Published in final edited form as:

J Cell Physiol. 2008 August ; 216(2): 486–497. doi:10.1002/jcp.21420.

Alpha-1 Adrenergic Receptor Transactivates Signal Transducer and Activator of Transcription-3 (Stat3) through Activation of Src and Epidermal Growth Factor Receptor (EGFR) in Hepatocytes

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Abstract

Hepatocytes express adrenergic receptors (ARs) that modulate several functions, including liver regeneration, hepatocyte proliferation, glycogenolysis, gluconeogenesis, synthesis of urea and fatty acid metabolism. Adrenergic hepatic function in adults is mainly under the control of α_1 -ARs; however, the mechanism through which they influence diverse processes remains incompletely understood. This study describes a novel α_1 -AR-mediated transactivation of signal transducer and activator of transcription-3 (Stat3) in primary and transformed hepatocytes. Treatment of primary rat hepatocytes with the α_1 -AR agonist, phenylephrine (PE), induced a rapid phosphorylation of Stat3. PE also increased Stat3 phosphorylation, DNA binding and transcription activity in transformed human hepatocellular carcinoma cells (Hep3B). The PE-induced Stat3 phosphorylation, DNA binding and reporter activity were completely blocked by the selective α_1 -AR antagonist, prazosin. In addition, transfection of Hep3B cells with human α_{1B} -AR expression vector also enhanced Stat3 phosphorylation and reporter activity. Moreover, overexpression of RGS2, a protein inhibitor of $G_{q/11}$ signaling, blocked PE-induced Stat3 phosphorylation and reporter activity. The observations that PE induced the formation of c-Src-Stat3 binding complex and phosphorylation of epidermal growth factor receptor (EGFR) and that inhibiting Src and EGFR prevented PE-induced Stat3 activation indicate the involvement of Src and EGFR. Taken together, these observations demonstrate a novel α_1 -AR-mediated Stat3 activation that involves $G_{q/11}$, Src and EGFR in hepatic cells.

Keywords

alpha1-adrenergic receptor; signal transducer and activator of transcription-3; hepatocyte; epidermal growth factor receptor; phenylephrine

INTRODUCTION

The liver constitutes a unique, multicellular organ whose functions are crucial for the overall physiology of humans. The natural catecholamines bind to adrenergic receptors (AR) in hepatic cells and regulate a wide variety of such functions including modulation of carbohydrate, lipid, and amino acid metabolism. Adult hepatocytes express both α_1 and β_2 -ARs, with distinct functions and signaling pathways (Exton, 1980). In addition to its role in metabolism, hepatic α_1 -AR is also involved in regulation of hepatocyte proliferation and liver regeneration after hepatic injury or partial hepatectomy (Cruise et al., 1985; Cruise et al., 1987; Michalopoulos and DeFrances, 2005; Michalopoulos and DeFrances, 1997). For example, norepinephrine is

one of the several substances that increase in the plasma following partial hepatectomy (Cruise et al., 1987; Michalopoulos and DeFrances, 2005; Michalopoulos and DeFrances, 1997). α_1 -AR agonists including phenylephrine have been shown to enhance hepatocyte proliferation in the presence of epidermal growth factor (EGF) or hepatocyte growth factor (HGF) (Cruise et al., 1985; Cruise et al., 1987; Cruise and Michalopoulos, 1985; Knopp et al., 1997; Lindroos et al., 1991; Michalopoulos and DeFrances, 1997; Spector et al., 1997). Norepinephrine also increases synthesis of HGF in fibroblasts (Brotan et al., 1999) and catecholamines also increase synthesis of EGF from Brunner's glands of the duodenum (Olsen et al., 1985). In view of all these direct and indirect effects enhancing hepatocyte proliferation after partial hepatectomy, it is not surprising that blockade of the α_1 -AR abolishes DNA synthesis for the first 24 hours after partial hepatectomy (Cruise et al., 1987). Recent work also suggests that norepinephrine may be a trophic factor for hepatic stellate cells and that it may also be produced by the stellate cells, acting in an autocrine manner (Oben et al., 2004; Sancho-Bru et al., 2006). All of these findings underscore the importance of norepinephrine as a regulator of liver regeneration and the complexity of pathways involved in these regulatory processes need to be better understood.

