Induction of LRF-1/ATF3 by Vasopressin in Hepatocytes: Role of MAP Kinases

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Key Words

LRF-1/ATF3 • Hepatocytes • G protein-coupled receptors • Vasopressin • Ca²⁺ • Mitogen-activated protein kinases

Abstract

Background/Aims: Liver regeneration factor 1 (LRF-1/ATF3) is an early response gene which is rapidly induced upon partial hepatectomy in rats, and by growth factors and G protein-coupled receptor (GPCR) agonists in cultured rat hepatocytes. The aim of the present study was to examine the mechanisms involved in induction of LRF-1/ATF3 by the GPCR agonist vasopressin. **Methods:** Primary cultures of rat hepatocytes were treated with vasopressin, TPA, and the Ca²⁺-elevating agents thapsigargin and A23187. LRF-1/ATF3 mRNA and protein were measured by Northern blot analysis or RT-PCR and immunoblotting. Signalling pathways were examined by immunoblots and kinase assays. **Results:** While elevation of intracellular calcium induced LRF-1/ATF3 expression, treatment with TPA did not. Inhibition of phospholipase C, protein kinase C, or pretreatment with calcium chelators did not affect vasopressin-induced expression of LRF-1/ATF3. Inhibition of each of the MAP kinases ERK1/2, JNK or p38 did not affect

vasopressin-induced LRF-1/ATF3 expression. Combined inhibition of JNK and p38, and of ERK1/2 and either JNK or p38 suppressed vasopressin-induced expression of LRF-1/ATF3. **Conclusion:** Vasopressin induces LRF-1/ATF3 expression by mechanisms that differ from those activated by Ca²⁺-elevating agents. The results suggest that partly redundant, complex MAP kinase networks are involved in induction of LRF-1/ATF3 by vasopressin in hepatocytes.

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Introduction

Liver regeneration factor 1 (LRF-1/ATF3) is an early response gene that is rapidly induced upon partial hepatectomy in rats [1]. It is also rapidly induced in cultured hepatocytes stimulated with growth factors [2] or agonists acting on G protein-coupled receptors (GPCR) [3]. LRF-1, the rat homolog of activating transcription factor 3 (ATF3), belongs to the basic leucine zipper (bZip) family of transcription factors, and may repress or activate gene transcription through formation of homodimers or heterodimers with other members of the bZip family [1, 4, 5]. Although a large number of studies have shown

that LRF-1/ATF3 is a stress-inducible gene (reviewed in [5]), there is also evidence that induction of LRF-1/ATF3 may prevent apoptosis [6-8], stimulate cell proliferation [9, 10], and tumour invasion [11, 12]. The signalling mechanisms involved in induction of LRF-1/ATF3 are incompletely characterized, however, the Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 MAP kinase pathways have been implicated in stress-induced induction [5, 13]. The promoter region of the human ATF3 gene contains binding sites for a number of transcription factors, including activator protein-1 (AP-1), ATF/CRE, NF- κ B, E2F, Myc/Max, Egr-1, C/EBP, and Smad proteins [14-17]. Several of these transcription factors are regulated by mitogen-activated protein (MAP) kinases. Stimulation of JNK leads to phosphorylation of ATF2 and c-Jun, enhancing their transcriptional activity and stabilizing them against breakdown [18-20]. Stimulation of p38 and extracellular signal-regulated kinase (ERK) MAP kinases may also lead to phosphorylation of ATF2 [21-23], as well as cAMP response element binding protein (CREB) [24], and ERK may also phosphorylate c-Jun, at least in JNK deficient cells [23]. In addition, transcription of c-jun and c-fos may be regulated by various MAP kinases [25].

