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Activation and Glucagon Regulation of Mitogen-Activated Protein Kinases (MAPK) by Insulin and Epidermal Growth Factor in Cultured Rat and Human Hepatocytes

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Many hepatocellular activities may be proximally regulated by intracellular signalling proteins including mitogen-activated protein kinases (MAPK). In this study, signalling events from epidermal growth factor (EGF) and insulin were examined in primary cultured human and rat hepatocytes. Using Western immunoblots, rat and human hepatocytes were found to produce a rapid tyrosine phosphorylation of the EGF receptor and MAPK following 0·5–1 min exposure to EGF. Phosphorylation of p42 and p44 MAPK was observed following 2·5 min exposure to EGF. Insulin treatment produced phosphorylation of the insulin receptor β subunit; shc phosphorylation was not observed. MAPK phosphorylation corresponded with a shift in molecular weight and an increase in kinase activity. Insulin-dependent activation of MAPK was unequivocally observed only in human hepatocytes, though a slight activation was detected in rat. Co-treatment with insulin and EGF produced phosphorylation and complete electrophoretic shift in molecular weight of MAPK, with an additive or synergistic increase in enzyme activity in rat but not human hepatocytes; human hepatocyte MAPK was maximally stimulated by EGF alone. Glucagon pretreatment blocked phosphorylation, gel mobility shift and kinase activity of MAPK induced by insulin but only partially blocked EGF-induced MAPK activation in human hepatocytes. Glucagon also reduced the activation of MAPK by EGF in rat hepatocytes. Pre-treatments with forskolin or cyclic AMP analogues diminished in the insulin-, EGF- and insulin plus EGF-dependent activation of MAPK in rat hepatocytes without effecting phosphorylation of receptors or MAPK. These results indicate that although EGF and insulin may both signal through the MAPK/ras/raf/MAPK pathway, the response for MAPK differs between these ligands and between species. Further, in both rat and human, glucagon exerts its effects through a cyclic AMP-dependent mechanism at a level in the insulin and EGF signal transduction pathways downstream of MAPK but promixal to MAPK. The partial inhibition of EGF-induced MAPK phosphorylation by glucagon in human hepatocytes provides further evidence for a raf-1-independent pathway for activation of MAPK. © 1998 John Wiley & Sons, Ltd.

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KEY WORDS — hepatocyte; protein kinase; insulin; glucagon; MAPK; EGF; epidermal growth factor; rat; human

INTRODUCTION

Significant gains have been made in the understanding of how ligand–receptor binding can activate and transduce intracellular signals, leading to alterations in gene expression and changes in cellular phenotype. In the liver, as in other tissues, hormonal regulation and cellular signal transduction pathways are crucial for maintaining metabolic and cell replicative balance. Disruption or activation of signalling events is thought to play a role in the hepatic response to injury, such as that promoted by ethanol,[1] oxidants such as H_2O_2[2] and hepatocellular stress such as heat shock.[3]

The liver is a major target for insulin, epidermal growth factor (EGF), and glucagon actions.[4–8] These agents modulate glucose metabolism, activities of key enzymes in intermediary metabolism, rates of protein, RNA and DNA synthesis, transcription of specific genes, cellular growth, differentiation and division in cultured hepatocytes.[9–15]

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Both insulin and EGF are known to initiate their effects through membrane receptors with intrinsic tyrosine kinase activity.[16–19]. Agonist-dependent receptor activation initiates receptor homo- or hetero-dimerization.[20] The insulin receptor is expressed as a dimer[21,22] and autophosphorylates at specific tyrosine residues on the intracellular domain following ligand binding.[19] Transduction of signal from the activated receptor to a number of proximal signalling proteins occurs through Src homology 2-domain (SH2) interactions[23,24] leading to $p21^{ras}$ activation. Through a subsequent cascade, insulin through $p21^{ras}$-GTP activates mitogen-activated protein kinases (MAPK).[25–28] The activation of MAPK by EGF and hepatocyte growth factor (HGF) in cultured rat hepatocytes has also been reported.[29,30] In isolated rat hepatocytes, EGF and insulin stimulate MAPK activity;[31] similar results were observed in liver following insulin injection.[24] Mitogen-activated protein kinases are a family of serine–threonine kinases first observed by Cooper *et al*. and described by Ray and Sturgil.[32,33] Subsequent work has shown that hormone- or growth factor-dependent activation of MAPK correlates with an altered phosphorylation state of various cytosolic enzymes and cell surface, structural, and nuclear proteins in a variety of cell types.[27,28] However, there have been no reported investigations on insulin- and EGF-stimulated MAPK activation in human hepatocytes as compared to rat hepatocytes or cell lines. Further, it has become apparent that insulin activation of MAPK is not involved in the regulation of gluconeogenesis,[34,35] and hence the role of this pathway may be restricted to growth regulation as opposed to regulation of metabolic physiology.[36] Glucagon, an antagonist to insulin that signals through cyclic AMP, affects hormone- and growth factor-dependent ketogenesis,[37] glycogen and glucose metabolism[12] and proliferation.[10,13,14] In addition, cyclic AMP blocks growth factor- and hormone-dependent activation of signal transduction proteins including MAPK in a variety of cell types.[38–42]