α_1 -ARs belong to the seven transmembrane domain G protein-coupled receptor superfamily and include three subtypes (α_{1A} , α_{1B} , and α_{1D}), all of which couple to $G_{q/11}$ and regulate phospholipase C (Bylund et al., 1994; Hieble et al., 1995; Minneman and Esbenshade, 1994). Activation of α_1 -AR by catecholamine leads to activation of phosphoinositide-specific phospholipase C and generation of inositol-1,4,5-trisphosphate and diacylglycerol, which mediate the activation of protein kinase C and intracellular Ca^{2+} release, respectively. This classical mechanism is thought to mediate α_1 -AR effect in different cells including hepatocytes. Additionally, α_1 -AR has also been linked to other intracellular cascades in several extrahepatic cell types, including activation of phospholipase A_2 (Xing and Insel, 1996), phospholipase D (Ruan et al., 1998) and MAP kinases (Williams et al., 1998), production of reactive oxygen species and activation of NADPH oxidase (Xiao et al., 2002). These responses appear to be tissue- and cell type-specific and it remains obscure whether alternative signaling mechanisms are involved in hepatic α_1 -AR actions.

In the liver, Stat3 (signal transducer and activator of transcription-3) has been shown to play a key role in acute phase response, protection against liver injury, promotion of liver regeneration, glucose homeostasis, and hepatic lipid metabolism (Gao, 2005). Stat3 in the liver is mainly activated by IL-6 and its related cytokines, IL-22, IL-10, EGF, HGF, and hepatitis B and C viral proteins (Gao, 2005; Gong et al., 2001; Schaper et al., 1997; Waris et al., 2001). Disruption of the Stat3 gene impairs liver regeneration (Li et al., 2002) and causes insulin resistance associated with increased hepatic expression of gluconeogenic genes (Inoue et al., 2004). Overexpression of constitutively activated Stat3 reduces blood glucose, plasma insulin concentrations and hepatic glucogenic gene expression in diabetic mice (Inoue et al., 2004) and protects against Fas-induced fulminant hepatitis via redox-dependent and -independent mechanisms (Haga et al., 2003). Mice with disruption of IL-6 show impaired liver regeneration (Cressman et al., 1996; Sakamoto et al., 1999) and are susceptible to liver injury induced by carbon tetrachloride (Kovalovich et al., 2000), Fas (Kovalovich et al., 2001) and ethanol (Hong et al., 2002). These findings suggest that IL-6/Stat3 signaling plays an important role in liver regeneration and protection against liver injury. In addition, treatment with IL-6 or overexpression of constitutively activated Stat3 *in vivo* ameliorates fatty liver disease (Hong et al., 2004; Inoue et al., 2004), indicating that IL-6/Stat3 signaling is also involved in fatty acid metabolism. Furthermore, activation of Stat3 and subsequent induction of anti-apoptotic and proliferation associated genes appear to contribute to the hepatoprotective and mitogenic effect of IL-22 in the liver (Radaeva et al., 2004).

Thus, evidence is compelling that both α_1 -AR and Stat3 importantly regulate various physiological functions in the liver. In this report, we describe a novel link between the α_1 -AR

and Stat3 signaling pathways in primary and transformed hepatocytes. Our findings show that activation of α_1 -AR by its selective agonist, phenylephrine, induced Stat3 phosphorylation, DNA binding and transcription activity, which were completely blocked by the selective α_1 -AR antagonist, prazosin. Accordingly, transfection of human α_{1B} -AR expression vector also enhanced Stat3 phosphorylation and reporter activity. Furthermore, our data provide evidence for the involvement of $G_{q/11}$, c-Src and EGFR in α_1 -AR-mediated Stat3 activation in hepatic cells.