The aim of the present study was to examine the signalling pathways involved in induction of LRF-1/ATF3 by agonists acting on G_q-coupled receptors in rat hepatocytes, using vasopressin as a typical agonist. It was previously shown that vasopressin, as well as angiotensin II, norepinephrine, and prostaglandins E₂ (PGE₂) and F_{2 α} (PGF_{2 α}), stimulate phospholipase C and the MAP kinases ERK1/2 and p38 in the hepatocytes [26-30]. Furthermore, norepinephrine activated JNK in freshly isolated hepatocytes [31]. Activation of phospholipase C results in breakdown of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (InsP₃) and diacylglycerol (DAG), leading to release of calcium from intracellular stores and activation of protein kinase C, respectively [32]. Both calcium and protein kinase C appear to be involved in ERK 1/2 activation by these agonists [28, 33, 34]. The mechanisms leading to JNK and p38 MAP kinase activation by G protein-coupled receptors in hepatocytes have not been extensively characterized, however, JNK activation by norepinephrine appeared to be dependent on protein kinase C [31]. Here we provide evidence that vasopressin induces LRF-1/ATF3 by mechanisms that differ from those activated by the Ca²⁺-elevating agents thapsigargin and A23187. The results suggest that partly redundant, complex MAP kinase networks are involved in induction of LRF-1/ATF3 by vasopressin.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), Williams' medium E, penicillin, and streptomycin were from Gibco (Grand Island, NY). Adenosine 5'-triphosphate, prostaglandin F_{2 α} (tris salt), norepinephrine bitartrate, vasopressin, angiotensin II, timolol maleate, dexamethasone, insulin, collagenase, fatty acid-free albumin, collagen, myelin basic protein, EGTA, GF109203X, and 12-O-tetradecanoylphorbol 13-acetate (TPA) were from Sigma Chemical Co. (St. Louis, MO). Prostaglandin E₂ was from Cayman Chemical Company (Ann Arbor, MI). Thapsigargin, A23187, U73122, U73343, BAPTA-AM, PD98059, and SB203580 were from Calbiochem (San Diego, CA). SP600125 was from BioSource Europe (Nivelles, Belgium). [α -³²P]Deoxycytidine 5'-triphosphate (3000 Ci/mmol), [γ -³²P] adenosine 5'-triphosphate (3000 Ci/mmol) and D-myo[³H]-inositol 1,4,5-trisphosphate (21 Ci/mmol) were from Amersham Pharmacia Biotech (Amersham, Buckinghamshire, UK). Antibodies against JNK and phosphorylated JNK (Thr183/Tyr185) were from Cell Signaling (Beverly, MA), and antibody against ATF3 (C-terminal) was from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of analytical grade.

Isolation and culture of hepatocytes

Male Wistar rats (170-220 g), fed ad libitum, were used. Hepatocytes were isolated by in vitro collagenase perfusion and low speed centrifugations [35, 36], with modifications as previously described [37]. Cell viability, measured as the ability to exclude trypan blue, was 95-97 %. The cells were suspended in culture medium and plated at a density of 20.000/cm² in Costar wells or flasks. The culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium and Williams' medium E; it was bicarbonate buffered and supplemented with penicillin (67 μ g/ml), streptomycin (100 μ g/ml), insulin (100 nM), dexamethasone (25 nM), and collagen (3 μ g/ml). Unless otherwise indicated, cells were cultured for 5 hours before stimulation with agonists.

RNA extraction, Northern blot analysis, and RT-qPCR

RNA was isolated by acid guanidium thiocyanate-phenol-chloroform extraction according to the procedure described by Chomczynski and Sacchi [38] and modified by Houge et al. [39]. For Northern blots, ten microgram of total RNA was electrophoresed in 1.5 % agarose gel in the presence of 6.6 % formaldehyde. The RNA was transferred to Biotrans nylon membranes, UV exposed for 1 min and baked for 1 h at 80 °C. The filters were prehybridized overnight in 50 % formamide, 5x Denhardt's solution, 6x SSC, and 1 % SDS at 42 °C, and hybridized overnight in the same solution. The probe (a 1.9 kB EcoRI fragment of rat LRF-1 [1]) was ³²P-dCTP labelled by random priming (Rediprime II DNA Labelling System, Amersham Biosciences, Buckinghamshire, UK). After hybridization the filters were washed 4 times in 2x SSC/0.1 % SDS at 25 °C for 5 min and twice in 0.1 x SSC/0.1 % SDS at 50 °C for 30 min. The hybridized bands were visualized and analyzed by Instant Imager Electronic Autoradiography (Packard Instrument Company, Medridan, CT).