In the present study we compared the activation of MAPK in cultured human and rat hepatocytes in response to insulin and EGF, separately and in combination, and the effects of glucagon on insulin and EGF activation of MAPK. Growth factor- and hormone-activated signal transduction pathways were analysed for changes in tyrosyl phosphorylation of proteins using an anti-phosphotyrosine antibody and MAPK mobilities (both isoforms) using anti-MAPK antibodies in conjunction with Western immunoblot techniques. Kinase activity was evaluated using an in-gel activity assay.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids, and gentamicin were purchased from Gibco, Grand Island, NT. Pierce BCA protein assay kit was purchased from Pierce, Rockford, IL, Electrophoresis reagents of Tris–glycine SDS running buffer, Tris–glycine transfer buffer, 2 × Tris–glycine sample buffer and pre-cast Tris–glycine polyacrylamide gels were purchased from NOVEX, San Diego, CA. Immobilon-P® PVDF membrane was from Millipore, Medford, MA. Anti-phosphotyrosine (4G10, monoclonal $IgG2b_k$) and anti-rat MAPK R2 (polyclonal rabbit IgG) antibodies were purchased from Upstate Biotechnology Inc, Lake Placid, NY. Anti-MAPK antibodies (rabbit IgG) were purchased from Transduction Laboratories, Lexington, KY. ECL-reagents, Hyperfilm-ECL®, sheep anti-mouse IgG and horse anti-rabbit IgG horseradish peroxidase-conjugated antibodies were purchased from Amersham Life Sciences, Arlington Heights, IL. Epidermal growth factor, glucagon, insulin, 8-bromo-cyclic AMP, dibutyryl-cyclic AMP, forskolin and all other chemicals were from Sigma Chemical Co, St. Louis, MO.

Hepatocyte Isolation and Treatment

Parenchymal liver cells from 200–250 g male Sprague-Dawley rats were isolated and cultured according to the methods of Seglan[43] as modified by Elliget and Kolaja.[44] Parenchymal cells from human livers were isolated by a modified method of Ulrich *et al*.[45] using 125–150 g encapsulated liver wedges. Human donor tissues for phosphotyrosine Western blots and in-gel kinase activity assays were from a 64-year-old female (HH-24) and a 34-year-old male (HH-29); cells from an additional donor (59-year-old male, HH-27) were also used for in-gel kinase activity assays. All isolations and cultures were conducted using serum-, hormone- and steroid-free DMEM. Medium was exchanged at 4 h post-isolation, and cells were maintained in DMEM at 37°C in a 95 per cent air/5 per cent CO_2

environment for 20 to 24 h before experimental treatments. Hormone and growth factor treatment concentrations were achieved using additions of 1000 × stocks to culture medium. After timed incubations, plates were quickly rinsed three times with a 2 ml of ice-cold Dulbecco's phosphate buffer saline (D-PBS), quick-frozen in liquid nitrogen and stored at −80°C. Lysates were prepared using 125 µl of ice cold lysis buffer (20 mM HEPES, pH 7·3, with 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 0·1 mM molybdic acid, 1 mM dithiothreitol, 10 mM MgCl, 10 mM β-glycerolphosphate, 5 mM sodium *p*-nitrophenyl-phosphate, 1 mM phenylmethyl sulfonyl fluoride, 5 µg ml^{-1} leupeptin, 5 µg ml^{-1} pepstatin A, 0·50 per cent NP-40, 1 mM sodium fluoride) per plate. A post-nuclear supernatant lysate was obtained by centrifugation at 2000 *g* for 5 min at 4°C. Protein concentrations were determined using a Pierce BCA protein assay kit with bovine serum albumin (BSA) as a standard. The remaining supernatant was transferred to a clean tube, mixed with one volume of 2 × sample buffer and placed in a boiling water bath for 5 min prior to electrophoresis.

