and hepatocellular carcinoma cells lines. Hypo-osmotic stress using hypotonic medium or liver perfusate (180–225 mOsm/L) is most commonly used as it induces cell swelling in a characteristic fashion. Studies in rat hepatocytes confirm that the initial movement of water into cells following exposure to hypotonic media occurs by simple diffusion, and is not dependent on water channels as is seen in cholangiocytes [Chen and Ingber, 1999]. When measured by electron microscopy, isolated rat hepatocytes exposed to hypotonic media (225 mOsm/L) for 5 min underwent a volume increase of 25% then a subsequent decrease to a final volume of 16% above control cells. When individual compartments were examined, (1) the cytosolic volume paralleled the overall cell volume, (2) the mitochondria underwent a 30% increase followed by a return to control volumes, and (3) the nuclear volume remained stable [Pfaller et al., 1993]. In addition, cell membrane surface increased nearly two-fold during these volume changes. As discussed earlier, various endogenous and exogenous compounds such as amino acids (glutamine, glycine) [Wettstein et al., 1990], insulin [Vom and Haussinger, 1996], bile acids [Haussinger et al., 1992], and ethanol have also been used to induce cell swelling.

The dynamic response of hepatocytes and cells to hypo-osmotic stress is biphasic, with an immediate cell-swelling phase followed by a RVD.

Immediate cell swelling. Cells exposed to hypo-osmotic medium or various physiologic compounds (e.g., amino acids, insulin, and bile acids) undergo rapid but transient cell volume increase (see Table I). In hypo-osmotic stress, swelling is due to simple osmotic shift of water across the cell membrane from a hypotonic pericellular environment [Yano et al., 1996; Lang et al., 1998b]. However, hepatocyte swelling induced by different stimuli involves different mechanisms. These include immediate K^+ influx and cell volume increase following exposure to various amino acids [Wettstein et al., 1990] (see below).

RVD. Following cell swelling, cells shrink to near baseline cell volume by RVD, a response mediated by various mechanisms which are dependent on the initial swelling stimulus. Many studies implicate the internal anchoring effects of the cytoskeleton (CSK) in both the restriction of cell swelling and subsequent RVD. In rabbit proximal convoluted tubule cells exposed to hypo-osmotic medium, RVD is abrogated by the disruption of microfilaments with cytochalasin B, thus suggesting that intact F-actin may not only contain cell volume but also shrink it [Linshaw et al., 1992]. In perfused rat livers exposed to various amino acids, RVD following initial swelling is mediated by K^+ efflux [Wettstein et al., 1990] (see section Cell membrane stretch, ion channels, and membrane potentials).

Some of the physiologic effects seen in swollen hepatocytes may be secondary to RVD rather

than the initial cell volume increase. The increased bile acid secretion in hypo-osmotically perfused rat livers occurs in a biphasic manner correlating with the initial volume increase followed by RVD. Since the second peak is abolished by both colchicine and $BaCl_2$, RVD-induced bile acid secretion is dependent on both microtubules and K^+ channels [Bruck et al., 1992].

Cell Structure, Tensegrity, and Mechanotransduction

Even before evidence associating hepatocellular volume with altered metabolism and gene expression mounted, a link between the physical changes and function had been sought. *Tensegrity* refers to the intracellular architecture composed of a filamentous network linking organelles, cell membrane, and the nucleus [Ingber et al., 1994]. This scaffolding not only allows the transfer of physical forces to chemical signals, or *mechanotransduction*, but also tethers cells to each other and to the extracellular matrix by membrane attachments [Ingber, 1997]. When physical stress is applied to the cell membrane, as occurs in swelling, tension is applied to these filaments with the following results: (1) the cytoskeletal anchor pulls on the membrane thereby containing expansion, (2) the membrane anchors undergo conformational change when pulled, and (3) CSK-bound molecules interact as the CSK reorganizes [Ingber, 1997]. Often, these changes in anchor shape activate them to initiate second messengers.