For real time quantitative RT-qPCR, RNA was isolated with the RNEasy Mini Kit from Quiagen (Quiagen Inc., Valencia, CA, USA), according to the manufacturer's instructions. First strand cDNA was synthesized from one μg total RNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Standard curves with RNA ranging from 0.25 to 2.5 μg were made to control for the reverse transcription and PCR quantification. Reactions without SuperScript III were run in parallel to control for contamination with chromosomal DNA. The cDNA was analyzed in triplicate by real time quantitative PCR on an ABI Prism 7700 Sequence detector (Applied Biosystems) with the following cycling parameters: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Raw data were collected and analyzed in Sequence Detector Software (SDS Version 1.7; Applied Biosystems) and threshold quantification cycle (Ctq) calculated from each amplification plot. Standard curves (Ctq value versus log initial RNA concentration) were used to calculate the relative input amount of RNA for each sample based on the Ctq value [40]. Satisfactory and comparable amplification efficiency was verified by the slopes of standard curves. Primers were designed using Primer Express® software v2.1 (ABI Prism, Applied Biosystems), and were validated by the production of single products of expected size on agarose gels, as well as uniformity of melting temperature, which was routinely performed on PCR products. LRF-1/ATF3 was detected with SYBR Green methodology and the following primers: forward 5'-GCG GCG GGA AAG AAA CA-3' and reverse 5'-TCT GCA GGC ACT CTG TCT TCT C-3'. PCR products were controlled using dissociation curve analysis. GAPDH cDNA was quantified using the dual hybridization probe Double Dye oligonucleotide 5' labelled with the fluorescent reporter dye Yakima yellow and quenched with Dark Quencher, 5'-CTC ATG ACC ACA GTC CAT GCC ATC ACT-3' and the following primers: forward 5'-CCA AGG TCA TCC ATG ACA ACT T-3' and reverse 5'-AGG GGC CAT CCA CAG TCT T-3'. Results for LRF-1/ATF3 were normalized to GAPDH, and vehicle-treated controls were assigned a value of 1, unless otherwise indicated. Similar results were obtained with normalization to β -actin (SYBR Green detection, forward primer 5'-TGA CCG AGC GTG GCT ACA-3', reverse primer 5'-GGC AAC ATA GCA CAG CTT CTC TTT A-3').

Immunoblots

Hepatocytes were exposed to agonists for the indicated time periods after 5 h of culturing. Aliquots of 20 μg cell protein (total cell lysate prepared in Laemmli buffer) were electrophoresed on 10 % (w/v) polyacrylamide gels (acrylamide:N'-N'-bis-methylene acrylamide 30:0.8) followed by immunoblotting with specific antibodies. Immunoreactive bands were visualized with ECL Western blotting detection reagents (Amersham International) and quantified with GeneTools (Syngene, Cambridge, UK).

Measurement of ERK activity

The measurement of ERK1/2 activity was performed as previously described [29]. In brief, the cells were exposed to agonists for 5 min before rinsing and scraping the cells into a

Fig. 1. LRF-1/ATF3 mRNA and protein expression after stimulation with agonists activating G protein-coupled receptors in hepatocytes. Hepatocytes were isolated and cultured for 5 hours before stimulation with agonists as described in Materials and Methods. The concentrations used were 1 μM for vasopressin (VP) and angiotensin II (AII), 50 μM for norepinephrine (NE, in the presence of 10 μM timolol to prevent activation of β -adrenoceptors), and 100 μM for prostaglandins E₂ (PGE₂) and F_{2 α} (PGF). Control cells (Ctrl) were added vehicle. **A:** mRNA expression determined by RT-PCR after 1 hour of stimulation. Results are from a typical experiment representative of a number of RT-PCR and Northern blot experiments. It should be noted that PGE₂ and PGF_{2 α} exerted similar effects on LRF-1/ATF3 expression in several experiments. Results are presented as mean $\pm$ SEM of 3 wells. **B:** LRF-1/ATF3 and actin protein expression, determined by immunoblotting after 3 hours of agonist exposure. Results are from a typical experiment which has been repeated 3 times.