Western Immunoblotting

Tyrosine phosphorylation levels for cellular proteins were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and Western analysis. Using 10–20 µg of protein per lane, samples were separated on 10 per cent gels and electrophoretically transferred to PVDF membrane.[46] Non-specific binding was blocked using TBS–BSA (Tris-buffered saline; 20 mM Tris–HCl pH 7·4, 0·9 per cent NaCl, 0·1 per cent Tween-20, 0·1 per cent BSA) supplemented to 3 per cent BSA. Membranes were then incubated with specific antibodies (diluted in TBS-BSA) at room temperature and with constant agitation. Immunoblots were washed three times with TBS–BSA and localized specific antibodies were detected using ECL Western blotting reagents and Hyperfilm-ECL® as described elsewhere.[47]

Antibody Stripping

Antibodies were removed from PVDF membranes by incubating in 62·5 mM Tris–HCl (pH 6·7) containing 100 mM β-mercaptoethanol and 2 per cent SDS at 60°C for 30 min with agitation.[48] Membranes were washed extensively in TBS, and non-specific membrane binding was blocked before re-incubating membranes with additional specific antibodies.

In-Gel Kinase Assay

Mitogen-activated protein kinases were assayed for kinase activity essentially as described.[49] Briefly, post-nuclear extracts were subjected to SDS–PAGE in 7·5 per cent gels in which myelin basic protein (600 µg) was included during polymerization. Following electrophoresis, the SDS was extracted and the proteins were denatured with guanidine. The proteins were permitted to refold after removal of the guanidine and phosphorylation-unincorporated ^{32}P, the activity was quantified using a Molecular Dynamics PhosphorImager™.

RESULTS

In rat hepatocytes, autophosphorylation of the EGF receptor occurred 0·5 to 1 min after treatment with 10 ng ml^{-1} EGF and was maximal by 2·5 min (Figure 1a). The insulin receptor (β-subunit) was autophosphorylated after 0·5 min and was maximal

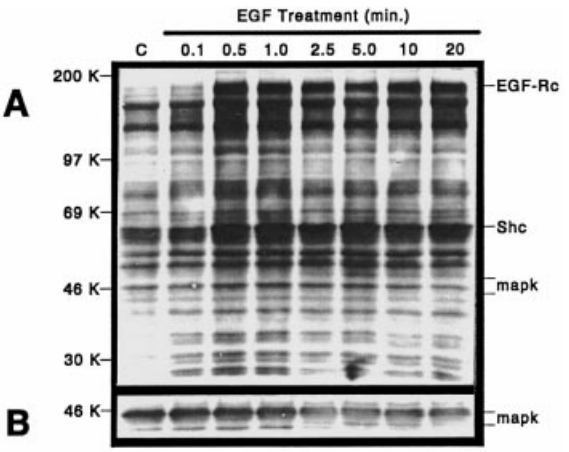

Figure 1. Western immunoblots showing time course of EGF signal transduction in rat hepatocytes. Hepatocytes were incubated for 20 h in serum-, hormone- and steroid-free medium, then treated with 10 ng ml^{-1} EGF for 0·1 to 20 min. Lane C is the no-treatment control. Cell lysates were analysed for tyrosyl phosphorylation using the monoclonal anti-phosphotyrosine (4G10) antibody (panel A), or MAPK protein using the rabbit anti-erk1-CT polyclonal antibody (panel B). EGF receptor (EGF-Rc), shc and MAPK proteins are indicated on right margin, molecular weight markers are indicated at the left margin.