MATERIALS AND METHODS

Materials

Minimum essential medium (MEM), fetal bovine serum, glutamine, antibiotics and the Lipofectamine plusTM reagent and LipofectamineTM 2000 reagent were purchased from Invitrogen (Carlsbad, CA). Phenylephrine hydrochloride (α_1 -adrenergic agonist), isoproterenol hydrochloride (β -adrenergic agonist), prazosin hydrochloride (α_1 -adrenergic antagonist) and pertussis toxin were purchased from Sigma (St Louis, MO). Recombinant murine IL-6 and human IL-6 were from Inclone System (Somerville, NJ). The Src-family tyrosine kinase inhibitor 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and the EGFR tyrosine kinase inhibitor AG 1478 were purchased from Calbiochem (San Diego, CA). The antibodies against Stat3, EGFR, c-Src, PARP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against phospho-Stat3 (Tyr⁷⁰⁵) or (Ser⁷²⁷), phospho-EGFR (Tyr⁸⁴⁵), (Tyr⁹⁹²) or (Tyr¹⁰⁶⁸) were purchased from Cell Signaling (Beverly, MA). Adrenergic receptor α_{1B} expression plasmid, adrenergic receptor β_2 expression plasmid and RGS2 expression plasmid were purchased from UMR cDNA Resource Center (Rolla, MO). Translucent STAT3(1)-Luc reporter vector which is designed to measure transcriptional activity of Stat3 and pCRE-Luc reporter vector which is designed to measure transcriptional activity of cAMP response element (CRE) were purchased from Panomics (Redwood City, CA). Horseradish peroxidase-linked streptavidin and chemiluminescence detection reagents were purchased from Amersham Pharmacia Biotech Inc (Piscataway, NJ).

Preparation of rat primary hepatocytes

Rat hepatocytes were isolated from male Fisher 344 rats (Harlan, Indianapolis, IN) using the modified two-step collagenase perfusion technique as previously described (Runge et al., 2000; Seglen, 1976). Freshly isolated hepatocytes of >90% viability, as assessed by trypan blue exclusion, were placed on rat-tail collagen I-coated culture dishes or plates in Williams' E medium supplemented with 5% calf serum. The cells were incubated at 37°C (5% CO₂) and checked for adherence of monolayers after 2 to 4 hours. Once adhered, the cells were subjected to treatment with phenylephrine, prazosin, PP2, or AG1478 as indicated in the text.

Hepatocyte DNA Synthesis

To determine *in vitro* DNA synthesis, 1 μ Ci [³H]thymidine (PerkinElmer, Boston) was added to the cultured hepatocytes in each well of 6-well plates. The cells were treated with specific reagents as indicated in the text. After overnight incubation, the hepatocytes were harvested and [³H]thymidine incorporation was measured using a scintillation counter (Wang et al., 1998).

Culture of Hep3B cells and transfection assays

The human hepatoma cell line, Hep 3B, was cultured according to our previously described methods (Han et al., 2006b; Leng et al., 2003). The cells were cultured in EMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine. The culture plates were maintained at 37°C in a humidified CO₂ incubator. For transient transfection assays, the cultured cells were

transfected with the α_{1B} -AR expression plasmid, β_2 -AR expression plasmid, RGS2 expression plasmid, or pcDNA control plasmid using Lipofectamine plusTM reagent.

Luciferase reporter assay

The cells were seeded at a concentration achieving 80% confluence in 12-well plates eighteen hours before transfection. The cells were transiently co-transfected with 0.2 μ g/per well translucent Stat3(1)-Luc reporter vector or pCRE-Luc reporter vector with or without 0.1 μ g of a plasmid containing a cytomegalovirus promoter-driven β -galactosidase gene (pIEP-LacZ). After transfection, the cells were treated with specific reagents as indicated. The cell lysates were then obtained with 1X reporter lysis buffer (Promega). The luciferase activity was assayed in a Berthold AutoLumat LB 953 luminometer (Nashua, NH) by using the luciferase assay system from Promega. β -Galactosidase activity was determined as recommended (Promega), using a 96-well multiplate reader with SOFTMAX software (Molecular Devices). The relative luciferase activity was calculated after normalized to either β -galactosidase activity or cellular proteins. All values are expressed as –fold induction relative to basal activity.

