α-Adrenergic inhibition of proliferation in HepG2 cells stably transfected with the α_{1B}-adrenergic receptor through a $p42^{MAPkinase}/p21^{Cip1/WAF1}$-dependent pathway

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Abstract **Activation of α_{1B} adrenergic receptors (α_{1B}AR) promotes DNA synthesis in primary cultures of hepatocytes, yet expression of α_{1B}AR in hepatocytes rapidly declines during proliferative events. HepG2 human hepatoma cells, which do not express α_{1B}AR, were stably transfected with a rat α_{1B}AR cDNA (TFG2 cells), in order to study the effects of maintained α_{1B}AR expression on hepatoma cell proliferation. TFG2 cells had a decreased rate of growth compared to mock transfected HepG2 cells as revealed by a decrease in [^{3}H]thymidine incorporation into DNA. Stimulation of α_{1B}AR with phenylephrine caused a further large reduction in TFG2 cell growth, whereas no effect on growth was observed in mock transfected cells. Reduced cell growth correlated with increased percentages of cells found in G_0/G_1 and G_2/M phases of the cell cycle. In TFG2 cells, phenylephrine increased $p42^{MAPkinase}$ activity by 1.5- to 2.0-fold for up to 24 h and increased expression of the cyclin dependent kinase inhibitor protein $p21^{Cip1/WAF1}$. Treatment of TFG2 cells with the specific MEK1 inhibitor PD98059, or infection with a −/− MEK1 recombinant adenovirus permitted phenylephrine to increase rather than decrease [^{3}H]thymidine incorporation. In addition, inhibition of MAP kinase signaling by PD98059 or MEK1 −/− blunted the ability of phenylephrine to increase $p21^{Cip1/WAF1}$ expression. In agreement with a role for increased $p21^{Cip1/WAF1}$ expression in causing growth arrest, infection of TFG2 cells with a recombinant adenovirus to express antisense $p21^{Cip1/WAF1}$ mRNA blocked the ability of phenylephrine to increase $p21^{Cip1/WAF1}$ expression and to inhibit DNA synthesis. Antisense $p21^{Cip1/WAF1}$ permitted phenylephrine to stimulate DNA synthesis in TFG2 cells, and abrogated growth arrest. These results suggest that transformed hepatocytes may turn off the expression of α_{1B}ARs in order to prevent the activation of a growth inhibitory pathway. Activation of this inhibitory pathway via α_{1B}AR appears to be $p42^{MAPkinase}$ and $p21^{Cip1/WAF1}$ dependent.**

1. Introduction

α_1-Adrenergic receptors (α_1AR) are members of the G protein-coupled receptor superfamily. Through pharmacological and molecular cloning studies multiple subtypes of α_1AR have been identified: $\alpha_{1A/C}$AR, α_{1B}AR and α_{1D}AR [1]. The rat liver only expresses the α_{1B}AR subtype, which is primarily coupled to phospholipase C (PI-PLC) via a pertussis toxin-insensitive G-protein [2,3]. Upon activation, PLC catalyzes the breakdown of polyphosphatidylinositol-4,5-bisphosphate (PIP_2) to yield two second messengers: inositol tris-phospate (IP_3) which subsequently releases intracellular calcium, and 1,2-diacylglycerol (DAG), which can activate protein kinase C (PKC) [4]. The α_{1B}AR is also coupled to several downstream signaling cascades such as the c-Jun NH_2-terminal kinase (JNK) and the mitogen activated protein kinase (MAPK) pathways [5,6].

The α_{1B}AR plays an important role in the sympathoadrenal response to stress, including the acute effects of catecholamines on liver carbohydrate, lipid, and amino acid metabolism [7]. In addition to these short-lived effects, the α_{1B}AR may also mediate the co-mitogenic effect of catecholamines in the liver. For example, activation of α_{1B}ARs increases DNA synthesis in primary cultures of hepatocytes [5,8] and their inhibition delays liver regeneration [9]. Activation of α_{1B}AR can also promote malignant cell growth, as suggested by the agonist-dependent focus formation and disordered growth observed in Rat-1 fibroblasts transfected with α_{1B}AR, or the tumorigenic effect of transplanting cells bearing transfected α_{1B}ARs into nude mice [10].

Activation of the MAP kinase (MAPK) cascade has been implicated in proliferative signaling in various cell types [11,12]. However, in primary cultures of rat hepatocytes the α_{1B}AR-induced increase in DNA synthesis was found to be relatively independent of MAPK activation, and it was instead coupled to activation of the p38 stress activated protein (SAP) kinase [5].

Paradoxically, quiescent hepatocytes express much higher levels of α_{1B}AR than proliferating hepatocytes, as observed in cells in primary culture [13,14], in the remnant liver following partial hepatectomy [7,15,16], and in fetal [17] or malignantly transformed liver [18]. The mechanism(s) by which proliferating hepatocytes lose α_{1B}AR expression are unclear. A number of clonal human liver tumor cell lines, including HepG2 cells, lack functional α_1ARs altogether [19]. These findings could suggest that despite the mitogenic effect of α_{1B}AR activation in the early stages of the regenerative response of the normal liver, continued activation of α_1AR may interfere with the maintenance of high rates of hepatocyte proliferation in liver tumor cells. In order to test this hypothesis, we generated a HepG2 hepatoma cell line that stably

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expresses the rat α_{1B}AR at high levels (TFG2 cells). We present evidence that stimulation of α_{1B}AR in TFG2 cells inhibits their growth via a $p42^{MAPkinase}$ and $p21^{Cip1/WAF1}$ dependent mechanism.

2. Materials and methods

2.1. Materials

Dominant negative (−/−) MEK1, $p21^{Cip1/WAF1}$ sense and antisense adenoviruses were generated as described [20–22]. Anti-p42 MAP kinase (sc-154AC), anti-cdk2 (sc-163), anti-$p16^{INK4a}$ (sc-1207), anti-$p21^{Cip1}$ (sc-397-G), anti-$p27^{Kip1}$ (sc-527-G), anti-cyclin A-(sc-596), anti-cyclin D-(sc-753), and anti-cyclin E-(sc-481) antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Radiolabelled [γ-^{32}P]ATP and [^{3}H]thymidine were from NEN (Boston, MA, USA). Protein preparations of other reagents were as in [5,23]. The specific MEK1/2 activation inhibitor PD98059 [24] was a gift from Parke Davis/Warner Lambert. Wild-type HepG2 human hepatoma cells were obtained from ATCC (Rockville, MD, USA) and cultured under conditions specified by the supplier. The TFG2 cell line was cultured under the same conditions as the naive HepG2 cells, except for the additional presence of 50 μg/ml geneticin to maintain selection pressure (see below).

