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Differential Regulation of the Mitogen-Activated Protein and Stress-Activated Protein Kinase Cascades by Adrenergic Agonists in Quiescent and Regenerating Adult Rat Hepatocytes

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To study the mechanisms by which catecholamines regulate hepatocyte proliferation after partial hepatectomy (PHX), hepatocytes were isolated from adult male rats 24 h after sham operation or two-thirds PHX and treated with catecholamines and other agonists. In freshly isolated sham cells, p42 mitogen-activated protein (MAP) kinase activity was stimulated by the α_1-adrenergic agonist phenylephrine (PHE). Activation of p42 MAP kinase by growth factors was blunted by pretreatment of sham hepatocytes with glucagon but not by that with the β_2-adrenergic agonist isoproterenol (ISO). In PHX cells, the ability of PHE to activate p42 MAP kinase was dramatically reduced, whereas ISO became competent to inhibit p42 MAP kinase activation. PHE treatment of sham but not PHX and ISO treatment of PHX but not sham hepatocytes also activated the stress-activated protein (SAP) kinases p46/54 SAP kinase and p38 SAP kinase. These data demonstrate that an α_1- to β_2-adrenergic receptor switch occurs upon PHX and results in an increase in SAP kinase versus MAP kinase signaling by catecholamines. In primary cultures of hepatocytes, ISO treatment of PHX but not sham cells inhibited [^{3}H]thymidine incorporation. In contrast, PHE treatment of sham but not PHX cells stimulated [^{3}H]thymidine incorporation, which was reduced by ~25 and ~95% with specific inhibitors of p42 MAP kinase and p38 SAP kinase function, respectively. Inhibition of the p38 SAP kinase also dramatically reduced basal [^{3}H]thymidine incorporation. These data suggest that p38 SAP kinase plays a permissive role in liver regeneration. Alterations in the abilities of catecholamines to modulate the activities of protein kinase A and the MAP and SAP kinase pathways may represent one physiological mechanism by which these agonists can regulate hepatocyte proliferation after PHX.

Partial hepatectomy (PHX) or acute dissociation and primary culturing of hepatocytes trigger their entry into the cell cycle, which is accompanied by alterations in the levels of expression of various liver-specific proteins (14, 34). Epidermal growth factor (EGF), hepatocyte growth factor, and insulin have been shown to induce DNA synthesis in cultured hepatocytes or in quiescent liver (14, 29, 34). Catecholamines have been shown to increase DNA synthesis in quiescent liver via stimulation of α_1-adrenergic receptors (ARs) (6, 14, 18, 24, 27, 34, 44, 51). The role(s) of β_2-ARs in the control of hepatic DNA synthesis is less clear, as both stimulatory and inhibitory effects have been described elsewhere (13, 15, 35). Previous studies have demonstrated that PHX or primary culture of rat hepatocytes significantly increases β_2-AR, and decreases α_1-AR, expression and function (24, 35, 38). However, the capacity of this receptor switch to modulate many of the recently discovered signal transduction pathways, such as the Raf/mitogen-activated protein (MAP) kinase cascade, and its role in the regulation of hepatocyte regeneration are unclear.

Raf-1 is a member of a family of serine/threonine protein kinases termed Raf-1, B-Raf, and A-Raf (41). Proto-oncogenes of the Raf family have been implicated in hepatic carcinogenesis (2, 11, 17, 34, 40, 41). Raf family members function in a signaling cascade that extends from the plasma membrane, via tyrosine kinase (16) and serpentine (22, 36) receptors, to the low-molecular-weight GTP-binding protein Ras. GTP-Ras binds and translocates Raf-1 to the plasma membrane, which leads to the activation of Raf-1 by multiple mechanisms (12, 16, 30, 36). Raf family members in turn activate MEK1/2, which in turn activates the p42/44 MAP kinases, whose activation leads to modulation of downstream transcription factor activities (30, 46). The MAP kinase pathway can be negatively regulated by agonists that elevate cyclic AMP and activate protein kinase A (PK-A), such as glucagon and β_2-AR agonists (8, 28, 32, 45). Activation of PK-A has been shown to inhibit or delay the activation of Raf-1 in response to growth factors by inhibition of the ability of Raf-1 to associate with GTP-Ras in the plasma membrane (28, 32, 45). Elevation of cyclic AMP and activation of PK-A have also been shown to reduce or delay mitogenesis in established fibroblast cell lines (32, 39, 45, 51). However, the precise role of activation-inactivation of p42/44 MAP kinases in the control of hepatocyte proliferation remains unclear.

The p46/54 stress-activated protein (SAP) kinase (also termed JNK1/2 and c-Jun NH_2-terminal kinase) and p38 SAP kinase (also termed p38-RK) cascades were initially discovered in yeasts, and both pathways have subsequently been described for mammalian cells (5, 7, 43, 47, 49). Activation of p46/54 SAP kinase and p38 SAP kinase is thought to be sequentially mediated by receptor clustering (37), low-molecular-weight GTP-binding proteins Ras and Cdc42/Rac/Rho, MEKK isoforms, and SAP kinase enzymes which in turn phosphorylate and activate the SAP kinases p46/54 and p38 (4, 5, 10, 19, 24, 33, 42, 43, 47, 49, 50).

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Treatment of cells with UV irradiation or exogenous ceramide causes robust activation of p46/54 SAP kinase, whose primary substrate is the immediate-early transcription factor, c-Jun (33, 37). In some systems, p46/54 SAP kinase has been proposed to transduce, in part, the cytotoxic actions of ceramide in the mediation of apoptosis, as judged by the ability of a dominant negative c-Jun mutant (TAM67) to prevent ceramide-induced apoptosis (19). Osmotic shock has been shown to activate p38 SAP kinase (37, 47). This enzyme was recently demonstrated to phosphorylate the transcription factor ATF-2 and also to be involved in regulating the synthesis of interleukin-6 (IL-6), a cytokine with a permissive role in liver regeneration (4, 10, 42, 50). Although some roles in cellular signaling have been proposed for these protein kinases in several cell types, the roles of both p46/54 SAP kinase and p38 SAP kinase in the regulation of growth, and potentially apoptosis, in hepatocytes are unclear.

In the regenerating liver, the stress of liver damage triggers complex regulatory mechanisms that provide a tightly controlled program of liver cell proliferation followed by growth inhibition as the liver regains its original mass. PHX triggers a major rise in the levels of circulating catecholamines, which exert a strong influence on hepatocyte functions. It would be expected that each of the above signaling pathways would play a number of important roles (14, 34, 37, 47). In order to better understand the cellular mechanisms involved in the early stages of liver regeneration and the roles that catecholamines can play in these processes, we analyzed the activities of the MAP and SAP kinase cascades in normal and regenerating hepatocytes and the modulation of these activities by α_1- and β_2-AR agonists.

MATERIALS AND METHODS

Materials. Male Sprague-Dawley rats (150 to 200 g) had access to food and water ad libitum. Anti-Raf family, p42 MAP kinase, p46/54 SAP kinase, and p38 SAP kinase antibodies (in solution and/or agarose conjugated) were from Santa Cruz Biotechnology (Santa Cruz, Calif.). Hormones and growth factors were purchased from Sigma Chemicals (St. Louis, Mo.). Forskolin, 1,9-dideoxy-forskolin, and dioctyl-cyclic AMP were purchased from Calbiochem. Glutathione *S*-transferase (GST)–c-Jun (amino acids [aa] 1 to 169) was prepared with GST-Sepharose as described elsewhere (25). Radiolabelled [γ-^{32}P]ATP, [^{3}H]thymidine, [^{14}C]glucose-1-phosphate, [^{3}H]prazosin, and ^{125}I-pindolol were from Dupont, NEN. Protein preparations of $(His)_6$-MEK1, kinase-defective K52R p42 MAP kinase, and all other reagents were as described in references 11, 12, 18, 25, and 32). Specific kinase domain inhibitors of classical, novel, and atypical protein kinase C (PK-C) isoforms, bisindolyl-maleimide/GF109203X and chelerythrine (3); the specific regulatory domain inhibitor of classical and novel PK-C isoforms, sphinganine (20); and the specific cyclic AMP protein kinase (PK-A) catalytic subunit inhibitor, U89 (21), were from Calbiochem (1). The specific inhibitors for p38 SAP kinase SB203580 and SKF106978 were kindly provided by Smith-Kline Beecham Pharmaceuticals. Photographic images of Western immunoblots on Fuji RX film were taken with a digital DC40 camera from Eastman Kodak (Rochester, N.Y.), processed on Microsoft PowerPoint, and printed in gray scale with a laser printer on standard bond paper.