Following hepatocyte swelling, striking increases in cell membrane surface area and changes in cytoskeletal organization have been noted [Theodoropoulos et al., 1992b; Pfaller et al., 1993]. These changes contribute to mechanotransduction and utilize the following components: (1) focal adhesion complexes (FACs), (2) CSK, (3) second messengers, and (4) stretch-sensitive ion channels. In vitro studies demonstrate that these physical and structural changes are both temporally and causally linked to the modulation of the hepatocytes physiologic response to swelling. In vivo, however, other mechanisms such as ligand-receptor binding may work in concert with mechanotransduction to effect liver growth. [Ingber, 1997].

FACs. One important mechanism of mechanotransduction involves FACs-clusters of integrin-associated signal transducers that tether the CSK to the cell membrane and that may undergo conformational change when force is applied to the cellular scaffold [Geiger et al., 1995; Kieffer et al., 1995; Schmidt et al., 1995]. More specifically, these complexes are called FACs when involved with cell-extracellular membrane (ECM) binding, and junctional complexes when involved with cell–cell interaction [Geiger et al., 1995; Huang and Ingber, 1999].

The interaction between hepatocytes and their ECM has been associated with altered cell shape and proliferative competence. Primary rat hepatocytes cultured on fibronectin, a known integrin ligand which supports cell-spreading, allowed the transient expression of junB and ras, and progression into S-phase. However, these same cells culture on arg-gly-asp (RGD), a separate integrin ligand which inhibits cell spreading, prevented cells from entering S-phase [Hansen et al., 1994]. Although the authors attribute these proliferative effects to ligand-integrin binding, FAC activation via membrane stretch and cytoskeletal pull in these stretched cells may also be contributing factors.

Microfilaments. Microfilaments are organized F-actin fibers which anchor intracellular components, contribute to cellular turgor by tethering the membrane, and apply stress onto FACs via integrins [Chen and Ingber, 1999]. This integrin–cytoskeletal linkage may be Ca^{2+}-dependent, as studies in mHepR1 hepatocytes treated with Ca^{2+} chelators showed a decrease in these connections [Nebe et al., 1996]. Changes in microfilament organization following hepatocyte swelling have been observed. When isolated rat hepatocytes are exposed to hypo-osmotic media, glutamine or insulin, the G-actin/total actin ratio decreased by 1 min while sustained exposure for 2 h increased actin mRNA levels [Theodoropoulos et al., 1992b]. Because G-actin (monomer) decreases while F-actin (filamentous) increases during actin polymerization, the decreased ratio suggests that hypo-osmotic stress induced the organization of actin microfilaments in swollen cells. In addition, cell swelling further promotes actin polymerization by making more actin available via transcription.

Other evidence linking actin organization to proliferation is found in Buffalo rat hepatocytes transformed with the Ha-ras-1 oncogene. The microfilaments in these transformed cells were

more stable than those found in wild type rats, thus suggesting that F-actin stability is conducive to proliferation [Theodoropoulos et al., 1992a].

Actin reorganization may be responsible for some of the physiologic changes induced by hepatocyte swelling. Hepatocytes exposed to hypo-osmotic stress increase intracellular Ca^{2+} and hyperpolarize their membranes, and this response is inhibited by actin dissociation with cytochalasin B [Khalbuss and Wondergem, 1991]. In addition, the interaction between microfilaments with FACs stimulates various cell proliferation signals [Ben-Ze'ev, 1991]. Recently, rat hepatocytes cultured on type I collagen (to induce spreading) showed decreased cyclin D1 expression upon exposure to cytochalasin D, thus suggesting that cell cycle regulation may require an intact CSK [Hansen and Albrecht, 1999]. Finally, our group has demonstrated that in HepG2 cells, hypoosmotic stress stimulated the activation of Focal Adhesion Kinase (FAK) and PKB with the translocation of AP-1. This signaling cascade was inhibited by cytochalasin D, thus suggesting that actin reorganization following hypoosmotic stress is essential for the FAK-mediated activation of the PI-3-K/PKB/AP-1 proliferative cascade [Kim et al., 2001b]. Collectively, these findings suggest that cell hydration may stimulate hepatocyte growth through actin organization.