2.2. Partial hepatectomy

Adult male Sprague-Dawley rats were subjected to 2/3 partial hepatectomy under aseptic conditions. The rats were anesthetized with sodium pentobarbital, 50 mg/kg i.p., and the median and left lateral lobes of the liver were ligated at their stem and excised. Control rats were subjected to sham operation, which consisted of laparotomy and a brief manipulation of the intestines but not the liver with a cotton swab prior to wound closure.

2.3. Primary culturing of rat hepatocytes

For isolation of primary cultured rat hepatocytes, male Sprague-Dawley rats weighing 80–120 g were anesthetized with sodium pentobarbital, 50 mg/kg intraperitoneally, and the portal vein cannulated under aseptic conditions. Liver cells were isolated by a collagenase perfusion protocol as described earlier [25]. The isolated cells were washed twice with hepatocyte wash medium (Gibco), and plated onto polylysine-coated culture dishes in attachment medium (Gibco). After 3 h, the medium was changed to DMEM containing 5% fetal bovine serum, 1×10^{-8} M dexamethasone, 10 ng/ml EGF, 5 μg/ml insulin, 2.5 μg/ml fungizone, 50 μg/ml geneticin, 67 μg/ml penicillin, and 100 μg/ml streptomycin.

2.4. Construction of plasmids

The α_{1B}AR expression vector was prepared by subcloning the rat α_{1B}AR cDNA (−240 to +1676) [26] into the pcDNA3 expression vector (Invitrogen, CA, USA).

2.5. Transient and stable transfections

Transient transfections were performed by using the lipofectin reagent (Gibco), as described previously [16,26]. Briefly, the HepG2 cells were grown in six-well culture plates to about 50–60% confluency. The DNA to be transfected was then introduced along with the lipofectin reagent to the cultures in reduced-serum medium OPTI-MEM (Gibco). After 16 h the medium was replaced with normal growth medium. Assays were performed 48 h post transfection. To achieve stable transfection, cells were treated in the same manner as in transient transfections, with a few exceptions. These cells were grown to 30–50% confluency, and after transfection they were exposed to a selective concentration (as determined by a 'killing curve') of 375 μg/ml of geneticin (G418-sulfate, Gibco) to isolate stably transfected cells. Positive colonies were passaged three more times before selecting unique clones. The stably transfected cell lines were maintained under constant selection pressure in the continued presence of 375 μg/ml geneticin.

2.6. Adenoviral infection of HepG2 cells

HepG2/TFG2 cells (in DMEM, 10% (v/v) fetal calf serum (FCS), 60-mm dishes, 10^6 cells) were washed with medium (DMEM), and the cells infected with either null recombinant adenovirus or with dominant negative (−/−) MEK1, $p21^{Cip1/WAF1}$ sense, or $p21^{Cip1/WAF1}$ antisense recombinant adenoviruses in a total volume of 1 ml and at a multiplicity of infection of 200. After 2 h at 37°C, the cells were washed with DMEM and cultured for a further 24 h prior to experimentation in DMEM containing 10% (v/v) FCS.

2.7. Treatment of HepG2 cells and primary hepatocytes for kinase assays

Primary hepatocytes (2×10^5 cells per well of a 12-well dish) were cultured in DMEM containing 100 nM insulin, 1 nM dexamethasone, 1 nM thyroxine [21]. Cells were cultured for 90 min after isolation prior to assay. This was to allow stabilization of protein kinase activities, which may have been affected by the isolation process. In addition, prolonged culture of primary hepatocytes (∼12–24 h) causes a decrease in α_{1B}-adrenergic receptor expression. HepG2 cells were grown to near confluency in 100-mm dishes, and 2 h prior to the kinase assays they were serum starved in DMEM containing 100 nM insulin, 1 nM dexamethasone and 1 nM thyroxine.

Drugs were added directly to the medium for the specified periods of time and final concentrations for each cell type. Thirty seconds prior to terminating the incubation, the medium was aspirated and the dishes were placed on dry ice. After the addition of 400 μl of ice-cold homogenization buffer (25 mM HEPES, pH 7.4, 5 mM EDTA, 5 mM benzamidine, 1 mM PMSF, 1 mg/ml soybean trypsin inhibitor, 40 μg/ml pepstatin A, 40 μg/ml aprotinin, 1 μM microcystin-LR, 0.5 mM sodium orthovanadate, 0.5 mM sodium pyrophosphate, 1% (v/v) Triton X-100, 0.1% 2-mercaptoethanol), cells were collected with a rubber policeman. Samples were stored on ice for 5 min prior to centrifugation ($14\,000\times g$, 5 min, 4°C).

2.8. Immunoprecipitations from cell homogenates

The protein A agarose slurry (25 μl bead volume) was washed with 1.0 ml PBS containing 0.1% (v/v) Tween 20, and resuspended in 0.4 ml of the same buffer. Antibodies (2 μg, 20 μl) were added to each tube and incubated (2 h, 4°C) to allow for their conjugation to the protein-A agarose beads, followed by a subsequent wash to remove non-conjugated antibodies. The supernatant from the homogenates (0.5 ml, 0.5 mg total protein for both HepG2 cells and primary hepatocytes) was mixed with protein A agarose-conjugated antibody and incubated on a rocking mixer (2 h, 4°C). The protein A agarose was recovered by centrifugation, the supernatant discarded, and the immunoprecipitates were washed twice with homogenization buffer and once with washing buffer (25 mM HEPES, pH 7.4, 15 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 0.1% 2-mercaptoethanol).