PHX and preparation of hepatocytes. PHX was performed under pentobarbital anesthesia and aseptic conditions as described elsewhere (24). The sham operation involved anesthesia and laparotomy, but the liver was left intact. Twenty-four hours postsurgery, rats were reanesthetized and hepatocytes were prepared by collagenase perfusion of the liver as described elsewhere (24). The cells were resuspended to 5 mg (wet weight) per ml in serum-free Krebs-Henseleit buffer containing 10 mM glucose and 1.5% (wt/vol) Difco gelatin and were incubated at 37°C under an atmosphere of 5% CO_2 in O_2 with continuous rotary shaking at 100 cycles per min. After a 30-min preincubation to stabilize basal levels of protein kinase activities, aliquots were taken from the first portion and used immediately for experiments.

Treatments of hepatocytes for protein kinase assays. Hormones were added to microcentrifuge tubes containing hepatocytes (5 mg [wet weight] in 1 ml of buffer; 2 mg of total protein per immunoprecipitate); hormones were added to give final specified concentrations (see text and figure legends), vortexed to ensure complete mixing, and incubated for the specified times at 37°C in a water bath. Cells were pretreated with glucagon or isoproterenol (2 min); forskolin, 1,9-dideoxy-forskolin, and dioctyl-cyclic AMP (10 min); or protein kinase inhibitors (20 min) prior to hormonal addition to the tube containing the hepatocytes. Thirty seconds prior to termination, tubes were removed from the bath and placed in a microcentrifuge, the cells were pelleted and placed on ice, and the supernatant was removed. Cells were homogenized in 1 ml of ice-cold buffer A (25 mM HEPES [pH 7.4 at 4°C], 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mg of soybean trypsin inhibitor per ml, 40 μg of pepstatin A per ml, 40 μg of E64 per ml, 40 μg of aprotinin per ml, 1 μM Microcystin-LR, 0.5 mM sodium orthovanadate, 0.5 mM sodium pyrophosphate, 0.05% [wt/vol] sodium deoxycholate, 1% [vol/vol] Triton X-100, 0.1% [vol/vol] 2-mercaptoethanol), with trituration to lyse the cells. Homogenates were stored on ice (5 min) prior to clarification by centrifugation (14,000 × *g*, 5 min, 4°C).

Immunoprecipitations from hepatocyte homogenates. Protein A-agarose slurry (25-μl bead volume) was washed with 1 ml of phosphate-buffered saline containing 0.1% (vol/vol) Tween 20 and resuspended in 0.1 ml of the same buffer. Antibodies (2 μg or 20 μl) or serum (20 μl) was added to each tube and incubated (3 h, 4°C). Clarified homogenates (0.5 ml; 2 mg of total protein) were mixed with protein A-agarose-conjugated antibody by gentle agitation (2.5 h, 4°C). Protein A-agarose was recovered by centrifugation, the supernatant was discarded, and the immunoprecipitates were washed (10 min) sequentially with each of the following: 0.5 ml of buffer A (twice), phosphate-buffered saline, and buffer B (25 mM HEPES [pH 7.4 at 4°C], 15 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 0.1% [vol/vol] 2-mercaptoethanol). Immunoprecipitations were performed in duplicate.

Assays of Raf-1 activity. Assays for Raf-1 activity were primarily performed with ^{32}P incorporation into $(His)_6$-MEK1 (12). In some assays, to determine that phosphorylation of $(His)_6$-MEK1 by Raf protein kinases in the immunoprecipitate was functional for $(His)_6$-MEK1 activation, coupled assays were performed to measure $(His)_6$-MEK1-stimulated activity versus K52R p42 MAP kinase as described elsewhere (12). No difference was observed between the two assay methods for Raf activity. ^{32}P incorporation in $(His)_6$-MEK1 or K52R p42 MAP kinase protein bands after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was quantified by liquid scintillation spectroscopy. Identical results were obtained in measuring Raf-1 activity with either $(His)_6$-MEK1 or K52R p42 MAP kinase in a coupled assay measuring stimulated $(His)_6$-MEK1 activity (12a).

Assay of p42 MAP kinase activity. Immunoprecipitates were incubated (final volume, 50 μl) with 50 μl of buffer B containing 0.2 mM [γ-^{32}P]ATP (5,000 cpm/pmol), 1 μM Microcystin-LR, and 0.5 mg of myelin basic protein (MBP) per ml, which initiated reactions. After 20 min, 40 μl of the reaction mixtures was spotted onto a square (2 by 2 cm) of P81 paper (Whatman, Maidstone, England) and immediately placed into 180 mM phosphoric acid. Papers were washed four times (10 min each) with phosphoric acid and once with acetone, and ^{32}P incorporation into MBP was quantified as described above. Preimmune control assays were performed to ensure that MBP phosphorylation was dependent upon specific immunoprecipitation of p42 MAP kinase in the assay (11, 12, 45).

Assay of p46/54 SAP kinase activity. Immunoprecipitates were incubated (final volume, 100 μl) with 2 μl (10 μg) of GST–c-Jun (aa 1 to 169), and reactions were initiated with 98 μl of buffer B containing 0.2 mM [γ-^{32}P]ATP (5,000 cpm/pmol) and 1 μM Microcystin-LR. After 30 min, reactions were terminated with sample buffer and prepared for SDS-PAGE (10% polyacrylamide gel) to quantify ^{32}P incorporation into excised, Coomassie blue-stained GST–c-Jun (aa 1 to 169) bands, as described above. Preimmune control assays were performed to ensure that GST–c-Jun (aa 1 to 169) phosphorylation was dependent upon specific immunoprecipitation of p46/54 SAP kinase in the assay.

Assay of p38 SAP kinase. Assays were performed in an identical manner as per those for p42 MAP kinase, with MBP as substrate (11, 12, 45).

Primary culture and assay for DNA synthesis in hepatocytes. Hepatocytes isolated from sham operation or PHX rats were cultured on rat tail collagen (Vitrogen)-coated plastic dishes (20-mm or 12-well plates) in William's E medium containing 1 nM dexamethasone and 100 nM insulin. Protein kinase inhibitors were added at the time of the medium change, and adrenergic drugs were added 30 min afterwards. Cells were then cultured for 48 h, after which time the cells were lysed with 0.5 N NaOH and DNA was precipitated with 12.5% (wt/vol) trichloroacetic acid (final concentration). The acid-precipitable material was transferred to glass fiber filters and washed with 5% (wt/vol) trichloroacetic acid, followed by quantitation of [^{3}H]thymidine incorporation into DNA by liquid scintillation spectrometry (26).

Assay for glycogen phosphorylase activity. Glycogen phosphorylase a activity in freshly isolated hepatocytes was measured by the incorporation of [^{14}C]glucose-1-phosphate into glycogen, as described elsewhere (34a). Exposure of aliquots of cells (4 mg [wet weight] in 1 ml of Krebs' buffer) to vehicle or agonist was for 1 min, which was previously determined to result in peak increases in enzyme activity, expressed as units (nanomoles of [^{14}C]glucose incorporated into glycogen per milligram of cytosolic protein per minute at 31°C).