Preparation of cellular protein

At the end of each indicated treatment, the cells were scraped off the plates and centrifuged, washed twice with cold phosphate-buffered saline (PBS) containing 0.5 mM PMSF and 10 μ g/ml leupeptin and resuspended in 5-fold volume of hypotonic buffer consisting of 50 mM HEPES pH 7.55, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail tablets (Roche Diagnostics GmbH) and phosphatase inhibitor. After sonication, the whole cell lysate was collected by centrifugation at the speed of $16,000 \times g$ at 4°C for 10 minutes to remove cell debris and stored in aliquots at –20°C until use. The protein concentrations in the cell extracts were determined by the Bio-Rad protein assay (Bio-Rad, CA) using BSA as a standard.

Preparation of nuclear protein

The nuclear proteins from control or stimulated cells were extracted with the Nuclear Extraction Kit from Chemicon International (Temecula, CA) according to the manufacturer's instructions. Briefly, the cultured cells were washed and scraped into phosphate-buffered solution and centrifuged at 4,500 rpm for 5 min. The pelleted cells were suspended in cytoplasmic lysis buffer at approximately 5 times the packed cell volume and lysed by gentle pipetting. Nuclei were recovered by centrifugation at $8000 \times g$ for 20 minutes. The nuclear proteins were extracted by gentle resuspension of the nuclei at approximately 2 times the packed nuclear volume of Nuclear Extraction Buffer, followed by 30 minutes of platform rotation. The nuclear protein suspension was cleared by centrifugation at $16,000 \times g$ for 5 minutes. The supernatants were collected and frozen at –80°C. All buffers contained DTT, protease inhibitor cocktail and phosphatase inhibitor. All the steps were carried out on ice or at 4°C. The protein concentrations in the nuclear extracts were measured by the Bio-Rad protein assay (Bio-Rad, CA) using BSA as a standard.

Immunoblotting

30 μ g of either cellular or nuclear proteins were subjected to SDS-PAGE on 4–20% Tris-glycine gels (Invitrogen, CA) for Stat3, phosph-Stat3 and β -actin; or on 6% Tris-glycine gels (Invitrogen, CA) for EGFR, phosph-EGFR, and PARP. The separated proteins were electrophoretically transferred onto the nitrocellulose membranes (BioRad, CA). Nonspecific binding was blocked with PBS-T (0.5% Tween 20 in PBS) containing 5% non-fat milk for 1 hr at room temperature. The membranes were then incubated overnight at 4°C with individual primary antibodies in PBS-T containing 1% non-fat milk at the dilutions specified by the manufacturers. Antibodies against phospho-EGFR (Tyr⁸⁴⁵), (Tyr⁹⁹²) and (Tyr¹⁰⁶⁸) were utilized for EGFR phosphorylation. Antibodies against phospho-Stat3 (Tyr⁷⁰⁵) and (Ser⁷²⁷)

were utilized for Stat3 phosphorylation. Following three washes with PBS-T, the membranes were then incubated with the horseradish peroxidase-conjugated secondary antibodies at 1:10,000 dilution in PBS-T containing 1% non-fat milk for 1 hour at room temperature. The membranes were then washed 3 times with PBS-T and the protein bands were visualized with the ECL Western blotting detection system according to the manufacturer's instructions.

Biotinylated oligonucleotide precipitation assays

These experiments were performed as previously described (Han et al., 2006a; Hata et al., 2000). The sequences of biotinylated oligonucleotides corresponding to Stat3 binding site are forward: 5'-

TGCTTCCCGAATTCCCGAATTCCCGAATTCCCGAATTCCCGAATTCCCGAACG
T-3'; and reverse: 5'-ACGTTTCGGGAAT

TCGGGAATTCGGGAATTCGGGAATTCGGGAATTCGGGAAGCA-3'. The 5'-biotinylated oligonucleotides were synthesized by Sigma-Genosys (Woodland, Texas). Cellular extracts were prepared as described above. Binding reactions in nuclear extracts were carried out at 4°C for 16 hours with 1 µg biotinylated double-strand oligonucleotides and 10 µg poly(dI-dC).poly(dI-dC). The DNA-bound proteins were precipitated using ImmunoPure streptavidin-agarose beads (Pierce, Rockford, IL) at 4°C for 1 hour and subjected to detect Stat3 by western blotting.