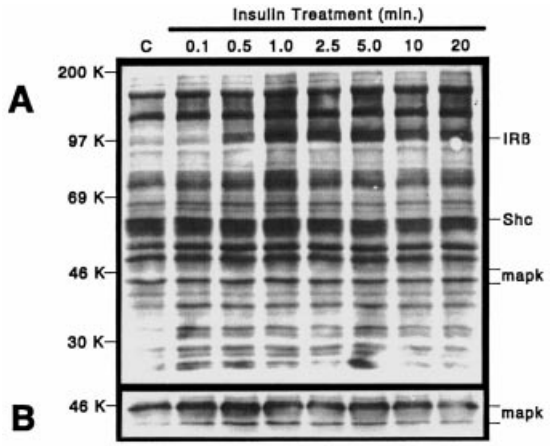

Figure 2. Western immunoblots showing time course of insulin signal transduction in rat hepatocytes. Hepatocytes were incubated for 20 h in serum-, hormone- and steroid-free medium, then treated with 100 nM insulin for 0·1 to 20 min. Lane C is the no-treatment control. Cell lysates were analysed for tyrosyl phosphorylation using the monoclonal anti-phosphotyrosine (4G10) antibody (panel A), or MAPK protein using the rabbit anti-erk1-CT polyclonal antibody (panel B).

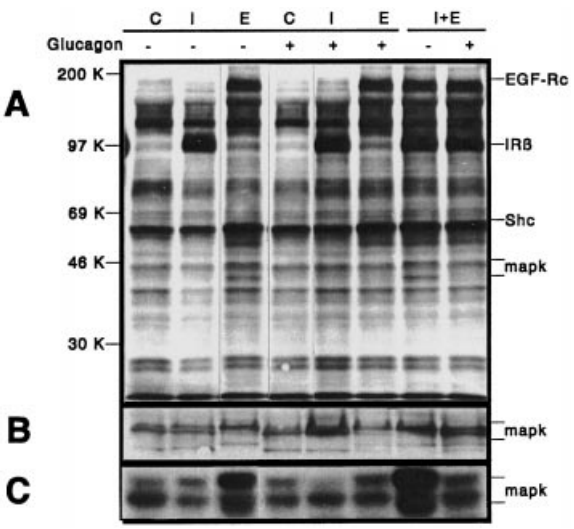

Figure 3. Western immunoblots showing the effects of glucagon pretreatment on insulin, EGF, insulin plus EGF signal transduction in rat hepatocytes. Hepatocytes were incubated for 20 h in serum-, hormone- and steroid-free medium, then pretreated (+) or not pretreated (−) for 2 min with 100 nM glucagon followed by a 5 min treatment with 100 nM insulin and/or 10 ng ml^{-1} EGF. Lanes C are the no treatment controls (+ or − glucagon); I, insulin; E, EGF. Cell lysates were analysed for tyrosyl phosphorylation using a monoclonal anti-phosphotyrosine (4G10) antibody (panel A), or MAPK protein using a polyclonal anti-erk1-CT antibody (panel B). Panel C shows MAPK activity as determined using an in-gel activity assay as described.[49]

at 1 to 2·5 min after 100 nM insulin treatment (Figure 2a). Shc showed initial and maximal phosphorylation at 0·5 to 1 min after EGF addition (Figure 1a); this level of phosphorylation was maintained through 20 min. Phosphorylation of MAPK was not detected in rat hepatocytes treated with insulin (Figure 2a). The phosphorylation of both p44 and p42 MAPK was observed 2·5 min after EGF treatment (Figure 1a). Negligible phosphorylation of MAPK was detected at 5 min after insulin addition (Figure 2b). The EGF-dependent phosphorylation of MAPK isozymes was further supported by Western immunoblot using a polyclonal antibody (anti-erk1-CT) which recognizes both MAPK isozymes (p44 and p42). This assay revealed a decreased gel mobility (due to phosphorylation) of both p44 and p42 isozymes in rat hepatocytes treated with EGF after 1·0 min (Figure 1b); a suggestion of similar changes was observed with insulin treatment at 2·5 min (Figure 2b). To establish a correlation between activation of MAPK (determined by tyrosine phosphorylation and decreased gel mobility), and increased kinase activity, the kinase activity of p42 and p44 MAPK was assayed by the method of Wang and Erikson.[49] By this method, rat hepatocytes treated with EGF for 5 min exhibited phosphorylation, mobility shift and increased kinase activity of p44 and p42 MAPK (Figure 3a–c). A similar insulin treatment (5 min) did not effect the activities of the MAPK isozymes, however, insulin and EGF co-treatment synergistically increased the p44 and p42 MAPK isozyme activity to a level greater than EGF alone (Figure 3c).