AR binding assays. The cellular density of α_1- and β_2-ARs was determined in isolated hepatocytes or in liver plasma membranes by the specific binding of a saturating concentration of [^{3}H]prazosin (1 nM) or ^{125}I-pindolol (0.4 nM), respectively, as described elsewhere (24, 34a). When isolated hepatocytes were used, they were subjected to one cycle of freeze-thawing to reduce nonspecific binding, which was determined in the presence of 2 μM phentolamine for α_1-AR or 1 μM propranolol for β_2-AR. Liver plasma membranes were prepared from intact liver tissue by a sucrose density centrifugation technique, as described elsewhere (34a). Equilibrium binding (60 min, 31°C) was determined in triplicate aliquots and was termi-

TABLE 1. Alterations in α_1-AR and β_2-AR antagonist binding to collagenase-isolated hepatocytes correlate with the abilities of α_1-AR and β_2-AR agonists to regulate glycogen phosphorylase[a]

Antagonist	Sham		PHX	
	Phosphorylase	Receptor binding	Phosphorylase	Receptor binding
Control	1.00		1.00	
α1 receptor	14.0 ± 0.50	28.50 ± 3.30	4.5 ± 0.50[b]	11.90 ± 4.50[b]
β2 receptor	2.5 ± 0.50	1.64 ± 0.19	16.0 ± 2.00[b]	4.03 ± 0.90[b]

[a] Phosphorylase a activity was determined in freshly isolated aliquots of sham or PHX cells treated with phenylephrine (10 μM) or isoproterenol (1 μM) as described elsewhere (34a). Baseline phosphorylase a activity (control) in sham and PHX hepatocytes was 12.3 ± 1.1 and 11.3 ± 0.9 U, respectively. Receptor binding was performed as described elsewhere (24). [^{3}H]prazosin (α_1-AR antagonist, 1 nM) and ^{125}I-pindolol (β_2-AR antagonist, 0.4 nM) were bound to freshly isolated aliquots of sham or PHX cells. Data shown are femtomoles of ^{3}H label bound per milligram of membrane protein. Means ± standard errors from three experiments are shown.
[b] Statistically significant differences between corresponding sham and PHX values ($P < 0.05$).

nated by vacuum filtration. Specific binding was expressed as femtomoles per milligram of protein (total cellular or membrane protein, as appropriate).

Northern blotting. Steady-state AR mRNA levels were determined by Northern blotting, with poly(A)$^+$ RNA prepared from sham or PHX livers, and probed with full-length ^{32}P-labelled cDNA probes for the rat α_{1B}-AR and β_2-AR, as described elsewhere (27).

Data analysis. Comparison of the effects of various hormone treatments was performed by one-way analysis of variance and a two-tailed *t* test. Differences with a *P* value of <0.05 were considered statistically significant. All bar graph fold values shown are ± standard deviations (SDs) from the mean.

RESULTS

Increased β_2- and decreased α_1-adrenergic signaling towards glycogen phosphorylase in hepatocytes upon PHX. Previous studies have demonstrated that PHX or primary culture of rat hepatocytes significantly increases β_2-AR, and decreases α_1-AR, expression and function (24, 35, 38). PHX reduced the functional ability of the α_1-adrenergic agonist phenylephrine, and increased the functional ability of the β_2-adrenergic agonist isoproterenol, to stimulate glycogen phosphorylase activity in freshly isolated hepatocytes (Table 1) (18, 24). These changes correlated with a reciprocal increase in β_2-AR and decline in α_1-AR binding site densities in PHX versus sham hepatocytes (Table 1). Similar increases in β_2-AR density (from 3.28 ± 0.73 to 7.09 ± 0.50 fmol/mg of protein; $n = 3$; $P < 0.02$) and decreases in α_1-AR density (from 721.3 ± 92.5 to 111.7 ± 13.9 fmol/mg of protein; $n = 3$; $P < 0.02$) were found when receptor binding was assayed in plasma membranes prepared from intact PHX versus sham livers, respectively. These changes in radiolabelled antagonist binding were also reflected in the expression of each receptor mRNA, as assessed by Northern blotting (Fig. 1). To determine whether functional adrenergic regulation of the MAP kinase cascade is similarly altered by PHX, we assessed the ability of phenylephrine, isoproterenol, EGF, and glucagon to modulate Raf-1 and p42 MAP kinase activities in these cells.

Increased β_2- and decreased α_1-adrenergic signaling towards the Raf/MAP kinase cascade in hepatocytes upon PHX. The concentration dependence of p42 MAP kinase activation by phenylephrine in sham cells was initially examined. Maximal activation of p42 MAP kinase occurred after 10 μM phenylephrine treatment, and activation was blocked by cotreatment with prazosin, a specific α_1-AR antagonist (Fig. 2A). Both p42 MAP kinase and Raf-1 were activated with rapid kinetics by phenylephrine treatment (Fig. 2B). Activation of p42 MAP kinase was temporally delayed compared to that of Raf-1, and both activities returned to basal levels by 30 min (Fig. 2B).

In general agreement with the results described in Table 1 and Fig. 1, phenylephrine-mediated activations of Raf-1 and p42 MAP kinase were reduced after PHX (Fig. 3 and 4) and were dependent upon the function of PK-C (Fig. 5) (3, 23, 48). Surprisingly, the basal activity of p42 MAP kinase was also reduced after PHX from 31,500 ± 1,000 to 10,600 ± 400 cpm ($n = 6$) (~70% reduction) without an apparent reduction in the amount of immunoblottable enzyme (Fig. 6). This also correlated with an observed decrease in the abilities of EGF (Fig. 3 and 4), hepatocyte growth factor, and insulin to stimulate p42 MAP kinase (12a). Previous studies have shown that the expression of several growth factor receptors rapidly decreases after PHX, whereas some others do not. The reasons why these changes occur are unclear (14, 24, 27, 34a).

Isoproterenol treatment of sham cells did not influence basal or growth factor stimulation of Raf-1 or p42 MAP kinase activities (Fig. 3A and 4A). In the same cells, glucagon, a hormone which elevates cyclic AMP levels and activates PK-A in a manner similar to that of isoproterenol, significantly blunted the basal activities for up to 60 min (12a), while also reducing phenylephrine- and EGF-induced activations of both protein kinases (39). In contrast to sham cells, isoproterenol treatment of PHX cells reduced basal activities for up to 60 min (12a) and EGF-stimulated Raf-1 or p42 MAP kinase activities (Fig. 3B and 4B). The inhibition of growth-factor-induced p42 MAP kinase activation by isoproterenol was blocked by cotreatment with the specific β_2-AR antagonist propranolol or by pretreatment of cells with the specific PK- inhibitor U89 (1

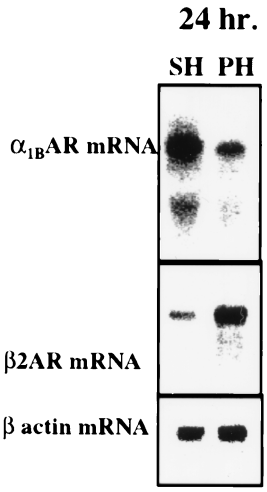

FIG. 1. Northern blot analysis of mRNA isolated from the livers of sham or PHX rats. Ten micrograms of poly(A)$^+$ mRNA isolated from the liver of a 24-h sham or PHX rat was sequentially hybridized with a ^{32}P-labelled full-length cDNA probe to either α_{1b}-AR, β_2-AR, or β-actin, followed by stripping after each cycle of probing. The intensity of the radioactive signal was quantified with a PhosphorImager. For sequential hybridization of the blots, stripping was performed by immersion of the blot in 0.1× Denhardt's solution containing 1 mM Tris HCl (pH 8.0) and 1 mM EDTA (pH 8.0) for 2 h at 75°C.