10 % ethylene glycol buffer. The lysate was centrifuged (15,800 x g) for 10 min, and the supernatant was mixed with phenyl-Sepharose which was washed twice in a 10 %, twice in a 35 % ethylene glycol buffer, and finally, ERK1/2 was eluted with a 60 % ethylene glycol buffer [41]. The eluate was assayed for ERK1/2 activity with myelin basic protein as substrate, thereafter spotted on P81 paper (Whatman, Maidstone, UK), which was washed, dried, and counted in a liquid scintillation counter.

Inositol 1,4,5-trisphosphate accumulation

After culturing for 5 h, medium was removed and Krebs-Ringer-Hepes buffer pH 7.4, containing 10 mM glucose, was added. Cells were preincubated in buffer at 37 °C for 30 min before stimulation for 1 min with 1 μM vasopressin. The reaction was stopped by removing the buffer and adding 0.4 M perchloric acid. Samples were processed as described previously [42], and the InsP₃ content was determined by a competitive radioligand binding assay [43].

Other methods

Protein was determined with the BCA Protein Assay (Pierce, Rockford, IL).

Results

The responsiveness of hepatocytes to agonists activating G_q-coupled receptors is down-regulated during

Fig. 2. Time course of vasopressin-stimulated LRF-1/ATF3 mRNA and protein expression. Hepatocytes were isolated and cultured for 5 hours before stimulation with 1 μ M vasopressin (VP). A: LRF-1/ATF3 mRNA expression, determined by Northern analysis. Results are presented as mean $\pm$ SEM of 3 experiments. B: Typical Western blot of LRF-1/ATF3 and actin protein expression with or without treatment with vasopressin. C: LRF-1/ATF3 protein expression. Results are presented as mean $\pm$ SEM of 5 experiments.

culturing [44, 45]. We therefore examined the effect of these agonists in hepatocytes that had been cultured for 5 hours. Consistent with previous results [3], the G protein-coupled receptor agonists vasopressin, angiotensin II, norepinephrine (in the presence of timolol to prevent activation of beta-adrenoceptors), and PGE₂ rapidly increased LRF-1/ATF3 mRNA expression in hepatocytes. Typical results are shown in Fig. 1A. Expression of LRF-1/ATF3 protein is shown in Fig. 1B. Vasopressin and angiotensin II were more efficacious than norepinephrine and PGF_{2 α} . In the subsequent studies we used vasopressin as agonist. The time course of vasopressin-stimulated expression of LRF-1/ATF3 mRNA and protein are shown in Fig. 2. Whereas mRNA expression peaked at 1-2 h (Fig. 2A) and then declined, the amount of LRF-1/ATF3 protein was slightly elevated at 1 h and continued to increase for at least 5 h after vasopressin addition (Fig. 2B, C). This is similar to the sustained expression of LRF-1/ATF3 protein observed in regenerating liver [4].

Fig. 3. Effect of phospholipase C inhibitor U73122 on vasopressin-stimulated InsP₃ accumulation and LRF-1/ATF3 expression. Hepatocytes were cultured for 5 hours before stimulation with 1 μ M vasopressin (VP), with DMSO (vehicle), 10 μ M U73122 or 10 μ M U73343 (inactive analog) present for 30 min before stimulation. A: Hepatocytes were stimulated with vasopressin for 1 min. InsP₃ was isolated and analyzed by a radioligand binding assay as described in Materials and Methods. Results are from one representative of 3 experiments, presented as mean $\pm$ SEM of 3 wells. B: LRF-1/ATF3 mRNA expression determined after 1 hour of vasopressin stimulation in the same cell preparation, determined by RT-PCR. Results from one experiment are presented as mean $\pm$ SEM of 3 wells. Similar results were obtained in 4 experiments using Northern analysis.