Binding of Src to Stat3

The binding complexes of Src and Stat3 were determined by immunoprecipitation and western blot. Serum starved cells were treated with phenylephrine in the presence or absence of prazosin as indicated in the text. Cellular extracts were prepared for immunoprecipitation with antibody against c-Src. The immunoprecipitants were then subjected to SDS-PAGE and Western blotting to detect Stat3.

RESULTS

Activation of α_1 -AR by phenylephrine induces Stat3 phosphorylation, DNA binding and transcription in hepatocytes

We first determined the effect of the α_1 -AR ligand, phenylephrine (PE), on Stat3 phosphorylation in rat primary hepatocytes. The isolated cells were utilized for experiments within 6 hours to prevent the possibility of AR down-regulation during prolonged culture. As shown in Figure 1A and B, activation of α_1 -AR by PE induced the phosphorylation of Stat3 in primary hepatocytes, which was observed 15 minutes after treatment. A similar effect was also observed in transformed hepatocytes (Hep3B) (Figure 1C and D). The phenylephrine-induced Stat3 phosphorylation was inhibited by co-treatment with prazosin, a selective α_1 -AR antagonist in both primary and transformed hepatocytes (Figure 1E and F). The level of Stat3 phosphorylation in PE-treated cells was approximately 40–50% of that in IL-6 treated cells (Figure 2A and B).

Transient transfection and reporter activity assay was next performed to determine the effect of α_1 -AR ligand activation on Stat3 transcription activity. As shown in Figure 3A, PE induced the binding of Stat3 to its DNA response element in Hep3B cells, as determined by the biotinylated chromatin immunoprecipitation assay. Accordingly, PE treatment also increased the Stat3-mediated luciferase reporter activity in these cells (Figure 3B). Both the Stat3 DNA binding and reporter activity induced by PE treatment were blocked by the α_1 -AR antagonist, prazosin. These findings suggest that Stat3 is a downstream signaling molecule of α_1 -AR.

Overexpression of α_{1B} -AR, but not β_2 -AR, increases Stat3 phosphorylation and transcription

We next performed transfection experiments to determine the direct effect of α_1 -AR overexpression on Stat3 activation in transformed hepatocytes. As shown in Figure 4, Hep3B cells transfected with α_{1B} -AR showed increased Stat3 phosphorylation as well as increased Stat3 transcription activity. Phenylephrine treatment of α_{1B} -AR overexpressing cells only marginally increased Stat3 reporter activity when compared with vehicle treatment of α_{1B} -AR overexpressing cells, which is likely due to autoactivation of the transfected receptor. The specificity of α_{1B} -AR transfection is further verified by the observation that transfection with β_2 -AR enhanced the CRE (cAMP response element) reporter activity but not Stat3 reporter activity (Figure 4). These findings provide further molecular evidence for the activation of Stat3 by α_1 -AR.

Inhibition of $G_{q/11}$ signaling by RGS2 prevents phenylephrine-induced Stat3 phosphorylation and transcription

Since α_1 -AR is coupled to $G_{q/11}$, further experiments were performed to determine whether overexpression of RGS2, which inhibits $G_{q/11}$ signaling, would alter α_1 -AR-mediated Stat3 activation. RGS2 is a member of the protein family termed Regulators of G-protein Signaling (RGS), which regulate G protein coupled receptors through binding $G\alpha$ -GTP to increase the rate of GTP hydrolysis and rapidly terminate responses; RGS2 selectively inhibits $G\alpha_{q/11}$ and thus blocks the function of $G\alpha_{q/11}$ -coupled receptors including α_1 -AR. As shown in Figure 5A and B, RGS2 overexpression in Hep3B cells inhibited PE-induced Stat3 phosphorylation and transcription activity. In parallel control experiments, RGS2 overexpression did not affect isoproterenol-induced CRE reporter activity, a process mediated by G_s activation (Figure 5C). Furthermore, pretreatment with pertussis toxin, which inhibits G_i , also had no effect on PE-induced Stat3 transcription activity (Figure 5D). These results suggest the involvement of pertussis toxin-insensitive $G_{q/11}$ in α_1 -AR-mediated Stat3 activation.