2.9. Assay of $p42^{MAPK}$ activity

The assay for $p42^{MAPK}$ activity was described previously [5,21]. Briefly, immunoprecipitates were incubated (final volume 50 μl) with 50 μl of washing buffer containing 0.2 mM [γ-^{32}P]ATP (5000 cpm/pmol), 1 μM microcystin-LR, and 0.5 mg/ml myelin basic protein (MBP) – the substrate for $p42^{MAPK}$. Following a 20-min incubation at 37°C, 40 μl of the reaction volume was spotted onto P81 paper (Whatman, Maidstone, UK) and immediately placed into 180 mM phosphoric acid. The papers were washed several times in 180 mM phosphoric acid, followed by a final wash with acetone. The ^{32}P incorporation into MBP was quantified by liquid scintillation spectrometry.

2.10. Assay of cyclin dependent kinase 2 (cdk2) activity

Cdk2 activity was measured in immunoprecipitates of whole cell lysates. TFG2 cells were rinsed with PBS/5 mM EGTA, then harvested in homogenization buffer (described above) containing 0.5% NP-40. Samples were sonicated (two 5-s bursts on ice), centrifuged at $13\,000\times g$ for 15 min at 4°C and either assayed immediately or stored at −70°C. In lysates prepared by sonication, over 90% of the total CaMK-II activity or pRb was solubilized, as measured by solution assays and immunoblots. Approximately 100 μg of cellular protein from whole cell homogenates was incubated sequentially with 1 μg of purified rabbit anti-cdk2 IgG, followed by 2 μg of biotinylated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) and finally streptavidin-magnespheres (Promega, Madison, WI, USA) on ice in 200 μl for at least 2 h each. Immune complexes were washed 3 times with 30 mM HEPES, pH 7.4, 15 mM $MgCl_2$, 80 mM β-glycerol phosphate, 2.6 mM EGTA, 1 μg/ml each of chymostatin, leupeptin, antipain, pepstatin and soybean trypsin inhibitor, 0.5% NP-40, and then resuspended in 25 μl of the same buffer without NP-40. Protein

kinase assays were conducted by assaying 10 μl of these immunoprecipitated samples in triplicate in a total volume of 25 μl, containing final concentrations of 30 mM HEPES, pH 7.4, 0.1 mM DTT, 25 mM $MgCl_2$, 30 mM β-glycerol phosphate, 0.5 μM protein kinase A inhibitor peptide (TTTADFIAGRRNAIHD), 0.5 μCi [γ-^{32}P]ATP and 20 μM ATP, 2 mM EGTA and 0.5 mg/ml histone H1. After 10–20 min at 32°C, assays were stopped by pipetting 20 μl of the mixture onto P81 phosphocellulose paper (Whatman, Clifton, NJ, USA), air dried for 1 min and then washed 5 times in 500 ml 1% phosphoric acid. The retained radioactivity was quantified by Cerenkhov counting.

2.11. [^{3}H]Thymidine incorporation

Following preincubation of the cells with an antagonist (if applicable), [^{3}H]thymidine (2–10 μCi/ml) was added to the culture along with the appropriate agonist at 24 h after plating. Cells were incubated for a further 3 h at 37°C. The cells were dissolved by the addition of 1.0 ml 0.5 N NaOH, collected, mixed with 1.5 ml H_2O, and precipitated with 0.5 ml of 50% trichloroacetic acid (TCA). The precipitated material was collected on glass fiber filters and washed twice with 5% TCA, followed by liquid scintillation counting of the filters.

2.12. Western blotting

Visualization of proteins by Western immunoblotting was done as described in detail elsewhere [5,16,21].

2.13. Preparation of crude nuclear extracts

Methods for preparing crude nuclear extracts from cultured cells were adapted from [27]. Briefly, cells were washed in PBS buffer and collected with a rubber policeman in 500 μl extract buffer (20 mM HEPES, 450 mM NaCl, 0.4 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF). The cells were subjected to three cycles of freeze-thawing, followed by centrifugation at $14\,000 \times g$ for 10 min. Protein in the supernatant was determined by using the Bio-Rad protein assay, using BSA as standard.

2.14. DNA gel mobility shift assay

DNA gel mobility shift assays (GMSA) were carried out as previously described [16,26].

2.15. Receptor binding assay

α_{1B}AR in stably transfected and mock transfected HepG2 cells were identified by an equilibrium binding assay using [^{3}H]prazosin as the radioligand [25]. Cells were subjected to one cycle of freeze-thawing to reduce non-specific binding, and were then suspended in 50 mM Tris-HCl/10 mM $MgSO_4$ buffer at pH 7.4. Triplicate aliquots containing 100 μg protein and a saturating concentration of [^{3}H]prazosin (20 nM) in the absence (total binding) or presence of 10 μM phentolamine (non-specific binding) were incubated for 50 min at 30°C in a total volume of 1.0 ml. Incubations were terminated by rapid vacuum filtration over Whatman GF/B filters presoaked in assay buffer, and the radioactivity retained by the filters was measured by liquid scintillation spectrometry. For generating displacement isotherms, cells were incubated with 1 nM [^{3}H]prazosin in the absence or presence of 8–12 concentrations of the unlabeled competing ligand. Competitor and radioligand were added to the assay simultaneously, except for chloroethylclonidine, which was added to the cell preparation 15 min prior to the addition of the radioligand. K_ds of the competitor ligands were derived by computerized curve-fitting, using the ALLFIT program.

2.16. Cell cycle analysis: propidium iodide staining of cells

Cells were grown on plates during various treatments and were isolated by tryptic digestion, followed by pelleting by centrifugation at 1500 rpm, 4°C for 5 min and resuspended in 1.5 ml of PBS followed by the addition of 3 ml of 100% ETOH (67% ETOH Fix) and incubated on ice at 4°C for at least 1 h. Cells were pelleted by centrifugation as above, supernatant removed and resuspended in 1.0 ml of propidium iodide stain containing 3.8 mM sodium citrate, 0.5 mg/ml RNase A and 0.01 mg/ml propidium iodide and incubated on ice at 4°C for 3 h. Cells were pelleted by centrifugation as above, supernatant removed and resuspended in 1.0 ml of PBS and ready for analysis. Cells were analyzed with a Becton-Dickinson FACScan flow cytometer and Verity Winlist software.

2.17. Data analysis

Comparison of the effects of various hormone treatments was performed using one way analysis of variance and a two tailed *t*-test. Differences with a *P*-value of < 0.05 were considered statistically significant. Experiments shown are the means of 3–6 individual experiments ± S.E.M. performed in duplicate.