Microtubules. Microtubules are organized fibers of tubulin which contribute to tensegrity. The changes in tubulin organization following cell swelling have been implicated in physiologic changes in hepatocytes. Experiment in hepatocytes demonstrate that microtubules may also act as internal struts to counteract the pulling effects of both cell membrane and microfilaments [Mooney et al., 1995].

In isolated rat hepatocytes, swelling induced by hypo-osmotic stress, glutamine, and insulin not only stabilized microtubules but also increased tubulin mRNA levels [Haussinger et al., 1994]. As stated earlier, swelling-induced changes in vesicular pH are linked to microtubular organization [Schreiber et al., 1994], an event thought to contribute to swelling-induced proteolysis [Busch et al., 1994]. In addition, the increased rate of taurocholate excretion seen following hypo-osmotic rat liver perfusion is microtubule-dependent [Bruck et al., 1992].

Cell membrane stretch, ion channels, and membrane potentials. The concept of stretch-induced ion channels was initially derived from observations in non-hepatocyte cells. Although the exact mechanism is unclear, actin-disruption studies suggest that these channels occur at CSK-membrane anchors, possibly involve FACs, and are activated by CSK pull during stress such as cell swelling and shear stress. In medullary thick ascending limb cells, hypo-osmotic stress caused an influx of Ca^{2+} via stretch-sensitive channels with subsequent efflux of K^+ [Taniguchi and Guggino, 1989]. In human epithelial T84 cells, swelling-induced activation of Na^+-K^+-Cl^- cotransporters was inhibited by phalloidin, thus suggesting that actin reorganization following volume increase influences these channels [Farokhzad et al., 1998].

In cultured saphenous vein smooth muscle cells (SMCs), pulsatile stretch increased DNA synthesis by 24 h and cell proliferation by six days [Predel et al., 1992]. Because experiments like this demonstrate that cell stretch causes proliferation, possibly by stretch-mediated ion flux, swelling-induced membrane stretch may also induce a growth response.

Recent evidence demonstrates that hypo-osmotic stress causes membrane stretch which in turn may influence ion channels. Depending on the stimulus used, hepatocellular swelling promotes different patterns of ion movement through these membrane channels, and these ion fluxes have significant effects on intracellular ion concentrations and membrane potentials — changes known to influence metabolism, transcriptional activation, and cell cycle progression.

As stated earlier, isolated hepatocytes exposed to hypotonic media doubled their surface area by electron microscopic measurements [Pfaller et al., 1993]. Although the exact mechanism for this surface area increase is unclear, it may involve a combination of swelling-mediated membrane stretch and exocytosis of vesicular membranes.

Recent evidence suggests that stretch-sensitive channels are important in hepatocellular swelling-mediated ion flux. As stated earlier, studies using mouse hepatocytes demonstrate that hypo-osmotic stress induces microfilament-mediated Ca^{2+}-channel opening and membrane hyperpolarization [Khalbuss and Wondergem, 1991]. Because cytosolic Ca^{2+} is

an important component in PKC activation, the opening of stretch-sensitive Ca^{2+}-channels following hepatocyte swelling may contribute to growth signaling via PKC.

A number of other studies have implicated hepatocyte swelling with changes in ion movement while not investigating the possible involvement of stretch-sensitive channels. In isolated rat liver perfusion, cell swelling induced by physiologic amino acid concentrations resulted in biphasic K^+ movement; 2 min of initial cation uptake followed by its release for 10 min [Wettstein et al., 1990]. In perfused rat livers, insulin-induced swelling was accompanied by activation of both Na^+/H^+ exchange and Na^+-K^+-$2Cl^-$ cotransport with resultant K^+ uptake and cytosolic acidification [Peak et al., 1992]. These findings were corroborated by studies which detected increased Na^+, K^+, and decreased pH of the effluent from rat livers perfused with hypotonic media, indicating the release of these substances from the liver [Lang et al., 1989a]. Recently, studies on human hepatocytes demonstrated that membrane-Cl^- permeability was affected by purinergic signaling via ATP-release when exposed to hypo-osmotic stress [Feranchak et al., 2000].