Fig. 4. Effect of TPA and protein kinase C inhibitor on ERK activity and LRF-1/ATF3 mRNA expression. Hepatocytes were cultured for 5 hours before stimulation with 1 μ M vasopressin (VP) or 1 μ M TPA. When present, GF109203X 3.5 μ M was added 30 min prior to agonists. A: ERK activity was determined after 5 min of stimulation as described in Materials and Methods. Results are presented as mean $\pm$ SEM of 3 experiments. B: LRF-1/ATF3 mRNA expression was determined after 1 hour of stimulation by Northern analysis. Results are presented as mean $\pm$ SEM of 3 experiments.

Role of calcium and protein kinase C in induction of LRF-1/ATF3

Pretreatment of hepatocytes with U73122, an inhibitor of phospholipase C [46], completely suppressed vasopressin-stimulated InsP₃ accumulation, whereas the inactive analogue U73343 did not affect InsP₃ levels (Fig. 3A). There was no apparent inhibitory effect of U73122 or U73343 on vasopressin-stimulated LRF-1/

Fig. 5. Effect of Ca^{2+} -elevating agents on LRF-1/ATF3 mRNA expression. Hepatocytes were cultured for 5 hours before stimulation with 1 μM thapsigargin (Thap, A) or 1 μM A23187 (A23, B). RNA was isolated after one hour of stimulation and LRF-1/ATF3 mRNA expression was determined by Northern analysis. Results are presented as mean $\pm$ SEM of 12 experiments (A) and 8 experiments (B).

ATF3 expression (Fig. 3B), however, due to the stronger U73122-induced elevation of basal than vasopressin-stimulated LRF-1/ATF3 induction, fold change after vasopressin was reduced in the presence of U73122. Because these results were not conclusive regarding the role of phospholipase C in the effect of vasopressin on LRF-1/ATF3 expression, we explored this issue further by examining signalling components downstream of phospholipase C. The phorbol ester TPA, which activates protein kinase C, as well as vasopressin, activated ERK in the hepatocytes [33]. ERK activation induced by vasopressin or TPA was completely blocked by pretreatment with the protein kinase C inhibitor GF109203X (Fig. 4A). However, pretreatment with GF109203X only slightly affected vasopressin-induced LRF-1/ATF3 expression (fold change after vasopressin was reduced from 3.0 to 2.2 after GF109203X) (Fig. 4B). Furthermore, TPA did not significantly induce LRF-1/ATF3 expression (Fig. 4B).

We next examined the effect of agents that elevate intracellular calcium on LRF-1/ATF3 expression. Both thapsigargin, which elevates intracellular calcium by inhibiting the SERCA pump [47], and the calcium ionophore A23187 strongly increased LRF-1/ATF3 mRNA expression (Fig. 5). Pretreatment of hepatocytes with EGTA to chelate extracellular calcium attenuated LRF-1/ATF3 induction by thapsigargin and A23187, but did not appear to inhibit vasopressin-stimulated expression (Fig. 6A). Furthermore, pretreatment with BAPTA to chelate intracellular calcium attenuated LRF-1/ATF3 induction by A23187, but did not affect vasopressin-stimulated expression (Fig. 6B). Thus,

Fig. 6. Effect of calcium chelators on LRF-1/ATF3 expression induced by vasopressin, thapsigargin, and A23187. Hepatocytes were cultured for 5 hours before stimulation with 1 μM vasopressin (VP), thapsigargin (Thap), or A23187 (A23). When present, 5 mM EGTA was added 5 min before agonists, and 40 μM BAPTA was added 30 min prior to agonists. RNA was isolated after one hour of stimulation and LRF-1/ATF3 mRNA expression was determined by Northern analysis. Results are presented as mean $\pm$ SEM of at least 3 experiments.