To evaluate the consequence of glucagon on insulin and EGF stimulation of MAPK, rat hepatocytes were treated for 2 min with glucagon followed by 5 min with insulin, EGF or insulin plus EGF. Rat hepatocytes pretreated with glucagon showed no change in insulin receptor, EGF receptor, or MAPK phosphorylation in tyrosine phosphate Western immunoblots. However, glucagon diminished the EGF-dependent phosphorylation, gel mobility shift, and in-gel kinase activity of p44 and p42 MAPK (Figure 3a–c). To determine a cyclic AMP involvement in glucagon pretreatment effects on EGS-dependent signalling, hepatocytes were pretreated with the glucagon agonist forskolin or cell permeable cyclic AMP analogues dibutyryl-cyclic AMP and 8-bromo-cyclic AMP. Lysates from hepatocytes pretreated with agents as described in Figure 4 showed changes in phosphorylation, gel

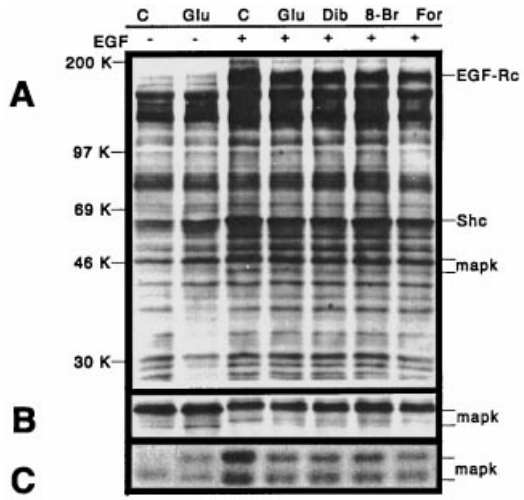

Figure 4. The effects of glucagon agonist and cyclic AMP analogues on EGF signal transduction in rat hepatocytes. Hepatocytes were incubated for 20 h in serum-, hormone- and steroid-free medium, then pretreated with: 100 nM glucagon for 2 min (Glu), 500 μM dibutyryl-cyclic AMP for 30 min (Dib), 1 mM 8-bromo-cyclic AMP for 30 min (8-Br) or 50 μM forskolin for 30 min (For) before 5-min treatments with (+) or without (−) 10 ng ml^{-1} EGF. Lanes C are no-pretreatment controls. Cell lysates were analysed for tyrosyl phosphorylation using the monoclonal anti-phosphotyrosine (4G10) antibody (panel A), or MAPK protein using the rabbit anti-erk1-CT polyclonal antibody (panel B). Panel C shows MAPK activity as determined using an in-gel activity assay as described.[49]

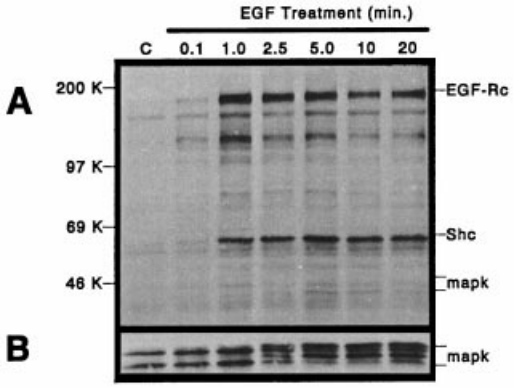

Figure 5. Time course of EGF signal transduction in staged human hepatocytes. Hepatocytes were treated with 10 ng ml^{-1} EGF for 0·1 to 20 min, lane C is the no-treatment control. Cell lysates were prepared as described and analysed as follows. Cell lysates were analysed for tyrosyl phosphorylation using the monoclonal anti-phosphotyrosine (4G10) antibody (panel A), or MAPK protein using the rabbit anti-erk1-CT polyclonal antibody (panel B).