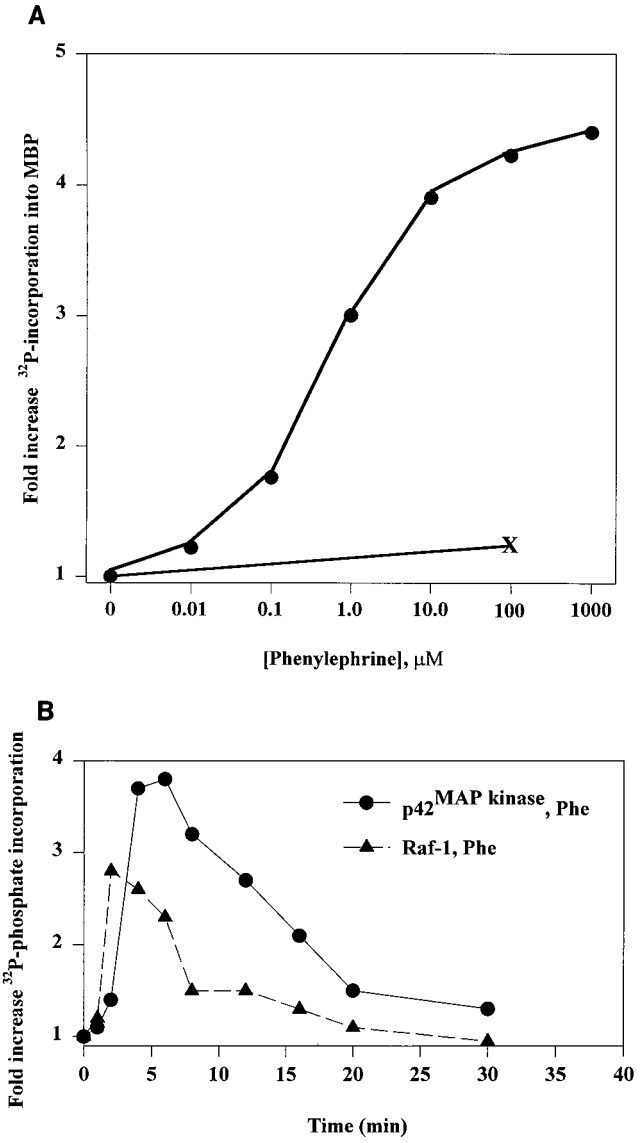

FIG. 2. Activation of Raf-1, p42 MAP kinase, p38 SAP kinase, and p46/54 SAP kinase by phenylephrine in sham freshly isolated hepatocytes. (A) Concentration-dependent activation of p42 MAP kinase by phenylephrine. Cells were exposed to the specified concentrations of phenylephrine for 6 min. X indicates p42 MAP kinase activity in the presence of 10 μM phenylephrine and 10 μM prazosin. Hormone-dependent p42 MAP kinase activation was determined, and values were expressed as fold increase over basal level. Data are means of duplicate values from a representative experiment ($n = 2$). (B) Time course of activation of Raf-1 and p42 MAP kinase. Hepatocytes were treated with phenylephrine (Phe) (10 μM) for the times indicated. Immunoprecipitation and immune complex kinase assays were performed as described in Materials and Methods. Data are means of duplicate values (differing by <10%) from a representative of three independent experiments. ^{32}P incorporation is expressed as a ratio (fold increase) to incorporation for buffer control treatment (Raf-1 with MEK1, 14,500 ± 550 cpm; p42 MAP kinase with MBP, 35,400 ± 1,560 cpm).

μM) (12a). The abilities of glucagon and isoproterenol treatments to reduce growth-factor-induced p42 MAP kinase activation were mimicked by cotreatment of hepatocytes with either (i) an activator of adenylate-cyclase, forskolin (50 μM), or (ii) a cell-permeable analog of cyclic AMP which activates PK-A in vivo, dioctanoyl-cyclic AMP (1 mM) (12a). Thus both glucagon and isoproterenol inhibit activation of p42 MAP kinase via a cyclic AMP–PK-A-dependent mechanism (39, 45, 46).

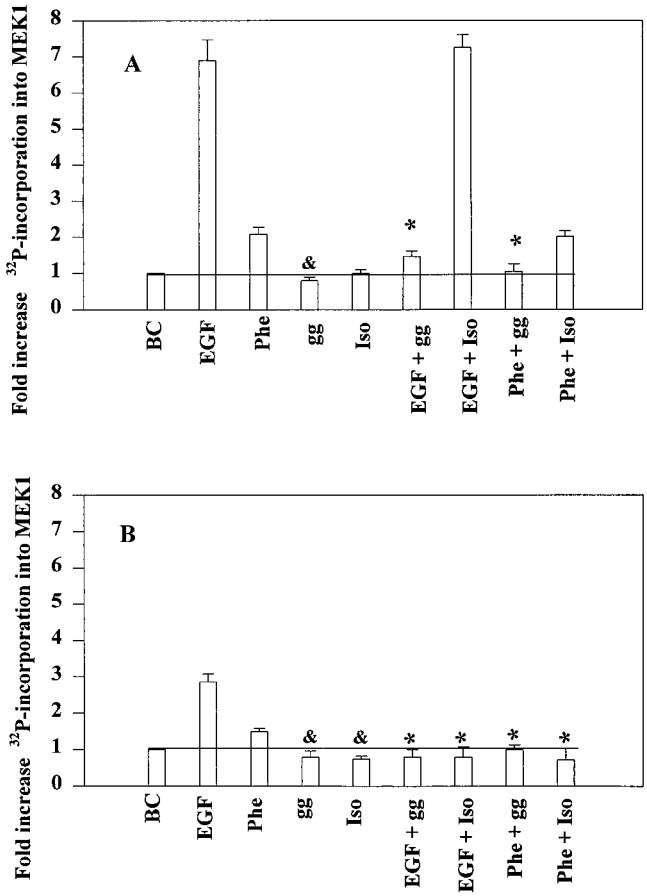

FIG. 3. Regulation of Raf-1 by growth factors in sham (A) and PHX (B) freshly isolated hepatocytes. Cells were exposed for 4 min to the following hormones or combinations thereof: phenylephrine (Phe, 10 μM), EGF (10 nM), glucagon (gg, 0.1 μM), isoproterenol (Iso, 1 μM). BC, buffer control. Raf-1 assays were performed as described in Materials and Methods. Data are expressed as fold increases in ^{32}P incorporation into MEK1 compared to incorporation for buffer control treatment. Values are means ± SDs from an average of experiments ($n = 3$ to 6 independent experiments). ^{32}P incorporation into MEK1 in buffer controls from sham and PHX cells was 11,800 ± 1,760 and 13,000 ± 1,950 cpm, respectively. Differences with a P value of <0.05 (* or &) in comparing single and cotreated cells and control and treated cells, respectively, were considered statistically significant.

Increased β_2- and decreased α_1-adrenergic signaling towards p46/54 SAP kinase and p38 SAP kinase in hepatocytes upon PHX. Since catecholamines are known to mediate acute stress responses, we tested the abilities of α_1-AR and β_2-AR agonists to activate the SAP kinase pathways in freshly isolated sham and PHX hepatocytes. Initial examination of basal SAP kinase activities demonstrated that PHX increased p38 SAP kinase activity from 21,100 ± 500 to 48,900 ± 1,300 cpm and p46/54 SAP kinase activity from 600 ± 35 to 1,550 ± 150 cpm ($n = 4$) (~230 and ~260% increases, respectively) without altering their immunoblottable protein levels (Fig. 6), the opposite to that observed with p42 MAP kinase.