Fig. 7. Phosphorylation of JNK stimulated by vasopressin and thapsigargin. Hepatocytes were cultured for 5 hours before stimulation with 1 μM vasopressin (VP) or 1 μM thapsigargin (Thap). A: Phosphorylated JNK after 30 and 60 min of stimulation and total JNK determined by Western blotting. Results from one typical of 3 experiments are presented. B: Time course of vasopressin-stimulated JNK phosphorylation. Total JNK was determined to demonstrate equal loading of the gel. Results from one typical of 3 experiments are presented. C: Effect of pretreatment with GF109203X on JNK phosphorylation. Cells were pretreated with 3.5 μM GF109203X for 30 minutes before stimulation with 1 μM vasopressin or 1 μM TPA for 60 minutes. Results are presented as mean $\pm$ SEM of 4 experiments. D: Effect of pre-treatment with 5 mM EGTA for 5 minutes before stimulation with 1 μM vasopressin or 1 μM thapsigargin for 60 minutes. Results are presented as mean $\pm$ SEM of 4 experiments.

Fig. 8. Effect of JNK inhibitor SP600125 on vasopressin- and thapsigargin-stimulated LRF-1/ATF3 expression. Hepatocytes were cultured for 5 hours before stimulation with 1 μ M vasopressin or 1 μ M thapsigargin for 60 min. 20 μ M SP600125 was added 30 min prior to agonists. LRF-1/ATF3 mRNA expression was determined by RT-PCR. Results represent mean $\pm$ SEM of 4 experiments.

whereas elevation of the intracellular level of Ca^{2+} activates pathways leading to LRF-1/ATF3 expression, these results suggest that inhibition of these pathways is not sufficient to inhibit the effect of vasopressin.

Vasopressin and thapsigargin stimulate JNK phosphorylation in the hepatocytes

Because JNK activity has been found to be involved in ATF3 induction by various stress stimuli, we examined if vasopressin could activate JNK in the hepatocytes. Both vasopressin and thapsigargin induced JNK phosphorylation, as shown in Fig. 7A. Phosphorylation of JNK was visible after 5 min of agonist exposure (data not shown), but was stronger at 30 min and 1 hour, and the response was sustained for up to 5 hours after agonist addition (Fig. 7B). TPA slightly increased JNK phosphorylation, and this effect was abolished by pretreatment with the PKC inhibitor GF109203X (Fig. 7C). Pretreatment with GF109203X enhanced basal and vasopressin-induced JNK phosphorylation (Fig. 7C), in agreement with published data from freshly isolated hepatocytes [31]. Pretreatment with EGTA enhanced basal and vasopressin-stimulated JNK phosphorylation, and reduced thapsigargin-stimulated JNK phosphorylation (Fig. 7D).

Role of MAP kinases in induction of LRF-1/ATF3

The above results suggested that vasopressin-induced phosphorylation of JNK as well as vasopressin-induced LRF-1/ATF3 expression were independent of Ca^{2+} and PKC. We therefore examined the role of JNK in induction of LRF-1/ATF3. Pretreatment of hepatocytes with the JNK inhibitor SP600125 [48] did not significantly

Fig. 9. Effect of MAP kinase inhibitors on vasopressin-stimulated LRF-1/ATF3 expression. Hepatocytes were cultured for 5 hours before stimulation with 1 μ M vasopressin for 60 min. LRF-1/ATF3 mRNA expression was determined by RT-PCR. A: 5 μ M SB203580 (SB) with or without 20 μ M SP600125 (SP) was added 60 min prior to vasopressin. Results represent mean $\pm$ SEM of 3 experiments. B: 75 μ M PD98059 alone (PD), or combined with 20 μ M SP600125 (SP) or 5 μ M SB203580 (SB) as indicated, was added 60 min before vasopressin. Immediately before vasopressin addition, medium was replaced with fresh medium containing inhibitors. Results are presented as mean $\pm$ SEM of 3 experiments.