Intracellular pH Changes

In association with ion fluxes, hepatocyte swelling induced changes in compartmental pH. In primary rat hepatocytes, hypo-osmotic stress lowers cytosolic pH while increasing vesicular pH, and this latter effect is not only reproduced with swelling induced by glutamine, histidine, and Ba^{2+}, but is also microtubule dependent [Schreiber et al., 1994]. This cytosolic acidification is due to RVD-mediated inhibition of Na^+/H^+ exchange with resultant H^+ retention [Gleeson et al., 1990]. In perfused rat livers, insulin-induced swelling also induces pH changes [Peak et al., 1992] (see below).

HEPATOCYTE SWELLING AND PROLIFERATIVE SIGNALING

Cell Swelling Activates Cytoplasmic Signals Involved in Cell Cycle Progression and Proliferation (See Table II)

MAPKs. The mitogen activated protein kinases (MAPKs) represent a family of eukaryotic protein kinases that are involved in various cellular processes including cell cycle progression, apoptosis, and cell survival [Seger and Krebs, 1995; Kutz and Burg, 1998; Mizunuma et al., 1998] Three parallel cascades are now commonly described, each of which is named after its end-moiety: p38, the extracellular signal regulated protein kinases 1 and 2 (ERK 1 and 2 or p42/p44), and the stress activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK). As their name implies, these

TABLE II. Effects of Hypo-Osmotic Stress on Liver Cells Signal Transduction

Cell type	Stimulus	Effect	Reference
Primary rat hepatocytes	Hypo-osmotic	⇑ Actin mRNA	Theodoropoulos et al. [1992]
		⇑ MRP2 mRNA	Kubitz et al. [1999]
		⇑ Bsep mRNA	Warskulat et al. [1999]
		⇑ STAT1 and 3 activation	Meisse et al. [1999]
		⇑ NF-κB activation	Kim et al. [2000]
		⇑ ERK-1 and 2, p38, JNK activation	Kim et al. [2000]
		⇑ PI-3-K and p70S 6 kinase activation	Krause et al. [1996]
	Glu, pro	⇑ PI-3-K and p70S6kinase activation	Krause et al. [1996]
	Insulin	⇑ p70S6kinase activation	Dixon et al. [1999]
Perfused rat liver	Hypo-osmotic	⇑ ERK-1 and 2 activation	Noe et al. [1996]
		⇑ G-protein and tyrosine kinase activation	
		⇑ p38 activation	Haussinger et al. [1999]
	Insulin	⇑ p38 activation	Haussinger et al. [1999]
	Glu/gly	⇑ p38 activation	Haussinger et al. [1999]
	Ethanol	⇑ p38 activation	Haussinger et al. [1999]
H4IIE hepatoma cells	Hypo-osmotic	⇑ ERK-1 and 2 activation	Schliess et al. [1995]
		⇑ c-jun phosphorylation	
		⇑ c-jun mRNA	Finkenzeller et al. [1994]
		⇑ NF-κB activation	Michalke et al. [2000]
		⇑ p38	Wiese et al. [1998]
		⇑ PI-3-K activation	Michalke et al. [2000]
HTC hepatoma cells	Hypo-osmotic	⇑ ATP release and Cl^- efflux	Feranchak et al. [1998]
H35 Cells	Insulin	⇑ c-fos, c-myc, β-actin mRNA	Taub et al. [1987]
Mouse hepatocytes	TNF-α	⇑ c-jun mRNA	Wiese et al. [1998]

proteins are associated with gene transcription and cell proliferation [Hilberg et al., 1993; Westwick et al., 1995].