In sham cells, phenylephrine treatment acutely activated p38 SAP kinase and p46/54 SAP kinase, and the activation profiles for each of these enzymes were similar (Fig. 7A). However, within 15 min, the activities of both p38 SAP kinase and p46/54 SAP kinase had returned to basal levels and continued to decrease to approximately 50 to 75% of the basal level by 45 min. The ability of phenylephrine treatment to activate either p38 SAP kinase or p46/54 SAP kinase in PHX cells was neg-

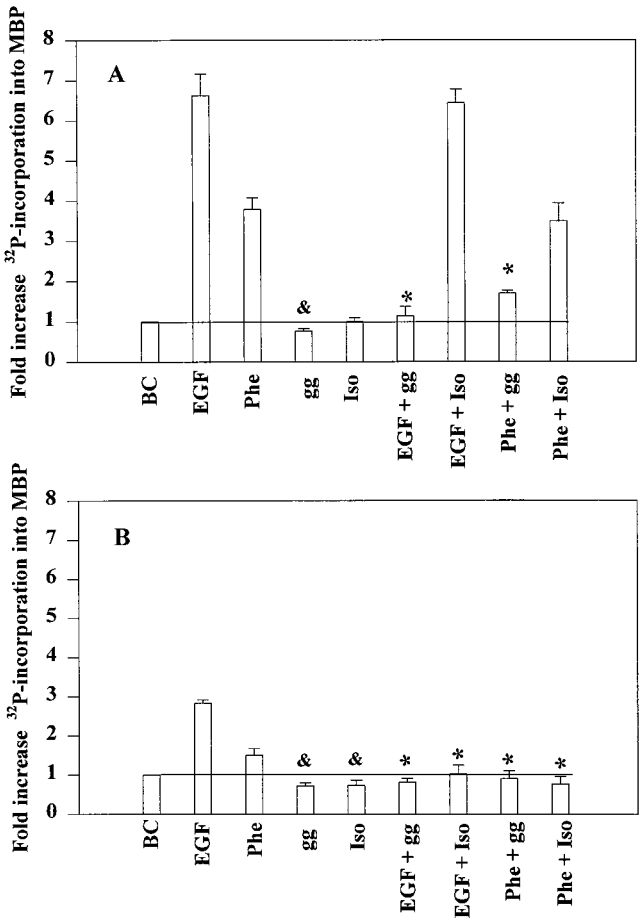

FIG. 4. Regulation of p42 MAP kinase by growth factors in sham (A) and PHX (B) freshly isolated hepatocytes. Cells were exposed for 6 min to the following hormones or combinations thereof: phenylephrine (Phe, 10 μM), EGF (10 nM), glucagon (gg, 0.1 μM), and isoproterenol (Iso, 1 μM). BC, buffer control. p42 MAP kinase activity was determined, and the values are expressed as described in the legend for Fig. 1 and in Materials and Methods. Data are expressed as fold increases in ^{32}P incorporation into MBP compared to incorporation for buffer control treatment. Values are means ± SDs from an average of experiments (three to eight independent experiments). ^{32}P incorporation into MBP in buffer controls from sham and PHX cells was 31,500 ± 1,700 and 10,600 ± 750 cpm, respectively. Differences with a P value of <0.05 (* or &) in comparing single and cotreated cells and control and treated cells, respectively, were considered statistically significant.

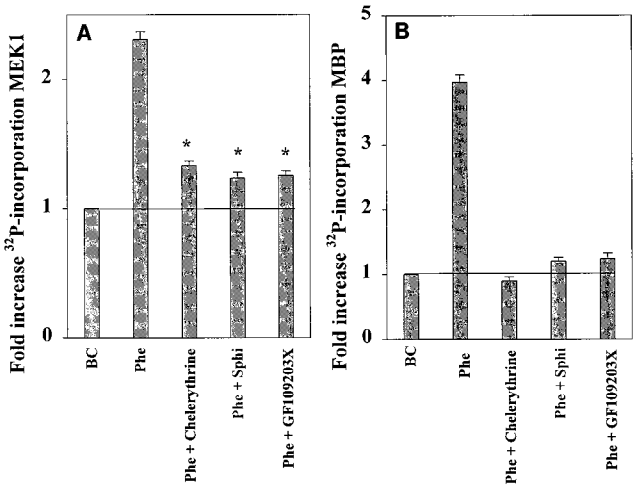

FIG. 5. Inhibition of Raf-1 (A) and p42 MAP kinase (B) activations induced by phenylephrine in sham freshly isolated hepatocytes with specific inhibitors of PK-C. Hepatocytes were pretreated with either sphinganine (Sphi) (20 μM), chelerythrine (5 μM), or bisindolyl-maleamide HCl (15 μM) for 20 min as described in Materials and Methods. Cells were then exposed to phenylephrine treatment (Phe, 10 μM) for 4 min (Raf-1) or 6 min (p42 MAP kinase). Raf-1 and p42 MAP kinase assays were performed as described in Materials and Methods. Data were expressed as fold increases in ^{32}P incorporation into MEK1 and MBP compared to buffer control (BC) treatment. Values are means ± SDs (n = 3) of a representative experiment from an average of duplicates. ^{32}P incorporation into MEK1 in sham buffer control was 12,450 ± 1,500 cpm and into MBP was 25,650 ± 2,400 cpm. *, significant difference between phenylephrine treatment of cells ± PK-C inhibitor.

ligible (12a). In contrast, isoproterenol also caused rapid activations of p38 SAP kinase and p46/54 SAP kinase in PHX, but not in sham, cells (Fig. 7B) (12a). Integration of the areas under each curve (Fig. 7) demonstrated that isoproterenol treatment caused 50 and 35% more total activation of p38 SAP kinase and p46/54 SAP kinase, respectively, than did phenylephrine treatment over the time course of the experiment.

In further studies, treatment of sham hepatocytes with phenylephrine and glucagon, but not isoproterenol, activated both p38 SAP kinase (Fig. 8) and p46/54 SAP kinase (Fig. 9). In contrast, treatment of PHX hepatocytes with glucagon and isoproterenol, but not phenylephrine, activated both p38 SAP kinase (Fig. 8) and p46/54 SAP kinase (Fig. 9). Inhibition of PK-A signaling by pretreatment of hepatocytes with the specific PK-A inhibitor U89 (1 μM) did not alter the ability of either glucagon or isoproterenol to activate p38 SAP kinase and p46/54 SAP kinase (12a). Furthermore, neither forskolin (50 μM) nor dioctanoyl-cyclic AMP (1 mM) treatment activated the SAP kinases in these cells (12a). This indicates that, in contrast to the PK-A-dependent inhibitory interaction between glucagon-isoproterenol and p42 MAP kinase, the stimulation of p46/54 SAP kinase and p38 SAP kinase activities is PK-A independent.

Inhibition of [^{3}H]thymidine uptake by isoproterenol is dependent upon PK-A. In order to test whether the observed alterations in the activities of the MAP and SAP kinase pathways play a role in the regulation of mitogenesis, we initially examined the ability of isoproterenol to alter proliferation in primary cultures of hepatocytes isolated from sham and PHX rats, as assessed by [^{3}H]thymidine uptake into DNA. In primary cells from sham rats, isoproterenol had no effect on [^{3}H]thymidine uptake (Table 2), whereas in cells from PHX rats, isoproterenol elicited a modest concentration-dependent inhibition of [^{3}H]thymidine uptake, which was blocked by the β_2-AR antagonist propranolol (Table 2). Incubation of hepatocytes with the specific PK-A inhibitor U89 (1 μM) also abrogated the ability of isoproterenol to inhibit [^{3}H]thymidine incorporation. These data demonstrate that isoproterenol inhibits proliferation through a PK-A-dependent mechanism.