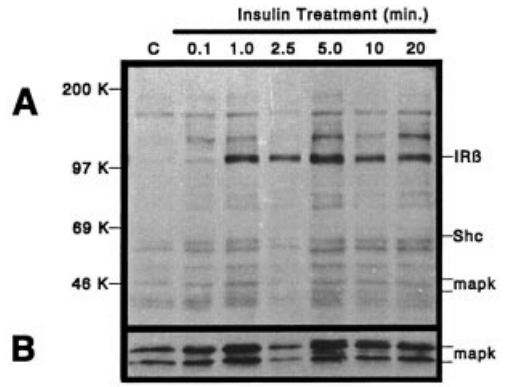

Figure 6. Time course of insulin signal transduction in staged human hepatocytes. Hepatocytes were incubated for 20 h in serum-, hormone- and steroid-free medium, then treated with 100 nM insulin for 0·1 to 20 min. Lane C is the no-treatment control. Cell lysates were analysed for tyrosyl phosphorylation using the monoclonal anti-phosphotyrosine (4G10) antibody (panel A), or MAPK protein using the rabbit anti-erk1-CT polyclonal antibody (panel B). (The 2·5 min lane was loaded with half the intended protein amount resulting in a reduced overall phosphorylation pattern).

mobility shift and in-gel kinase activity of the p44 and p42 MAPK isozymes comparable to those observed with glucagon pretreatment (Figure 4a–c).

Western immunoblot analysis of the growth factor or hormone-dependent MAPK signal cascade in human hepatocytes treated with 10 ng ml^{-1} EGF showed receptor autophosphorylation by 1 min (Figure 5a). Similar kinetics were observed with 100 nM insulin treatment (Figure 6a). Essentially maximal EGF- and insulin-receptor phosphorylation was observed through 20 min. Consistent with rat hepatocytes, human hepatocytes did not phosphorylate MAPK after insulin treatment (Figure 6a), whereas MAPK phosphorylation occurred by 1 min after EGF treatment (Figure 5a). Phosphorylation of both MAPK isozymes was observed after 5 min of insulin (Figure 6a) or 2·5 min of EGF (Figure 5a) treatment. Phosphorylation of MAPK correlated with a decreased gel mobility of both p44 and p42 isozymes (insulin, Figure 6b; EGF, Figure 5b). Co-treatment of human hepatocytes with EGF and insulin for 5 min produced an increase in MAPK phosphorylation, gel mobility shift, and in-gel kinase activity similar to that observed with EGF alone. Pretreatment with glucagon for 2 min diminished the insulin-, EGF- and EGF plus insulin-dependent activation of MAPK (Figure 7a–c). No changes were observed in the phosphorylation of insulin receptor, EGF receptor, or MAPK with glucagon pretreatment. Lysates from forskolin, dibutyryl-cyclic AMP and 8-bromo-cyclic AMP pretreated hepatocytes showed

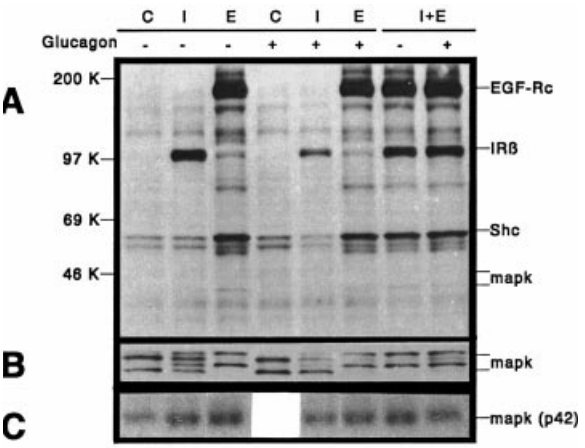

Figure 7. The effects of glucagon pretreatment on insulin, EGF and insulin plus EGF signal transduction in human hepatocytes. Hepatocytes were incubated for 20 h in serum-, hormone- and steroid-free medium, then pretreated (+) or not pretreated (−) for 2 min with 100 nM glucagon followed by a 5-min treatment with 100 nM insulin and/or 10 ng ml^{-1} EGF. Lanes C are the no-treatment controls (+ or − glucagon); I, insulin; E, EGF. Cell lysates were analysed for tyrosyl phosphorylation using the monoclonal anti-phosphotyrosine (4G10) antibody (panel A), or MAPK protein using the rabbit anti-erk1-CT polyclonal antibody (panel B). Panel C shows MAPK activity as determined using an in-gel activity assay as described;[49] no glucagon-pretreated control activity was assayed in this example.