Aniso-osmotic stress has been shown to activate various MAPKs. Recently, investigators have shown that upon exposure to hypo-osmotic stress, primary rat hepatocytes activated ERK-1 and 2, JNK, and p38 within minutes [Kim et al., 2000]. In perfused rat livers, hypo-osmotic stress-induced taurocholate excretion is dependent on ERK-1 and 2 activation [Noe et al., 1996]. In H4IIE hepatoma cells, hypo-osmotic stress activates p38, JNK, and ERK-1 and 2; the latter are activated in a G-protein-dependent, but PKC and calcium independent fashion [Schliess et al., 1995; Kane et al., 1999]. The antiproteolytic effects of hepatocyte swelling induced by hypotonic media, insulin, glutamine/glycine, and ethanol are dependent on the activation of p38 [Haussinger et al., 1999].

Phosphatidylinositol-3-OH-kinase. Phosphatidylinositol-3-OH-kinase (PI-3-K) is a cytoplasmic protein involved with cell salvage and proliferation. This heterodimer, consisting of p110 (catalytic) and p85 (regulatory) subunits, is activated by interaction with phosphotyrosine sequences located on receptor or non-receptor proteins. In one cascade, PI-3-K activates protein kinase B (PKB or also known as Akt), which in turn delays apoptosis via BAD. In another cascade, PI-3-K activates target of rapamycin (TOR), which subsequently induces cell proliferation via p70S6kinase or 4E-binding protein 1 (4E-BP1) [Krasilnikov, 2000] PI-3-K has also been implicated in MAPKs and PKC activation.

Various studies have implicated hepatocellular swelling with activation of the PI-3-K cascade. In primary rat hepatocytes, swelling induced by hypo-osmotic stress, glutamine, or proline activated both PI-3-K and p70S6kinase downstream [Krause et al., 1996]. Following insulin exposure, these cells also underwent increased proliferation in a p70S6kinase-dependent fashion, suggesting that hormone-induced swelling may also induce PI-3-K proliferative activity [Dixon et al., 1999]. In H4IIE rat hepatoma cells, swelling-mediated NF-κB activation was abolished by PI-3-K inhibition [Michalke et al., 2000] with LY294002, thus showing PI-3-K involvement in transcriptional activation. In HTC hepatoma cells, hypo-osmotic stress induced ATP release, and subsequent Cl^- efflux was PI-3-K dependent [Feranchak et al., 1998].

Protein kinase C (PKC). PKC is a family of second messenger proteins consisting of three classical and four novel isotypes. PKC is activated by two mechanisms: (1) diacylglycerol and inositol-1,4,5-triphosphate, the phospholipase C-mediated breakdown products of phosphatidylinositol and (2) phosphorylation at three priming sites by PDK1 [Parekh et al., 2000]. Because PDK-1 and other members of the PI-3-K signaling cascade have been implicated in PKC activation, PKC is thought to be involved in cell survival and proliferation either independent of or in concert with PI-3-K [Roman et al., 1998; Parekh et al., 1999; Ziegler et al., 1999].

PKC is involved in cellular processes that are commonly seen in mechanotransduction such as cytoskeletal change and ion flux. In human T84 intestinal epithelial cells, cytochalasin D potentiated whereas phalloidin inhibited PMA-induced basolateral endocytosis, thus implicating actin organization in PKC-mediated membrane activity [Song et al., 1999]. Additional experiments in these cells linked PKC to Na^+-K^+-Cl^- cotransporter activation [Farokhzad et al., 1998].

In rat hepatocytes, PKC is important in the Ca^{2+}-mediated redistribution of F-actin from a pericanalicular region to the cell body, thus demonstrating the interaction of this important second messenger with the CSK [Roma et al., 1998]. This interaction may explain the findings H4IIE rat hepatoma cells exposed to hypo-osmotic stress, in which swelling-mediated NF-κB activation was abolished by an PKC inhibition with Go6850 and was prolonged with PMA stimulation [Michalke et al., 2000]. Collectively, these two studies suggest that in the liver, swelling mediates both PKC and transcription factor activation via mechanotransduction.