3. Results

3.1. Expression of α_{1B}AR in TFG2 cells

To measure the cellular density of α_{1B}AR, ligand binding assays were performed using TFG2 cells (HepG2 cells stably transfected with α_{1B}AR cDNA) as well as mock transfected HepG2 cells. In TFG2 cells, α_{1B}ARs were expressed at a high level (2150 ± 150 fmol/mg total cellular protein) compared to either negligible levels of α_{1B}AR in mock transfected HepG2 cells (< 5 fmol/mg protein) or lower levels of expression in freshly isolated hepatocytes (125 ± 5 fmol/mg protein). Displacement of the radioligand by unlabeled competitor drugs displayed binding specificity expected from α_{1B}AR (Fig. 1). The α_{1B}AR-selective ligand WB-4101 displaced [^{3}H]prazosin with high affinity (K_d of 10 nM), whereas the affinities of the α_{1A}AR-selective ligands, niguldipine, oxymethazoline and 5-methylurapidil, were much lower (K_ds > 1–30 μM). The α_{1B}AR-selective irreversible antagonist, chloroethylclonidine, was able to completely displace [^{3}H]prazosin binding, with an apparent K_d of 10 μM.

3.2. Activation of α_1AR in TFG2 cells inhibits DNA synthesis and cell proliferation

We next examined the ability of phenylephrine (PE) to affect the rate of DNA synthesis and cell division in TFG2 cells, and compared these effects to those observed in primary cultured rat hepatocytes [28,29]. Treatment of primary cultures of adult rat hepatocytes with different concentrations of PE resulted in a concentration-dependent increase in [^{3}H]thymidine incorporation into primary hepatocyte DNA (Fig. 2A), which is in agreement with previously published findings [5]. In contrast, treatment of TFG2 cells with PE,

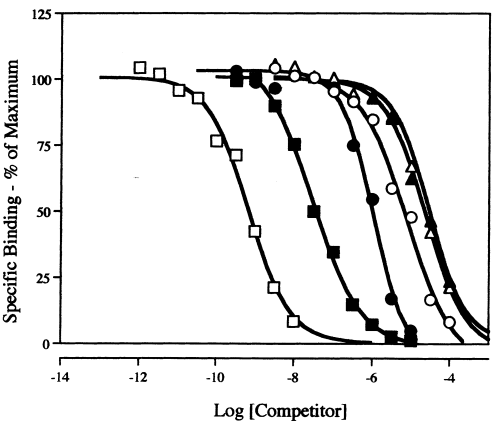

Fig. 1. Ligand binding to α_{1B}-adrenergic receptors stably transfected in TFG2 cells. TFG2 cells were cultured and radioligand binding displacement studies were performed as in Section 2. Note that the α_{1B}-adrenergic receptor-selective ligand WB-4101 (■) displaced [^{3}H]prazosin with high affinity (K_d of 10 nM), whereas the affinities of the α_{1A}AR-selective ligands, niguldipine (●), oxymethazoline (○) and 5-methylurapidil (▲), were much lower (K_ds > 1–30 μM). Complete displacement by chloroethylclonidine (△, apparent K_i: 10 μM) is also compatible with a pure population of α_{1B} receptors. Data shown are means of 3 independent experiments.

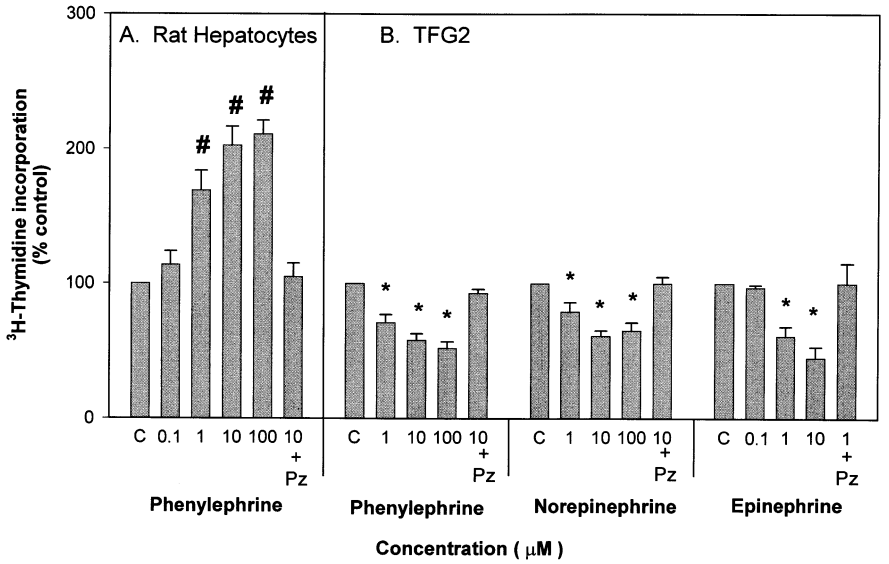

Fig. 2. Phenylephrine increases DNA synthesis in primary cultured rat hepatocytes and inhibits DNA synthesis in TFG2 cells. Freshly isolated rat hepatocytes (A) and TFG2 cells (B) were cultured as in Section 2. Cells were treated with either vehicle, the indicated adrenergic agonist, or agonist plus prazosin (Pz, 1 μM) as indicated, together with 2 μCi [^{3}H]thymidine. Forty eight hours after the addition of drug(s) and [^{3}H]thymidine, cells were harvested and [^{3}H]thymidine incorporation into DNA determined. Means and S.E. of 3 separate experiments are shown. # indicates significant increase and * indicates significant decrease ($P < 0.05$) from corresponding drug-free control (C).

EPI or NE caused concentration-dependent decreases in [^{3}H]thymidine incorporation into DNA, which were completely prevented by 1 μM prazosin (Fig. 2B). The ability of PE treatment to inhibit DNA synthesis was not blocked by pertussis toxin pre-treatment (100 ng/ml, 24 h) of the cells (not shown).