Fig. 10. Pathways involved in vasopressin-induced LRF-1/ATF3 expression in hepatocytes.

affect vasopressin-stimulated LRF-1/ATF3 expression, while the thapsigargin-stimulated expression was significantly attenuated (Fig. 8). Inhibition of MAPK/ERK kinase (MEK)1/2 with PD98059 [49] or inhibition of p38 MAP kinase with SB203580 [50] also did not affect LRF-1/ATF3 expression induced by vasopressin (Fig. 9). It has been reported that both ERK and p38 MAP kinases may substitute for JNK in phosphorylation of ATF2 in cell lines deficient in JNK [22, 23], and we therefore

examined how combined inhibition of JNK and p38 affected LRF-1/ATF3 expression. Combined inhibition of p38 and JNK significantly reduced LRF-1/ATF3 expression stimulated by vasopressin (Fig. 9A). Furthermore, combined inhibition of ERK and either JNK or p38 MAP kinase significantly suppressed LRF-1/ATF3 expression induced by vasopressin (Fig. 9B). Similar results were obtained with another inhibitor of MEK1/2, U0126 (data not shown). The pathways examined for their role in vasopressin-induced LRF-1/ATF3 expression are summarized in Fig. 10.

Discussion

The results of the present study suggest that vasopressin-induced LRF-1/ATF3 expression is mediated by multiple MAP kinases, and no single MAP kinase appears to be sufficient for this induction. Furthermore, in view of the results with inhibitors of the different MAP kinases it is likely that their effects may converge on the same transcription factors and may be partly redundant. In contrast, JNK activation appeared to be more important in the induction of LRF-1/ATF3 induced by elevation of intracellular calcium, since inhibition of JNK alone significantly attenuated thapsigargin-stimulated expression of LRF-1/ATF3. Induction of ATF3 has previously been found to be mediated either by increased stability of the message [8, 14, 51], or by increased transcription [10, 13, 52]. In preliminary experiments, we found no evidence that vasopressin affected mRNA stability of LRF-1/ATF3 (data not shown).

Sustained Ca^{2+} influx and calcium signalling induces immediate/early gene expression via multiple mechanisms, including phosphorylation of transcription factors [53]. While the elevation of intracellular calcium induced by thapsigargin or A23187 resulted in increased LRF-1/ATF3 expression that was suppressed by calcium chelators, chelation of calcium did not prevent vasopressin-stimulated LRF-1/ATF3 expression. This might suggest that the sustained calcium response induced by thapsigargin or A23187, in contrast to the physiological calcium response induced by vasopressin, results in activation of stress-induced pathways. On the other hand, the results may suggest that G_q -coupled receptors activate downstream signals independent of phospholipase C and Ca^{2+} /PKC in the hepatocytes. Calcium is involved in vasopressin-stimulated ERK activation in the hepatocytes [28, 34]. Also, we have previously shown that G_q -coupled receptors activate the calcium-dependent tyrosine kinase

Pyk2 in the hepatocytes [42]. While Pyk2 has been implicated in the activation of JNK [54-57], the present results suggest that vasopressin-stimulated phosphorylation of JNK is independent of both Ca^{2+} and PKC. G_q -coupled receptors have been reported to activate Rho family members and JNK through PLC-independent mechanisms that may include G protein $\beta\gamma$ subunits or $\text{G}_{12}/\text{G}_{13}$ [58]. The role of Ca^{2+} in the mechanisms leading to p38 MAP kinase activation by G-protein-coupled receptors in the hepatocytes has not been examined. While the mechanisms leading to JNK and p38 MAP kinase activation by G-protein-coupled receptors have not been as extensively characterized as the mechanisms leading to ERK activation, they appear to be equally diverse [58].