Cell Swelling Activates Transcription Factors Involved in Cell Cycle Progression and Proliferation

Transcription factors are proteins that communicate cytoplasmic signals to the nucleus to initiate gene expression. These factors, which are constitutively present in an inactive form, undergo postranslational modification in order to translocate into the nucleus. As their names imply, these factors then bind promoter regions

on target genes to initiate transcription [Taub, 1996].

NF–κB. NF-κB is a transcription factor important in the initiation of hepatocyte growth [Taub, 1996]. Consistent with its role as a primer in proliferation, NF-κB binds to the κB site of various gene promoter regions within minutes following partial hepatectomy [Tewari et al., 1992; FitzGerald et al., 1995]. Recently, our group has shown that hypo-osmotic cell swelling, either in it of itself or in conjunction with other factors, induces the nuclear translocation and activation of NF-κB in cultured quiescent rat hepatocytes [Kim et al., 2000]. The target genes of NF-κB in hepatocyte growth are not known, but it appears that the early activation of NF-κB is closely related to proliferative competence [FitzGerald et al., 1995] with subsequent transcription of the immediate early genes (IEGs) of hepatocyte cell cycle progression [Taub, 1996]. Recently, investigators have shown that hypo-osmotic stress caused NF-κB activation via IκB-α degradation in H4IIE rat hepatoma cells [Michalke et al., 2000] — a process separate from the concurrent activation of ERK-1/2 and p38 [Schliess et al., 1995; Wiese et al., 1998].

AP-1. AP-1 is a transcription factor complex composed of c-Jun and c-Fos proteins. Upon phosphorylation of a specific residue on the c-Jun subunit by JNK, AP-1 enters the nucleus to bind and initiate various genes involved in cell cycle progression. In regenerating livers following partial hepatectomy, activated JNK phosphorylates AP-1 at its c-Jun activation domain. Rat hepatocyte studies both in vitro and in vivo demonstrate that these events result in transcription of IEGs of growth [Westwick et al., 1995].

Although the cascade of signaling events culminating in AP-1 activation has been associated with cytokine-receptor binding, recent evidence suggests that hepatocyte swelling may be another important initiating event. One study in rats revealed that TNF-α is an important mitogen in liver regeneration as pretreatment with anti-TNF-α antibody prior to partial hepatectomy inhibits both DNA synthesis and JNK activation [Brenner, 1998]. Since TNF-α causes hepatocyte swelling [Feingold and Grunfeld, 1987], part of its mechanism of JNK/AP-1 activation may be through increasing cell volume. In another study, LPS potentiated the HGF-induced activation of JNK and AP-1 with resultant increases in hepatocyte replication (AP-1). As separate studies demonstrate that endotoxin induces hepatocyte volume increase [Qian and Brosnan, 1996], this LPS-mediated activation may also be due to swelling.

Signal transducer and activator of transcription. Signal transducers and activators of transcription (STATs) are a group of six transcription factors thought to initiate gene transcription important for cell proliferation [Taub, 1996]. Upon phosphorylation of specific tyrosine residues by the tyrosine kinase JAK, STATs translocate into the nucleus and bind target genes for subsequent transcription [Wu and Bradshaw, 2000]. Although initially found to be activated by various stimuli such as cytokines and growth factors in various cell types, recent studies indicate that STATs participate in the malignant transformation in many human cancers by rescuing cells from apoptosis [Catlett-Falcone et al., 1999].

When primary rat hepatocytes were exposed to hypo-osmotic medium, both STAT 1 and 3 are activated by 30 min with a peak at 1 h [Meisse et al., 1999]. These observations suggest that hepatocellular swelling is a mitogenic event that promoting proliferative and oncogenic transcription factors.