Inhibition of p38 SAP kinase and p42 MAP kinase decreases [^{3}H]thymidine incorporation. Isoproterenol treatment reduced hepatocyte [^{3}H]thymidine incorporation and resulted in activation of the SAP kinases and inhibition of p42 MAP kinase. Thus both activation of the SAP kinases and inhibition of p42 MAP kinase correlated with the decrease in [^{3}H]thymidine uptake. To test whether these phenomena were causally related, we incubated hepatocytes in the presence of specific inhibitors of MEK1 (PD98059), the upstream activator of p42 MAP kinase, and of p38 SAP kinase (SB203580, or its inactive analog SKF106978), the upstream activator of MAPKAPK2 (Table 3) (4, 10). Inhibition of p38 SAP kinase function dramatically decreased [^{3}H]thymidine incorporation, whereas inhibition of p42 MAP kinase function lowered [^{3}H]thymidine incorporation to a much lesser extent. Thus the isoproterenol-

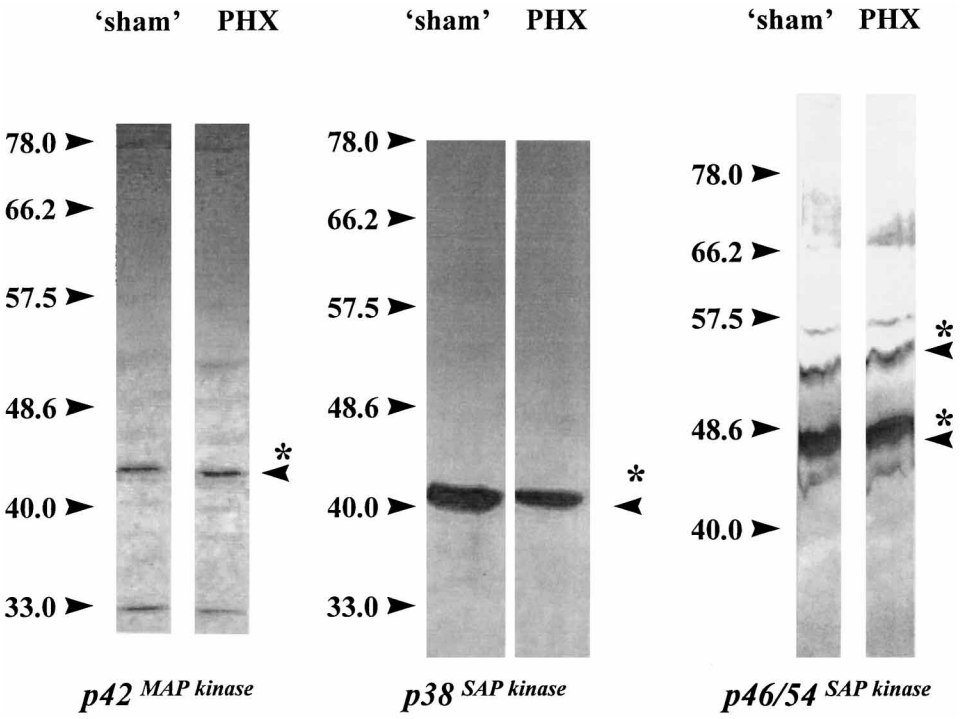

FIG. 6. Western immunoblotting of Raf-1, p42 MAP kinase, p38 SAP kinase, and p46/54 SAP kinase in sham and PHX freshly isolated hepatocytes. Five milligrams of total cell lysate for each of the protein kinases was prepared for SDS-PAGE in 5× running buffer containing bromophenol blue, and 1 mg was loaded onto the gel (20 cm by 20 cm by 1.5 mm). Proteins were transferred to nitrocellulose, and immunoblotting was performed as described in references 12 and 25 by enhanced chemiluminescence (Amersham). X-ray film was photographed with a Kodak DC40 digital camera, and gray-scale images were manipulated in Microsoft Powerpoint. Molecular weight standards (in thousands) are marked by arrowheads; p42 MAP kinase, p38 SAP kinase, and p46/54 SAP kinase are marked by arrowheads with asterisks.

mediated decrease in [^{3}H]thymidine incorporation was dependent upon PK-A function and weakly correlated with the decrease in p42 MAP kinase function, but not the function of p38 SAP kinase.

Inhibition of p38 SAP kinase blunts phenylephrine-stimulated [^{3}H]thymidine incorporation. In contrast to isoproterenol, phenylephrine treatment of sham, but not PHX, hepatocytes enhanced [^{3}H]thymidine incorporation (Table 4). As

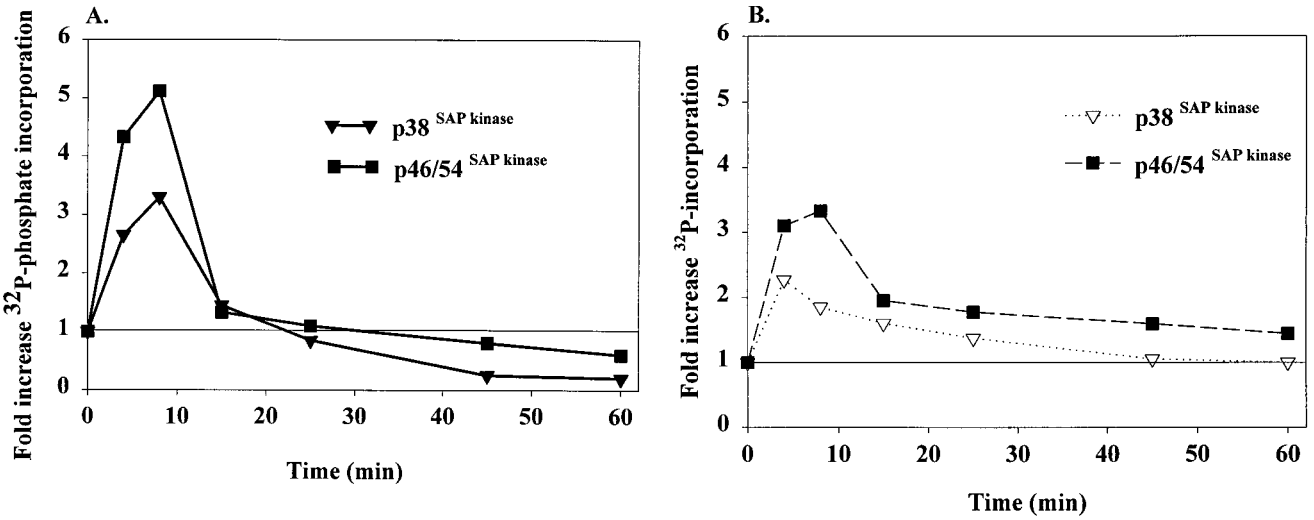

FIG. 7. (A) Time course of activation of p38 SAP kinase and p46/54 SAP kinase by phenylephrine in sham hepatocytes. Hepatocytes were treated with phenylephrine (10 μM) for the times indicated. Immunoprecipitation and immune complex kinase assays were performed as described in Materials and Methods. ^{32}P incorporation is expressed as a ratio (fold increase) to incorporation for buffer control treatment (p38 SAP kinase with MBP, 19,900 ± 1,000 cpm; p46/54 SAP kinase with GST–c-Jun [aa 1 to 169], 700 ± 70 cpm). Data are means of duplicate values (differing by <10%) from a representative of five independent experiments. (B) Time course of activation of p38 SAP kinase and p46/54 SAP kinase by isoproterenol treatment (1 μM) of PHX freshly isolated hepatocytes. Hepatocytes were treated with isoproterenol for the times indicated. Immunoprecipitation and immune complex kinase assays were performed as described in Materials and Methods. ^{32}P incorporation is expressed as a ratio (fold increase) to incorporation for buffer control treatment (p38 SAP kinase with MBP, 20,900 ± 1,200 cpm; p46/54 SAP kinase with GST–c-Jun [aa 1 to 169], 670 ± 50 cpm). Data are means of duplicate values (differing by <10%) from a representative of four independent experiments.