The promoter region of the human ATF3 gene contains binding sites for a number of transcription factors, including AP-1 [14], also known as TREs (TPA response elements), based on their ability to mediate transcriptional induction in response to the phorbol ester tumour promotor [59, 60]. Furthermore, ATF3 was initially identified as a TPA-inducible early response gene in HeLa cells [61]. However, TPA did not significantly affect LRF-1/ATF3 expression in the hepatocytes. On the contrary, inhibition of PKC, as well as inhibition of PLC, enhanced the basal expression of LRF-1/ATF3. We have not examined the mechanism of this enhancement, but it might possibly be due to an inhibitory effect of PKC on JNK activity that has been observed in various cells, including hepatocytes [31, 56, 62-64]. The lack of effect of TPA may also suggest that LRF-1/ATF3 induction in the hepatocytes is mediated by activation of the ATF/CRE element, which is preferentially activated by Jun and ATF2, rather than the AP-1 element, which is preferentially activated by Jun and Fos [21].

The role of MAP kinases in the induction of LRF-1/ATF3 appears to depend on cell type and stimulus. Whereas ERK 1/2 activation was involved in ATF3 expression induced by insulin in a hepatoma cell line [65], and by serum in fibroblasts [10], TNF α -induced ATF3 expression in endothelial cells was negatively regulated by ERK activity [52]. p38 MAP kinase was involved in ATF3 induction following wounding in cultured keratinocytes [66], and by various stress signals [13, 15]. Furthermore, both ERK and JNK activity contributed to ATF3 induction by GnRH in $\alpha\text{T3-1}$ gonadotrope cells [67].

Phosphorylation and transcriptional activation of c-jun, ATF2, and CREB are mediated by multiple MAP kinases in a cell-specific and stimulus-specific manner [22-24, 68]. Optimal induction of cyclin D1, a downstream

target of ATF2, by pp60^{v-src} required collaboration between ERK, p38, and JNK, involved an ATF/CRE response element and phosphorylation of CREB by p38 and ATF2 by JNK [69]. It is conceivable that the MAP kinase pathways converge on the phosphorylation of jun, ATF2 and CREB in the hepatocytes. In addition to direct phosphorylation of transcription factors, MAP kinases may also regulate the transcription of c-jun and c-fos [25]. The pathways linking G protein-coupled receptor agonists to the c-jun promoter involved the integration of numerous signals transduced by a network of MAP kinases, including JNK, p38 and ERK5 [70]. In this context, it should be noted that the MEK inhibitors PD98059 and U0126 may also inhibit ERK5 [71]. Thus, we can presently not exclude the possibility that ERK5, and not ERK1/2, contributes to induction of ATF3 in the hepatocytes.

Agonists activating G protein-coupled receptors in the hepatocytes exert a comitogenic effect early in G₁ [3, 27]. The mechanisms involved in their comitogenic effect are incompletely understood. Rapid induction of mRNA for LRF1/ATF3, junB, c-Fos as well as c-Myc protein has been reported in cultured hepatocytes stimulated with various growth factors or hormonal agents [2, 3, 72]. A growth stimulatory role of LRF1/ATF3 has been demonstrated in various cell lines [9, 10, 73, 74], and LRF1/ATF3, which is barely detectable in normal rat liver, is dramatically induced upon hepatectomy [1].

Thus, a role for LRF1/ATF3 in the growth-promoting effects of agonists activating G protein-coupled receptors is conceivable. Studies examining the role of LRF1/ATF3 in growth stimulatory pathways in the hepatocytes are currently ongoing in our laboratory.

Conclusions

Although Ca²⁺-induced LRF-1/ATF3 transcription appears to depend on JNK activity in the hepatocytes, the results suggest that Ca²⁺-dependent pathways alone do not mediate vasopressin-induced LRF-1/ATF3 transcription. It is likely that multiple converging signalling mechanisms involving several MAP kinases contribute, in a partly redundant manner, to the induction of LRF-1/ATF3 by vasopressin in hepatocytes.

Acknowledgements

We thank Eva Østby and Siri Johannesen for technical assistance, and Anders Molven for providing the LRF-1/ATF3 probe. This work was supported by the Norwegian Cancer Society, The Research Council of Norway, The Anders Jahre Foundation, and Helse Øst Research Fund.

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