CCAAT/enhancer-binding proteins. CCAAT-enhancer binding proteins (C/EBPs) are a family of transcription factors (consisting of α, β, and δ isoforms) involved in immediate early gene expression in regenerating hepatocytes following partial hepatectomy and mitogenic stimulation [Diehl, 1998]. Although data in rat and mouse models have been inconsistent, it is believed that C/EBPα is anti-proliferative whereas C/EBPβ and δ are proliferative, and during the pre-replicative period, the ratio of alpha/non-alpha isoforms decreases in the nucleus reflecting differential DNA-binding activity [Greenbaum et al., 1998; Soriano et al., 1998]. Specifically, C/EBP inhibits hepatocyte proliferation by stabilizing p21/WAF, a protein inhibitor of G1 to S cell cycle progression [Hendricks-Taylor and Darlington, 1995]. In contrast, C/EBPβ may be proliferative by both decreasing C/EBPα gene transcription and by increasing proliferative genes [Diehl, 1998].

Although no studies to date show a direct correlation between hepatocellular swelling and altered C/EBP activity, there is indirect evidence to suggest a relationship. In rats

pretreated with anti-TNF-α antibody, decreased nuclear translocation of C/EBPβ as compared to control rats was noted 3 h following partial hepatectomy [Diehl et al., 1995]. This activation of C/EBPβ by TNF-α may in part be mediated by the cytokine-induced swelling [Feingold and Grunfeld, 1987]. In another study, LPS, a known inducer of liver volume increase [Qian and Brosnan, 1996], increased both C/EBPδ mRNA and DNA-binding activity [Rabek et al., 1998]. Similarly, in mouse livers, LPS decreased C/EBPα and increased C/EBPβ activity as seen in proliferating hepatocytes [An et al., 1996]. Finally, in rabbit livers treated with LPS, C/EBP forms heterodimers with the p65 subunit of NF-κB that bind with varying affinity to both C/EBP and NF-κB regions of the serum amyloid A promoter [Ray et al., 1995]. This data demonstrates a functional link between C/EBP and NF-κB, the latter being an important proliferative transcription factor upregulated in hepatocellular swelling.

Cell Swelling Stimulates Transcription of IEGs of Regeneration

The IEGs refer to a group of over 70 genes that are expressed within minutes in the regenerating liver following partial hepatectomy. These genes are able to initiate the cell's proliferative machinery by being activated by transcription factors present constitutively within the cells, thus obviating the need for initial gene products [Taub, 1996]. Their protein products are diverse, ranging from transcription factors, growth factors, and metabolic proteins.

One group of IEGs consist of proto-oncogenes such as c-jun, c-fos, and c-myc: mRNA levels of c-jun and c-fos increase immediately following partial hepatectomy, peak by 30 min, and return to baseline by 2 h. The mRNA levels c-myc peak by 2 to 4 h. The protein products of these IEGs are found early in regenerating cells, and mark G0 toG1 cell cycle progression. Some of these proteins then potentiate further proliferative activity: for instance, c-Jun and c-Fos form AP-1 to act as a potent transcription factor. Other IEGs include insulin-like growth factor binding protein, liver regeneration factor 1 (LRF-1), regenerating liver inhibitory factor 1 (RL/IF-1), C/EBP, and partial hepatectomy factor (PHF/NF-κB).

Various studies suggest that swelling via different stimuli may initiate IEG transcription in hepatocytes. In H35 cells, insulin induced c-fos, c-myc, and β-actin mRNA levels [Taub et al., 1987], suggesting that insulin-induced swelling may contribute to this transcription [Vom and Haussinger, 1996]. Rat livers exposed to LPS may have increased C/EBPδ mRNA via LPS-mediated cell swelling [Rabek et al., 1998]. When mice were pre-treated with anti-TNF-α antibody before CCl4-induced liver damage, c-jun, c-fos, and proliferating cell nuclear antigen staining were significantly decreased [Bruccoleri et al., 1997]; this inhibition may be partially due to the prevention of TNF-α-induced cell swelling [Feingold and Grunfeld, 1987]. In H4IIE hepatoma cells, hypo-osmotic stress causes a five-fold increase in c-jun and MKP-1 mRNA levels [Finkenzeller et al., 1994; Wiese et al., 1998]- events which are preceded by the activation of various MAPKs.