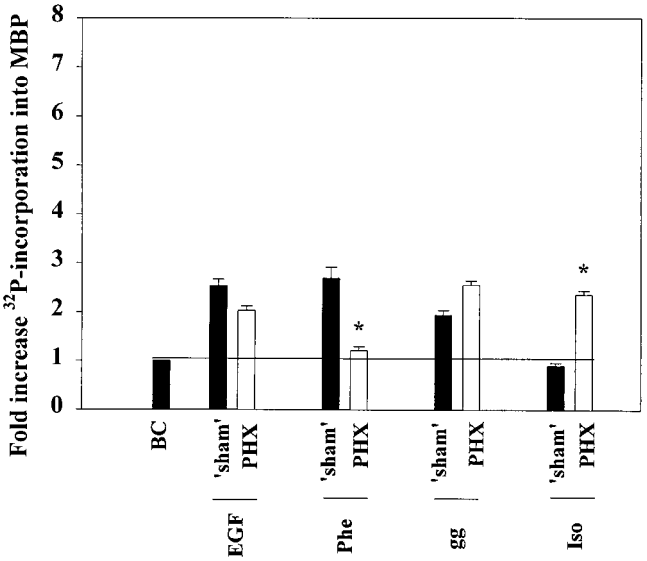

FIG. 8. Phenylephrine, glucagon, and isoproterenol treatments activate p38 SAP kinase in freshly isolated hepatocytes. Hepatocytes (sham [open bars] or PHX [solid bars]) were treated with EGF (10 nM), phenylephrine (Phe, 10 μM), glucagon (gg, 100 nM), or isoproterenol (Iso, 1 μM) for 6 min as described in Materials and Methods. p38 SAP kinase assays were performed as described in Materials and Methods. Values are fold increases in ^{32}P incorporation into MBP compared to incorporation in buffer controls (BC), defined as 1.0. Values are means ± SDs of a representative experiment from an average of duplicates (n = 4 independent experiments). ^{32}P incorporation in buffer controls was 21,100 ± 1,850 cpm in sham hepatocytes and 48,900 ± 2,300 cpm in PHX hepatocytes. *, significant difference between corresponding treatments in comparing sham and PHX cells.

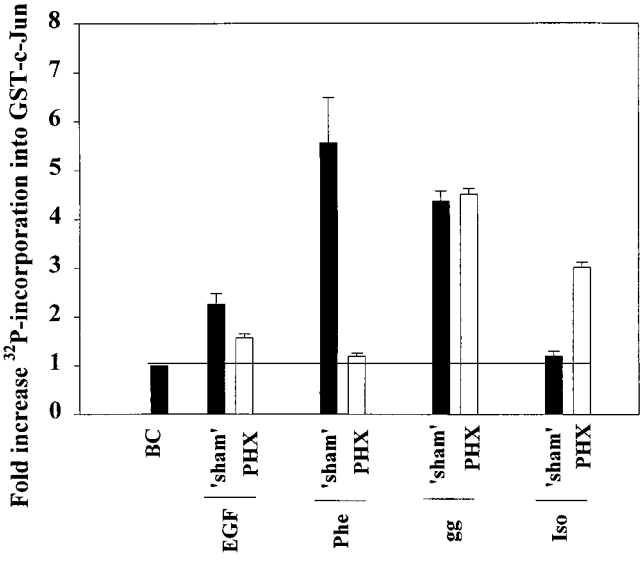

FIG. 9. Phenylephrine, glucagon, and isoproterenol treatments activate p46/54 SAP kinase in freshly isolated hepatocytes. Hepatocytes (sham [open bars] or PHX [closed bars]) were treated with EGF (10 nM), phenylephrine (Phe, 10 μM), glucagon (gg, 100 nM), or isoproterenol (Iso, 1 μM) for 6 min as described in Materials and Methods. p46/54 SAP kinase assays were performed as described in Materials and Methods. Values are fold increases in ^{32}P incorporation into GST–c-Jun (aa 1 to 169) compared to incorporation in buffer controls (BC), defined as 1.0. Values are means ± SDs of a representative experiment from an average of duplicates (n = 4 independent experiments). ^{32}P incorporation in buffer controls was 600 ± 60 cpm in sham hepatocytes and 1,550 ± 150 cpm in PHX hepatocytes.

noted previously, phenylephrine activated both p42 MAP kinase and p38 SAP kinase, and inhibition of these enzymes both by PD98059 and by SB203580 reduced [^{3}H]thymidine incorporation. To determine which of these pathways or enzymes mediates the ability of phenylephrine to stimulate [^{3}H]thymidine incorporation in hepatocytes, we treated cells with the inhibitors and phenylephrine. Prior treatment of hepatocytes with PD98059 significantly inhibited (80 to 90%) the ability of phenylephrine to activate p42 MAP kinase (12a) but only partially blocked (by ~25%) the ability of phenylephrine to stimulate [^{3}H]thymidine incorporation (Table 4). In contrast, prior treatment of hepatocytes with SB203580 completely blocked (by ~95%) the ability of phenylephrine to enhance [^{3}H]thymidine incorporation in these cells, reducing [^{3}H]thymidine incorporation to near the level of SB203580 treatment alone (Table 4). These data suggest that p38 SAP kinase plays a major role in the proliferative response of hepatocytes after PHX.

DISCUSSION

These studies were performed to determine whether the previously reported switch in catecholamine signaling towards glycogen phosphorylase, from an α_1- to β_2-AR-mediated event after PHX, similarly altered the ability of catecholamines to modulate the MAP and SAP kinase signaling cascades. Our data demonstrated that PHX decreased α_1-adrenergic signaling, reducing the ability of phenylephrine to activate p42 MAP kinase, p38 SAP kinase, and p46/54 SAP kinase. Conversely, PHX increased β_2-adrenergic signaling and enhanced the ability of isoproterenol to activate both p38 SAP kinase and p46/54 SAP kinase and to blunt activation of p42 MAP kinase. Furthermore, after PHX we unexpectedly also observed a decrease in basal MAP kinase and increases in basal SAP kinase activities which correlated with the alteration in the balance of adrenergic regulatory signals. Presumably, catecholamine signaling plays a partial role in altering the basal activities of the cascades but will be only one component, since other potent activators of the SAP kinase cascades, e.g., tumor necrosis factor alpha and other cytokines, are also known to be released after PHX (14, 14a).

Previous studies by others have demonstrated that phenylephrine, via α_1-ARs, leads to activation of the MAP kinase cascade. The present studies confirm and extend these findings

TABLE 2. Effect of isoproterenol treatment on DNA synthesis in sham and PHX primary cultured rat hepatocytes[a]

Treatment	Sham	PHX
Vehicle	1.00 ± 0.11	1.00 ± 0.08
Isoproterenol (0.1 μM)	1.06 ± 0.14	0.75 ± 0.05[b,c]
Isoproterenol (1 μM)		0.67 ± 0.13[b]
Isoproterenol (0.1 μM) plus propranolol (1 μM)	0.98 ± 0.13	1.05 ± 0.13
Isoproterenol (1 μM) plus U89 (100 nM)		1.02 ± 0.02
U89 (100 nM)		1.00 ± 0.10

[a] Hepatocytes isolated from sham operation or PHX rats were cultured on rat tail collagen (Vitrogen)-coated plastic dishes (20 mm) in William's E medium containing 2 μCi of [^{3}H]thymidine. Values shown are means ± standard errors from three independent experiments, expressed as fold changes compared to buffer-treated control cells. [^{3}H]thymidine uptake in buffer-treated controls was 360 ± 30 cpm (sham) and 2,100 ± 300 cpm (PHX).

[b] Significant difference from corresponding vehicle buffer control value ($P < 0.05$).

[c] Significant difference from corresponding value in sham cells ($P < 0.05$).