In additional experiments, cell counts over a five-day period also indicated corresponding changes in cell growth. TFG2 cells grew at a significantly slower rate (69 ± 3%) than mock transfected HepG2 cells, and their rate of growth was further significantly reduced in the presence of PE alone, but not in the presence of PE plus the antagonist prazosin (60 ± 5% and 100 ± 4%, respectively, of untreated TFG2 cells). Since the expression and function of the α_{1B}AR declines within a few hours of primary culturing in rat hepatocytes [5,16], whereas it always remains high in TFG2 cells, the above findings suggest that the prolonged ability of adrenergic agonists to signal through α_{1B}AR may be anti-proliferative in hepatocytes.

To confirm that cell cycle arrest in response to phenylephrine treatment had occurred, flow cytometric analysis was performed on TFG2 cells 48 h after addition of phenylephrine to the media. In untreated cells, 63 ± 4% of cells were in G_0/G_1; 31 ± 2% of cells were in S phase; and 6 ± 1% of cells were in G_2/M phase. However, in TFG2 cells treated with 10 μM phenylephrine, 83 ± 3% of cells were in G_0/G_1 phase; 3 ± 1% of cells were in S phase; and 14 ± 2% of cells were in G_2/M phase. These data demonstrate that phenylephrine causes cell cycle arrest in both G_0/G_1 and G_2/M phases of the cell cycle.

We recently demonstrated that PE acting through the α_{1B}AR increases the activity of p42^{MAPK} in primary cultured rat hepatocytes [5]. Others have reported that prolonged activation of the MAPK cascade can inhibit DNA synthesis in various types of cells [30], including primary cultures of rat hepatocytes [5]. We next tested whether PE can stimulate the activity of the MAPK cascade in TFG2 cells, and whether this activation may account for the ability of PE to inhibit DNA synthesis in these cells.

3.3. Phenylephrine causes prolonged activation of the MAPK cascade in TFG2 cells

We tested the effect of PE on p42^{MAPK} activity in primary cultures of rat hepatocytes, as well as in TFG2 and mock transfected HepG2 cells. HepG2 cells had a very high basal MAPK activity in comparison to primary hepatocytes without significant alteration in the relative protein expression of p42^{MAPK} (Fig. 3, data not shown). Transfection of HepG2 cells with the α_{1B}AR had little effect on basal MAPK specific activity. PE treatment of TFG2, but not mock transfected, cells caused a further ~2-fold increase in p42^{MAPK} specific activity, which remained elevated for up to 24 h (Fig. 3). In contrast, primary hepatocytes which had been in culture for 90 min possessed a much lower basal p42^{MAPK} activity than HepG2 cells, and responded to PE treatment by a transient, ~5-fold increase in MAPK specific activity which lasted ~20 min before returning to basal levels (Fig. 3). These data are in general agreement with those obtained earlier in freshly isolated hepatocytes [5]. The PE-dependent activation of p42^{MAPK} was similarly increased in TFG2 cells by NE and EPI, and the effects of all 3 agonists were blocked by prazosin (data not shown).

3.4. Prolonged activation or prolonged inhibition of MAPK signaling has anti-proliferative effects in TFG2 cells

To determine if the agonist-induced changes in growth rate in TFG2 cells were p42^{MAPK} dependent, TFG2 cells were treated with PE in the presence of either the specific MEK1 inhibitor, PD98059 (50 μM), or in cells infected with a dominant negative (−/−) MEK1 recombinant adenovirus. Basal p42^{MAPK} activity was decreased by ~80% after either the addition of 50 μM PD98059 or infection with the −/− MEK1 adenovirus, which confirms that signaling by the

p42^{MAPK} pathway was blocked by both of these treatments (data not shown). Treatment of TFG2 cells with PD98059 or expression of −/− MEK1 decreased DNA synthesis, suggesting that MAPK activity plays a role in maintaining the basal rate of growth in these cells (in agreement with data in [5,21]). Treatment of TFG2 cells with PE also decreased DNA synthesis (Fig. 4A). In contrast, co-treatment of TFG2 cells with PE and either PD98059 or −/− MEK1 returned DNA synthesis back to near-control levels (Fig. 4A). Thus a reduction in the ability of PE to stimulate p42^{MAPK} activity prevented PE from inhibiting DNA synthesis, and reversed the inhibitory effects of PD98059 and −/− MEK1 on basal DNA synthesis. Based on our data in primary hepatocytes, it is possible that other PE-stimulated pathways, e.g. p38^{SAPkinase}, play a role in the ability of the agonist to stimulate proliferation when MAPK activity is reduced [5]. These observations suggest that the α_{1B}AR-mediated inhibition of DNA synthesis in TFG2 cells is p42^{MAPK} dependent.

3.5. *Prolonged activation of the MAPK cascade in TFG2 cells induces the cyclin dependent kinase inhibor (CKI) protein, p21$^{Cip1/WAF1}$, by a MAPK-dependent mechanism*

Prolonged elevation of MAPK activity has been reported to decrease DNA synthesis via induction of cyclin dependent kinase inhibitor (CKI) proteins in other cells [21,28,29]. Since PE treatment of TFG2 cells also caused prolonged MAPK activation and inhibited DNA synthesis, we next examined the ability of PE to increase expression of CKI proteins via the MAPK cascade. PE treatment of TFG2 cells, but not mock transfected cells or primary hepatocytes, increased expression of p21$^{Cip1/WAF1}$ (Fig. 4B), but not p16^{INK4A}, and reduced p27^{Kip1} expression (data not shown), as tested by Western blotting. Pretreatment of cells with either PD98059 or expression of −/− MEK1 adenovirus blocked the ability of PE to increase p21$^{Cip1/WAF1}$ expression. Treatment of the cells