Cell Swelling Acts Mitogenically to Increase Proliferation

Although there are few well-controlled studies designed to show a direct relationship between swelling and increased proliferation in hepatocytes, studies in other cell lines suggest that physical perturbation may induce cell cycle progression and growth.

One study examined the proliferative competence of ras+ NIH 3T3 fibroblasts — cells known to undergo serum-independent proliferation with increased cell volume and cytoskeletal reorganization. Upon exposure to the calcium channel blockers bepridil and nifedipine, there was a decrease in the normally upregulated cell volumes and proliferation [Dartsch et al., 1995], suggesting a link between swelling and growth. These changes were accompanied by inhibition of microfilaments, further suggesting that cytoskeletal-mediated osmosensing is required for proliferative signaling. Pulsatile stretch has also been shown to increase proliferation in cultured saphenous vein SMCs (see membrane stretch), again delineating the importance of physical perturbation on growth. For mouse embryos cells, those cells cultured in low NaCl (85 mM) induced more embryos entering the 8-cell stage and a 2.4-fold increase in protein synthesis versus cells cultured in high NaCl (125 mM) [Anbari and Schultz, 1993]. Although the authors state that these changes are due to the more physiologic intracellular sodium levels created by the low NaCl media, another factor

may be that this hypo-osmotic medium may induce greater cell volume. In fetal spleen cells, exposure to mitogens resulted in both an increase in cell volume and increased DNA synthesis, again linking cell swelling with proliferation [Settmacher et al., 1993]. In human T cells, treatment with anti-TcR antibodies WT31 and Anti-CD3 for 23 h resulted in membrane depolarization, increased cell volume, and DNA synthesis [Gupta et al., 1991]. These data suggest that TcR-mediated growth stimulation involves cell volume increase. In culture dog coronary artery SMCs, fetal calf serum induced volume increases between Days 1 to 3 with subsequent increased cell numbers by Day 6 as compared to controls [Feltes et al., 1993].

More direct studies implicating hepatocyte volume increase with proliferative competence have been performed. Both in vitro and in vivo studies of rat hepatocytes have examined system A, the sodium-dependent neutral amino acid transporter that is activated following partial hepatectomy. When system A was inhibited 60 min prior to partial hepatectomy, DNA synthesis was decreased 45% and liver mass was reduced by 46% at 24 h [Freeman et al., 1999]. Similarly, cultured hepatocytes with inhibited system A showed a 56% decrease in DNA synthesis with decreased cell volume. These data demonstrate that cell volume increase is an important factor in hepatocyte proliferative competence. Finally, recent studies suggests that upon exposure to hypo-osmotic stress for 10 min (200 mOsm/L), HepG2 human hepatoma cells show increased proliferation by 48 h and increased DNA synthesis within 12 h [Kim et al., 2001b]. These changes may represent the phenotypic result of physical and chemical changes that have been described.

CONCLUSION

Cells are susceptible to countless environmental factors, and no other organ is exposed to a wider range of endogenous and exogenous insults as the liver. Many of these factors have been shown to increase hepatocellular hydration with resultant swelling. Because recent studies have demonstrated that cell swelling is a potent metabolic signal regulating gene expression, and because these same signals have been implicated in hepatocyte regeneration, it is likely that hepatocyte swelling is an important component of growth and repair.

From initial cell volume increase to proliferation, a number of cytoskeletal, cell membrane, cytoplasmic, and nuclear events work in concert to run the machinery of cell growth. By defining further the relationship between these signaling events, liver growth may be exploited to help patients with marginal liver function.

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