TABLE 3. Effect of inhibition of p42 MAP kinase and p38 SAP kinase function on DNA synthesis in sham and PHX primary cultured rat hepatocytes[a]

Condition	Sham	PHX
Vehicle	1.00 ± 0.06	1.00 ± 0.06
SB203580 (5 μM)	0.53 ± 0.09[b]	0.39 ± 0.10[b]
SKF106978 (5 μM)	0.96 ± 0.03	1.05 ± 0.10
PD98059 (50 μM)	0.85 ± 0.07[b]	0.83 ± 0.05[b]
Isoproterenol (1 μM)	1.01 ± 0.03	0.78 ± 0.04[b,c]

[a] Hepatocytes isolated from sham operation or PHX rats were cultured on rat tail collagen (Vitrogen)-coated plastic dishes (12-well plates) in William's E medium containing 10 μCi of [^{3}H]thymidine. Values shown are means ± standard errors from 12 independent experiments, expressed as fold changes compared to buffer-treated control cells. [^{3}H]thymidine uptake in buffer-treated controls was 3,800 ± 250 cpm (sham) and 14,100 ± 1,100 cpm (PHX).
[b] Significant difference from corresponding buffer control value ($P < 0.05$).
[c] Significant difference from corresponding value in sham cells ($P < 0.05$).

to freshly isolated and primary cultured rat hepatocytes and also show that the α_1-adrenergic effect in these cells is PK-C dependent. In contrast to the α_1-adrenergic-mediated activation of the MAP kinase cascade, isoproterenol via β_2-ARs, or glucagon, inhibited activation of the cascade by growth factors. Since the cyclic AMP-generating hormone glucagon was able to reduce basal levels of p42 MAP kinase and to inhibit activation by EGF in sham hepatocytes, our data suggest that the lack of similar effects for isoproterenol in the same cells was due to the absence of functional β_2-AR, and not to a defect in the cyclic AMP-dependent protein kinase cascade (Table 1 and Fig. 1). Other workers using different cell types have obtained both equivalent (39, 45) and slightly different data. For example, Crespo et al. reported that isoproterenol treatment of COS-7 cells had an acute stimulatory and chronic inhibitory effect on p42 MAP kinase activity, the former being mediated by G protein βγ subunits and the latter by G protein α subunits via PK-A (8). In contrast, our data demonstrated that glucagon and isoproterenol treatments both acutely and chronically inhibited p42 MAP kinase activation and were dependent upon PK-A function. Nevertheless, inhibition of PK-A by U89 while cotreating hepatocytes with either glucagon or isoproterenol permitted activation of p42 MAP kinase (12a). These data imply that PK-A-mediated signaling predominates over Gβγ signaling in hepatocytes 24 h after PHX. Differences in the subtype or cellular density of the β-ARs involved, or other tissue-specific factors, may also account for the absence of the stimulatory component in the remnant liver at this time or under our assay conditions (after 1 to 60 min of cotreatment). It is possible that the stimulatory effect of isoproterenol upon p42 MAP kinase activity may be observed at other stages of the regenerative process in hepatocytes.

TABLE 4. Inhibition of p38 SAP kinase blocks basal and phenylephrine-mediated stimulation of DNA synthesis in sham primary cultured rat hepatocytes[a]

Condition	Sham	PHX
Vehicle	1.00 ± 0.03	1.00 ± 0.02
Phenylephrine (10 μM)	1.35 ± 0.06[b,c]	0.96 ± 0.09
SB203580 (5 μM)	0.53 ± 0.06[b,c]	0.39 ± 0.10[b]
Phenylephrine (10 μM) plus SB203580 (5 μM)	0.60 ± 0.08[b]	
PD98059 (50 μM)	0.85 ± 0.07[b]	0.83 ± 0.05[b]
Phenylephrine (10 μM) plus PD98059 (50 μM)	1.20 ± 0.10[b]	

[a] Hepatocytes isolated from sham operation or PHX rats were cultured on rat tail collagen (Vitrogen)-coated plastic dishes (12-well plates) in William's E medium containing 10 μCi of [^{3}H]thymidine. Values shown are means ± standard errors from 12 independent experiments, expressed as fold changes compared to buffer-treated control cells. [^{3}H]thymidine uptake in buffer-treated controls was 3,800 ± 250 cpm (sham) and 14,100 ± 1,100 cpm (PHX).
[b] Significant difference from corresponding buffer control value ($P < 0.05$).
[c] Significant difference from corresponding value in comparison of sham and PHX cells ($P < 0.05$).

Another important finding was that PHX decreased the basal activity of p42 MAP kinase and increased the basal activities of p38 SAP kinase and p46/54 SAP kinase in hepatocytes from remnant livers. The decrease in basal p42 MAP kinase activity was associated with a decreased ability of not only phenylephrine, but also multiple growth factors such as EGF, hepatocyte growth factor, and insulin, to stimulate p42 MAP kinase in PHX cells, in agreement with the data of others (Fig. 3 and 4) (12a, 14, 18, 24, 34). The reasons why expression of multiple growth factor receptors is reduced after PHX is unknown. The decrease in basal p42 MAP kinase activity also correlated with the increased ability of isoproterenol to inhibit p42 MAP kinase function. Stimulation of β_2-ARs leads to generation of cyclic AMP and activation of PK-A, which have been shown to be antiproliferative, as shown by our studies with hepatocytes (Tables 2 and 3), and to induce senescence and/or apoptosis in some cell types (8, 31). Thus, the increased ability of PHX versus sham hepatocytes to activate PK-A via β_2-ARs and thus inhibit proliferation would appear to be counterintuitive based on our data (8, 31, 32). However, catecholamines are not primary mitogens in the liver, and their possible indirect effects on gene expression via increased signaling through the SAP kinase cascades may be more important in determining their net effect on the process of liver regeneration.

The decline in p42 MAP kinase activity contrasts with the marked increase in basal [^{3}H]thymidine incorporation in hepatocytes 24 h following PHX, which suggests that signaling via p42 MAP kinase may not be part of the primary mitogenic pathway at this stage of liver regeneration. This conclusion is further supported by the observation that near-maximal inhibition (80 to 90%) of p42 MAP kinase activity by PD98059 reduced basal [^{3}H]thymidine uptake by only ~20% and did not significantly block the ability of phenylephrine to stimulate [^{3}H]thymidine incorporation in sham cells (Table 3). The physiological reasons for, as well as the consequences of, the reduced signaling towards p42 MAP kinase upon regeneration via these multiple mechanisms remain unclear.

In contrast to the decline in basal p42 MAP kinase activity, the basal activities of the SAP kinases increased upon PHX, which correlated with an increase in basal [^{3}H]thymidine incorporation (Fig. 8 and 9) (Tables 2 to 4). This agrees with data of others suggesting that agonists such as tumor necrosis factor alpha, which can activate both SAP kinase pathways, are increased after PHX and appear to play a permissive role in liver regeneration (14, 14a). The present findings demonstrated that catecholamines can also activate the SAP kinases via either α_1-ARs or β_2-ARs. Activation of the SAP kinases by isoproterenol, which was PK-A independent, appeared to be slightly more efficacious than that induced by phenylephrine, although the role that this difference plays in the overall increase in basal SAP kinase activity is unclear.

Although simple correlation does not prove cause and effect, inhibition of p38 SAP kinase function significantly reduced basal [^{3}H]thymidine incorporation and blocked the ability of phenylephrine to increase [^{3}H]thymidine incorporation, suggesting a permissive role for this kinase in proliferative signaling within the regenerating liver (Tables 3 and 4). Such a possibility is compatible with recent findings that IL-6 is essen-

tial for the proliferative response after PHX (9) and that p38 SAP kinase function appears to be an essential player in the stimulation of IL-6 production (4). Presumably, inhibition of p38 SAP kinase in our primary cultures deprives these cells of an essential autocrine growth factor. Isoproterenol also activated p38 SAP kinase but had an antiproliferative effect (Table 2). However, these two effects were dissociated by the ability of the PK-A inhibitor U89 to prevent the decrease in [^{3}H]thymidine incorporation but not the increase in p38 SAP kinase activity by isoproterenol. These data suggest that PK-A plays a direct role in the inhibition of proliferation, such as by direct phosphorylation of transcription factors, e.g., the CREB/ATF family and the C/EBP family (14, 36a).

Our data suggest that hepatocyte proliferation, in part, is determined by the balance of signals emanating from p42 MAP kinase, p38 SAP kinase, p46/54 SAP kinase, and PK-A. Further studies are needed to elucidate the full physiological relevance of differential growth factor and catecholamine signaling through these cascades in the regulation of liver regeneration.

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