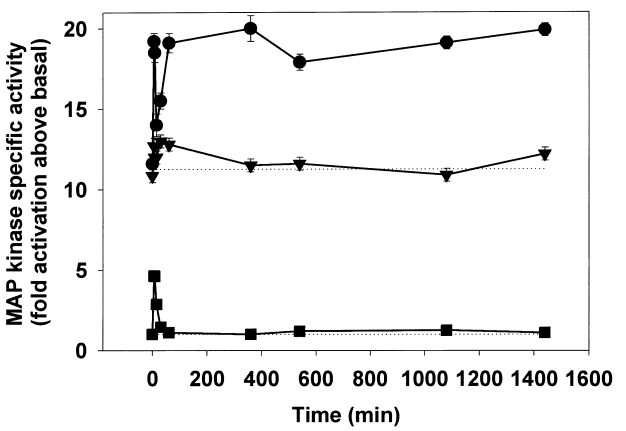

Fig. 3. Phenylephrine causes transient or prolonged activation of MAPK in primary hepatocytes or TFG2 cells, respectively. Primary hepatocytes (■), control transfected HepG2 cells (▼), and TFG2 cells (●) were cultured as described in Section 2. Cells were treated with phenylephrine (10 μM) and p42^{MAPK} was determined at the indicated times (0–24 h), as described in Section 2. Data are means of duplicate values (differing by $<10\%$) from a representative of three independent experiments and are expressed as -fold increase in ^{32}P-incorporation into MBP/min/mg cell protein (specific activity) over the specific activity of MAPK in primary hepatocytes at zero time. Basal MAPK specific activity was 0.14 ± 0.01 pmol/min/mg in primary hepatocytes, 1.52 ± 0.03 pmol/min/mg in control HepG2 cells, and 1.63 ± 0.02 pmol/min/mg in TFG2 cells.

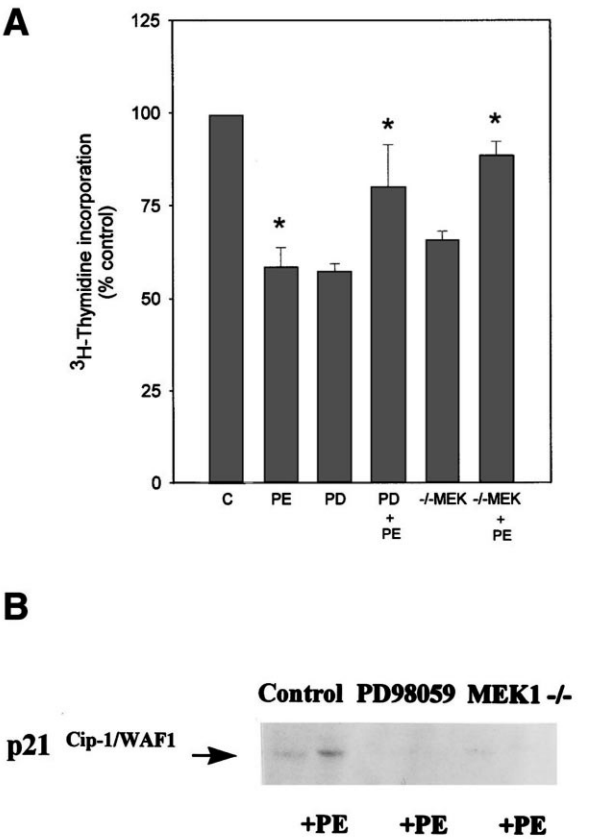

Fig. 4. Phenylephrine, via an MAPK dependent mechanism, inhibits DNA synthesis in TFG2 cells by increasing expression of the cyclin dependent kinase inhibitor protein p21$^{Cip1/WAF1}$. TFG2 cells were cultured as in Section 2. Twenty four hours prior to hormone addition, a portion of the cells was infected with a recombinant adenovirus to express dominant negative MEK1 (−/− MEK). Thirty minutes prior to addition of 10 μM phenylephrine (PE)+2 μCi [^{3}H]thymidine, aliquots of the cells were treated with either vehicle or 50 μM PD98059 (PD). Cells were harvested 48 h later, with one aliquot used for determination of [^{3}H]thymidine incorporation into DNA (A), and a second aliquot used for Western blotting with anti-p21^{Cip1} (B), as described in Section 2. Note that PE inhibits DNA synthesis in TFG2 cells, which is overcome by MAPK inhibition (A), and increases the expression of the cdk inhibitor protein p21$^{Cip1/WAF1}$ in an MAPK-dependent fashion (B). Data in A are the mean of 3 separate experiments.

with PE, PD89059, or −/− MEK1 adenovirus had little effect upon the expression of cyclins A, D1 or E (data not shown).

To further analyze the observed inhibitory pathway, we examined the ability of PE to modulate the activity of the cyclin dependent kinase cdk2, whose function is essential for progression from G1 to S phase [31]. Treatment of TFG2 cells with PE dramatically reduced cdk2 activity in assays using histone H1 as substrate, from 5.40 ± 0.5 to 0.70 ± 0.06 pmol/mg/min, which is in agreement with the observed induction of p21$^{Cip1/WAF1}$ by the same treatment. In further agreement with the data in Fig. 4, treatment of TFG2 cells with PD98059 alone decreased cdk2 activity to 1.10 ± 0.02 pmol/mg/min. However, co-treatment of cells with PE and PD98059 increased cdk2 activity to 1.90 ± 0.20 pmol/mg/min. These data suggest that PE stimulates a growth promoting pathway that is masked in TFG2 cells by increased growth inhibitory MAPK signaling.