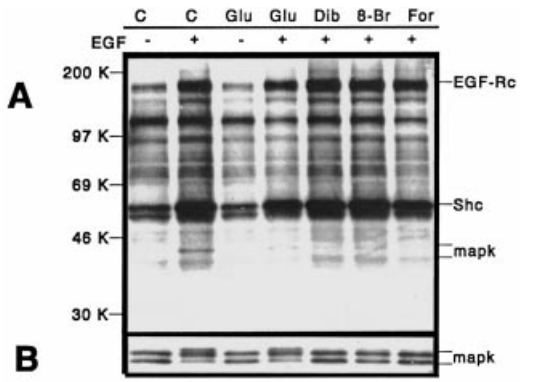

Figure 8. The effects of glucagon agonist and cyclic AMP analogues on EGF signal transduction in human hepatocytes. Hepatocytes were incubated for 20 h in serum-, hormone- and steroid-free medium, then pretreated with: 100 nM glucagon for 2 min (Glu), 500 μM dibutyryl-cyclic AMP for 30 min (Dib), 1 mM 8-bromo-cyclic AMP for 30 min (8-Br) or 50 μM forskolin for 30 min (For) before 5-min treatments with (+) or without (−) 10 ng ml^{-1} EGF. Lanes C are no-pretreatment controls. Cell lysates were analysed for tyrosyl phosphorylation using the monoclonal anti-phosphotyrosine (4G10) antibody (panel A), or MAPK protein using the rabbit anti-erk1-CT polyclonal antibody (panel B).

diminished EGF-dependent MAPK phosphorylation, decreased gel mobility without effecting receptor or MAPK phosphorylation (Figure 8a–b).

DISCUSSION AND CONCLUSIONS

Normal hepatic growth and function are tightly regulated through a balance of hormonal signals. These signals are transduced from hepatocellular surface receptors to the nucleus via a protein signalling network, and result in the adjustment or alteration of gene transcription to meet physiological demands. When injured, the liver has a remarkable capacity to grow and repair, events that are also regulated through signalling networks. The MAPK enzymes are key components of this signalling network, and are known to be activated in rat hepatocytes exposed to various regulators of hepatic growth and function. For insulin, activation of the MAPK cascade does not appear to be required for regulation of gluconeogenesis,[34,35] though it does have an apparent function in early gene induction and mitogenesis.[50,51] We have demonstrated the responsiveness of the hepatocellular MAPK signal transduction pathway to EGF and insulin in cultured human and rat hepatocytes. The responses observed for human hepatocytes were somewhat different from those observed for rat. Insulin stimulated a slight phosphorylation of MAPK in rat hepatocytes compared to the EGF-induced MAPK phosphorylation, and showed additive or synergy effects with EGF stimulation of MAPK. In human hepatocytes, however, insulin provided a distinct but partial phosphorylation of MAPK isozymes, whereas EGF stimulated complete phosphorylation. Glucagon and cyclic AMP-dependent inhibition of EGF- and insulin-dependent activation of the MAPK signal transduction pathway were demonstrated.

Treatment of rat or human hepatocytes with EGF initiated a rapid increase in autophosphorylation of the 170 kDa receptor. Epidermal growth factor receptor autophosphorylation is known to occur at multiple carboxy-terminal sites, however phosphorylation at tyrosine 1173 within the MAPK binding motif is thought to signal receptor–MAPK interaction. The interaction occurs at the SH2 (Src homology 2) domain and is thought to transduce signal through a number of intermediate proteins to activate $p21^{ras}$.[52,53] The kinetics of MAPK phosphorylation we observed in EGF-stimulated human and rat hepatocytes was consistent with previous reports for rat, [24,52] multiple

isoforms were observed as reported by others.[24,54] Guglielmo *et al.*[24] have also demonstrated that autophosphorylation of the hepatic insulin receptor β-subunit occurs in insulin-treated rats. In our studies, treatment of human and rat hepatocytes with insulin caused a rapid tyrosine autophosphorylation of the 95 kDa insulin receptor β-subunit without detectable phosphorylation of MAPK. By contrast, MAPK phosphorylation has been shown to be involved in signalling for both EGF and insulin in fibroblasts.[51]