To more conclusively test the role of the CKI p21$^{Cip1/WAF1}$

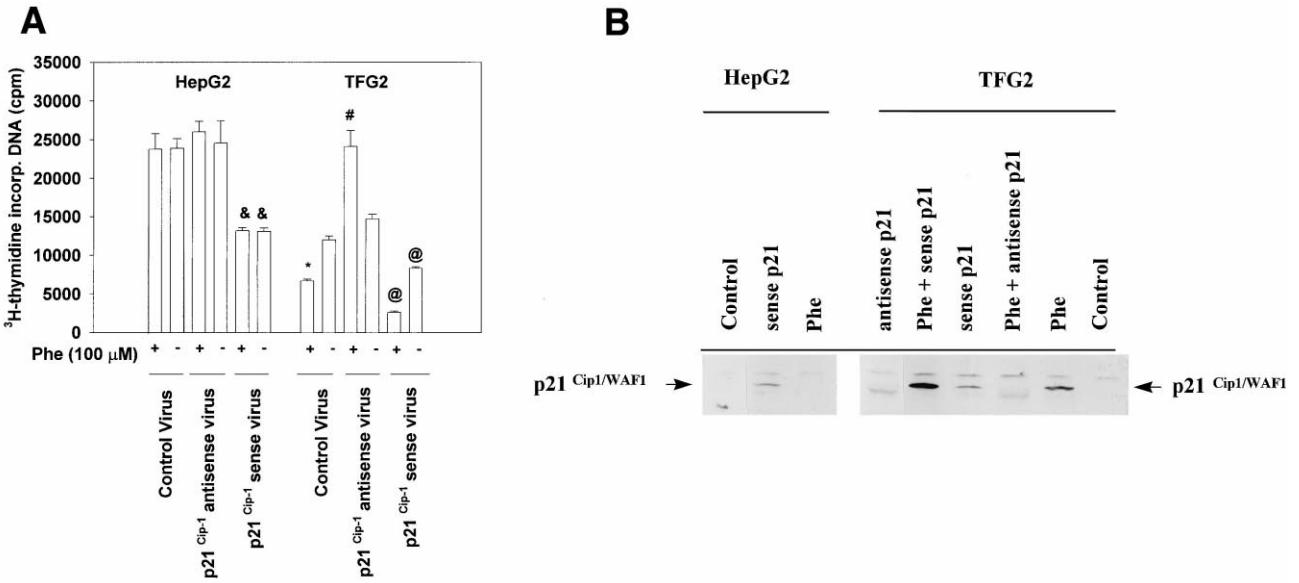

Fig. 5. Phenylephrine increases DNA synthesis in TFG2 cells infected with a recombinant adenovirus to express antisense p21$^{Cip1/WAF1}$. Mock transfected HepG2 cells and TFG2 cells were cultured as in Section 2. Cells were infected with either control adenovirus or p21$^{Cip1/WAF1}$ recombinant adenoviruses at a multiplicity of infection of 200, 24 h prior to the experiment. Cells were treated with either vehicle (−), or 100 μM of PE (+) as indicated, with 10 μCi [^{3}H]thymidine (A) or with vehicle (B). Twenty four hours after the addition of PE, cells in A were harvested and [^{3}H]thymidine incorporation into DNA determined, and cells in B were harvested and processed for SDS-PAGE followed by immunoblotting to determine p21$^{Cip1/WAF1}$ expression. Data are the means ± S.E.M. of 3 separate experiments. & indicates significant difference from corresponding values in control virus-infected HepG2 cells ($P < 0.05$); * and # indicate significant decrease or increase, respectively, from corresponding vehicle-treated TFG2 cells ($P < 0.05$), and @ indicates significant decrease ($P < 0.05$) from corresponding control virus-infected TFG2 cells.

in the PE/MAPK-mediated growth arrest response, we infected cells with recombinant adenoviruses to express either p21$^{Cip1/WAF1}$ antisense mRNA, p21$^{Cip1/WAF1}$ protein, or with control virus (Fig. 5). In HepG2 cells, expression of p21$^{Cip1/WAF1}$ protein blunted DNA synthesis compared to cells infected with control virus (Fig. 5B), whereas expression of p21$^{Cip1/WAF1}$ antisense mRNA had little effect on growth (Fig. 5A). In these cells, PE did not influence thymidine uptake in any of the 3 treatment groups (Fig. 5A), and failed to induce p21$^{Cip1/WAF1}$ expression, as tested in the control group only (Fig. 5B).

However, in TFG2 cells infected with the control virus, PE inhibited DNA synthesis (Fig. 5A) and increased p21$^{Cip1/WAF1}$ expression (Fig. 5B). In TFG2 cells expressing the p21$^{Cip1/WAF1}$ antisense mRNA, PE failed to induce p21$^{Cip1/WAF1}$ expression (Fig. 5B) and stimulated rather than inhibited DNA synthesis (Fig. 5A). Expression of p21$^{Cip1/WAF1}$ protein in TFG2 cells inhibited baseline DNA synthesis, which was further inhibited by PE treatment (Fig. 5A). These data suggest that inhibition of DNA synthesis via activation of α_{1B} receptors is a MAPK/p21$^{Cip1/WAF1}$-dependent event.

To confirm by an independent methodology that expression of p21$^{Cip1/WAF1}$ mRNA abrogates cell cycle arrest in response to phenylephrine treatment, flow cytometric analysis was performed on TFG2 cells 48 h after addition of phenylephrine to the media. In untreated cells infected with antisense p21$^{Cip1/WAF1}$, 62 ± 6% of cells were in G_0/G_1; 30 ± 2% of cells were in S phase; and 8 ± 2% of cells were in G_2/M phase. This data is very similar to that obtained in uninfected TFG2 cells. In TFG2 cells infected with antisense p21$^{Cip1/WAF1}$ and treated with 10 μM phenylephrine, 53 ± 2% of cells were in G_0/G_1 phase; 37 ± 4% of cells were in S phase; and 10 ± 2% of cells were in G_2/M phase. These data demonstrate that in the presence of antisense p21$^{Cip1/WAF1}$, PE does not cause cell cycle arrest in TFG2 cells.

4. Discussion

The major finding in the present study is that stimulation of the G protein-coupled α_{1B}AR has an anti-proliferative effect in a malignant hepatoma cell line continuously expressing the α_{1B}AR. HepG2 cells, stably transfected with the α_{1B}AR, respond to PE treatment with a decrease in DNA synthesis. The receptor specificity of these effects is indicated by their inhibition by the α_1AR antagonist prazosin.

PE treatment of TFG2 cells caused a prolonged activation of the MAPK cascade. Inhibition of the MAPK cascade by a selective inhibitor, PD98059, or by the over-expression of dominant negative −/− MEK1 reduced basal TFG2 cell DNA synthesis and reversed the effect of PE treatment on DNA synthesis from inhibition to stimulation. This indicates that the inhibitory effect of PE upon DNA synthesis in TFG2 cells is mediated via the MAPK cascade, which masks a pro-proliferative, MAPK-independent signal. The independence of this latter response from MAPK activity is in agreement with our earlier observations in primary cultured rat hepatocytes, in which the α_1-adrenergic stimulation of DNA synthesis was significantly blunted by an inhibitor of the p38^{SAPkinase}, but only weakly attenuated by an inhibitor of the MAPK pathway [5]. Our data suggest that prolonged activation or prolonged inhibition of the MAPK cascade can both reduce DNA synthesis in TFG2 cells.