Peak *et al.*[31] demonstrated that EGF and insulin increased MAPK enzyme activity in cultured rat hepatocytes. Results from the present studies show that treatment of human or rat hepatocytes with EGF initiated a time-dependent phosphorylation of both MAPK isoforms which was initially observed at 2·5 min and continued through 20 min. By contrast, in rat hepatocytes insulin only slightly increased the phosphorylation of MAPK at 5 min and in human hepatocytes phosphorylation occurred 5 to 10 min after addition and declined to 50 per cent by 20 min. The changes in phosphorylation and gel mobility shift of MAPK correlated with increased enzyme activity in rat and human hepatocytes as determined by in-gel assays. However, in human hepatocytes only the p42 MAPK kinase exhibited activity in the in-gel assay. This is consistent with previous observations using human muscle cells lysates, where in-gel kinase activity was only observed for the p42 MAPK isoform following insulin treatment.[55] Co-treatment with insulin and EGF produced a further increase of kinase activity in rat hepatocytes, possibly reflecting an additive or synergistic effect on MAPK by activation through separate signalling pathways. However, the same co-treatment of human hepatocytes did not increase the activity of p42 MAPK kinase above that observed with EGF alone. One possible explanation for this discrepancy is that p44 isoform of MAPK exhibited the greater effect to insulin or EGF activation. The kinase activity increases of p44 were also greater than that of p42 after EGF treatment of rat hepatocytes. In addition, it has recently been demonstrated that EGF and insulin activate p44 and p42 MAPK isoforms to different degrees, with the overall increases in activity of both MAPK isoforms being much lower from insulin treatments.[31] It is also possible that in human cells, EGF alone provides maximal stimulation of MAPK suggesting differences in upstream regulation.

In various cell types the inhibition of insulin- or EGF-activation of MAPK signalling pathway by cyclic AMP has been demonstrated.[38–42,56,57] We evaluated the consequence of glucagon on insulin and EGF stimulation of MAPK in rat and human hepatocytes. In agreement with data from cell lines,[41,42] glucagon pretreatment did not effect insulin- or EGF-dependent receptor autophosphorylation or EGF-dependent MAPK phosphorylation in human or rat hepatocytes. However, glucagon pretreatment decreased the insulin-dependent, and partially decreased the EGF-dependent phosphorylation, mobility shift and in-gel kinase activity of p44 and p42 MAPK in rat and human hepatocytes. Pretreatment with forskolin, 8-bromo-cyclic AMP or dibutyryl-cyclic AMP inhibited EGF-stimulated MAPK activity without effecting the phosphorylation pattern of the EGF receptor or MAPK. These effects were comparable to the effects of glucagon pretreatment on EGF-dependent activation of MAPK, and are consistent with reported effects of cyclic AMP on MAPK activation in rat hepatocytes.[57] Though cyclic AMP is known to exert its effects through protein kinase A at the level of raf,[38–42,59,60] the incomplete inhibition of EGF-stimulated MAPK activation by glucagon and cyclic AMP provides further evidence for distinct ras/raf dependent and independent MAPK activation pathways.[57,58]

In conclusion, our data suggest insulin to be only a weak activator of MAPK, with complete activation perhaps requiring additional signals. Insulin-dependent activation of MAPK, characterized by phosphorylation and a partial shift in molecular weight, was unequivocally observed only in human hepatocytes, though a slight activation was detected in rat. We did not detect MAPK phosphorylation in response to insulin, though it was readily detected in response to EGF in both rat and human cells. This raises the possibility that insulin signalling through MAPK phosphorylation only occurs at a low (perhaps difficult to detect) level, stimulating a subsequently partial or low level of MAPK activation through the ras/raf pathway and providing a modest mitogenic or growth maintenance response. The role of insulin signalling in maintaining metabolic physiology may be directed through the IRS proteins,[36] while MAPK signalling may contribute to insulin's role in liver regeneration.[10,61] The hepatic mitogenic response to EGS, important for liver regeneration[62,63] is indeed more robust, which coincides with phosphorylation of MAPK and complete activation of MAPK as observed in

this study. The reasons behind or implications of the differences in response, in terms of MAPK phosphorylation, between rat and human hepatocytes is not currently known.

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