Of note, however, is that over-expression of the α_{1B}AR did

not significantly increase basal MAPK activity, whilst it did cause a significant decrease in proliferative capacity. Thus our data also argue that increased expression of the unstimulated α_{1B}AR inhibits HepG2/TFG2 cell growth by an additional, MAPK-independent, mechanism.

Three lines of evidence suggest that the major anti-proliferative effect of α_{1B}AR activation in TFG2 cells is mediated via MAPK activation and increased protein levels of the CKI $p21^{Cip1/WAF1}$. First, treatment of cells with PD98059 or the expression of −/− MEK1 reversed the response to α_{1B}AR stimulation from a growth inhibitory to a growth stimulatory one, and also increased expression of $p21^{Cip1/WAF1}$. Second, increased expression of $p21^{Cip1/WAF1}$ in TFG2 cells resulted in decreased cdk2 activity and reduced DNA synthesis. Third, expression of $p21^{Cip1/WAF1}$ antisense mRNA abrogated both the α_{1B}AR-induced increase in $p21^{Cip1/WAF1}$ expression, the parallel inhibition of DNA synthesis, and cell cycle arrest. These findings strongly suggest that α_{1B}AR stimulation inhibits its DNA synthesis in TFG2 cells via the MAPK pathway and $p21^{Cip1/WAF1}$.

In mammalian cells, the cell division cycle is controlled by the formation and activation of protein kinase complexes, consisting of cyclins and cyclin dependent kinases (CDKs) [32–34]. The CDKs control the cell cycle checkpoints in a coordinated way, thus ensuring the integrity of the genome by preventing the replication of damaged DNA [35]. By inhibiting the kinase activity of the CDKs, CKI proteins interfere with phosphorylation events critical for cell cycle transitions. For example, it has been shown that over-expression of $p21^{Cip1/WAF1}$ can cause cell cycle arrest at the G1/S and G2/M interfaces, most likely as a result of decreased activity of cyclins D and E/cdk2-cdk4 complexes and cyclins A and B/cdc2 complexes, respectively [32–34].

Several studies have suggested that $p21^{Cip1/WAF1}$ is an important mediator of growth arrest [36]. Protein levels of $p21^{Cip1/WAF1}$ can be elevated by an over-expression of a number of transcription factors, such as p53 [37], C/EBPα [38], C/EBPβ [39], Sp1 [40], and MyoD [41], but also by treatment of cells with high concentrations of growth factors and mitogens [21,28]. In particular, prolonged activation of the MAPK cascade in a variety of transformed and established cell types has recently been shown to increase $p21^{Cip1/WAF1}$ expression [21,28–30]. In some of these studies it was also suggested that the ability of MAPK signaling to either increase or decrease proliferation depended upon the amount of signal generated [21,28]. Tombes et al. recently demonstrated in primary hepatocytes that a transient elevation of MAPK activity increased DNA synthesis whereas a prolonged activation of the cascade caused an increase in $p21^{Cip1/WAF1}$ expression, and a decrease in DNA synthesis. The reason why transient and prolonged MAPK signaling have disparate abilities to increase and decrease growth, respectively, is unclear.

It has also been shown that the MAPK-induced, $p21^{Cip1/WAF1}$ dependent cell cycle arrest in G1 can be overcome in various cell types by co-expression of the SV40 large T antigen [42] or by use of $p21^{Cip1/WAF1}$ null cells [30]. These data suggest that transformation of a primary cell requires the loss of function of multiple oncogenes/signaling proteins. For example, malignant transformation is frequently associated with mutations resulting in constitutively active membrane receptors and Ras/Raf kinase signaling [43–45]. Our studies in HepG2 cells suggest that additional mutations resulting in either receptor inactivation or loss of α_{1B}AR expression may also have additional growth advantages for hepatoma cells. Removal of a potential growth inhibitory signal in hepatoma cells, such as signaling by the α_{1B}AR, will reduce the ability of these cells to cell cycle arrest. In this respect, it is interesting to note that a number of malignant hepatoma cell lines have all been found to be devoid of α_1AR [19], which is in sharp contrast to the high level of α_1AR expression in normal liver tissue. These data are also similar to those obtained in A431 squamous carcinoma cells exposed to epidermal growth factor (EGF) [46,47]. A431 cells express high levels of the EGF receptor, and respond to this agonist by undergoing a $p21^{Cip1/WAF1}$ mediated growth arrest. Further studies are aimed to test whether stable transfection of α_{1B}AR into hepatoma cells reduces their in vivo tumorigenic potential.

Basal levels of $p42^{MAPK}$ activity were found to be considerably higher in HepG2/TFG2 cells than in primary cultures of hepatocytes (Fig. 4). As a result, even though the peak stimulation by PE was greatest in the primary cells, the total amount of MAPK specific activation induced by catecholamines was actually greater in HepG2/TFG2 cells than in primary hepatocytes. This increase in the amount of MAPK specific activity in HepG2/TFG2 cells may be responsible for switching the effect of catecholamine exposure from being mitogenic to anti-proliferative, in agreement with other published observations [5,30].

Data presented by Tombes et al. suggested that a sustained ∼4-fold elevation in MAPK activity, to ∼0.50 pmol/min/mg, can increase $p21^{Cip1/WAF1}$ expression in primary hepatocytes and inhibit DNA synthesis [5]. However, the *specific activity* of MAPK in primary hepatocytes which induced $p21^{Cip1/WAF1}$ in those studies is ∼3-fold less than the basal unstimulated specific activity of MAPK in HepG2/TFG2 cells (∼1.6 pmol/min/mg), which do not express significant levels of $p21^{Cip1/WAF1}$ protein (Fig. 5B). Together, these findings suggest that the threshold at which MAPK signaling can elevate $p21^{Cip1/WAF1}$ expression has increased in transformed vs. primary hepatocytes. An increase in this threshold will potentiate the ability of MAPK signaling to cause proliferation vs. cell cycle exit in the transformed hepatocytes. The change in threshold is unlikely to be due to altered p53 function since both primary hepatocytes and HepG2/TFG2 cells express wild-type p53 [48]. Since both primary hepatocytes and HepG2 cells also express $p21^{Cip1/WAF1}$, these data suggest that −53-independent mechanisms downstream of MAPK signaling and upstream of $p21^{Cip1/WAF1}$ expression may have become defective during the process of hepatocellular transformation.

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