Activation of α_1 -AR by phenylephrine enhances Src binding to Stat3

Further experiments were carried out to identify the possible down-stream molecules that mediate Stat3 activation in our system. Since Src can bind and activate Stat3 in other cells, we performed immunoprecipitation and western blot analysis to determine whether α_1 -AR might influence Src-association with Stat3. As shown in Figure 6, low level of Src-Stat3 binding complex is detected under basal conditions; activation of α_1 -AR by PE enhanced Src binding to Stat3 (the protein levels of Stat3 and Src were not altered). The PE-induced Src-Stat3 binding was completely blocked by cotreatment with prazosin. These results suggest that Src is a down-stream target of α_1 -AR that may regulate Stat3 activity in hepatocytes. The importance of Src in α_1 -AR-induced Stat3 activation is further supported by the observation that PE-induced Stat3 phosphorylation, DNA binding and transcription activity were blocked by the Src inhibitor, PP2 (Figure 8).

Activation of α_1 -AR by phenylephrine induces the phosphorylation of EGFR

Since EGFR is a key receptor tyrosine kinase that is involved in Stat3 activation, we next examined whether EGFR might be involved in α_1 -AR-induced Stat3 activation in hepatocytes. As shown in Figure 7, treatment of primary and transformed hepatocytes with phenylephrine induced a rapid phosphorylation of EGFR (within 15 minutes). The PE-induced Stat3 phosphorylation was blocked by the EGFR kinase inhibitor, AG1478, in both primary hepatocytes and Hep3B cells (Figure 8A and B). Similarly, the PE-induced Stat3 DNA binding and transcription activity were also blocked by the AG1478 (Figure 8C and D). These findings suggest the involvement of EGFR in α_1 -AR-induced Stat3 activation.

Hepatocyte DNA synthesis is inhibited by prazosin, PP2 and AG1478

To further determine the role of α_1 -AR-mediated signaling in hepatocyte proliferation, the effect of prazosin, PP2 and AG1478 on [3 H]thymidine incorporation was examined in rat primary hepatocytes. As shown in Figure 9, rat hepatocytes treated with PE alone showed only slight increase of [3 H]thymidine incorporation (by approximately 20%); this phenomenon has been previously documented (maximal mitogenic effect is achieved when the hepatocytes are treated with α_1 -AR agonists plus EGF or HGF) (Cruise et al., 1985; Cruise et al., 1987; Cruise and Michalopoulos, 1985; Knopp et al., 1997; Lindroos et al., 1991; Michalopoulos and DeFrances, 1997; Spector et al., 1997). It is of note that rat hepatocytes treated with prazosin, PP2 or AG1478 showed significantly decreased DNA synthesis when compared with cells treated with vehicle or PE. These findings, along with the previous reports, further indicate the role of α_1 -AR, Src and EGFR in hepatocyte proliferation.

DISCUSSION

The central and peripheral actions of catecholamines are mediated by several AR subtypes, which control a variety of functions. Based on their pharmacology, structure and signaling mechanisms, the ARs are classified as α_1 , α_2 , and β , and each class is further subdivided into three subtypes. These receptors belong to the family of seven transmembrane domain receptors, coupled to G proteins (Bylund et al., 1992; Bylund et al., 1994; Minneman, 1988). Classically, one signaling pathway has been associated with each AR type as follows. α_1 -ARs are coupled to $G_{q/11}$ protein, phospholipase C activation and increase of intracellular calcium. α_2 -ARs are coupled to G_i protein, causing inhibition of adenylyl cyclase and decreased cAMP production. β -ARs are coupled to G_s protein, thus increasing adenylyl cyclase activity and cAMP levels. On the other hand, studies have also shown that each AR type may activate signaling pathways other than classical ones in various cells and tissues.

α_1 -AR plays an important role in the sympatho-adrenal response to stress, such as peripheral vasoconstriction, increased cardiac contractility and hepatic glycogenolysis. Besides such short term effects, α_1 -AR also modulates the growth of a number of cells, including hepatocytes (Cruise et al., 1985; Cruise et al., 1987; Cruise and Michalopoulos, 1985; Spector et al., 1997), cardiomyocytes (Boluyt et al., 1997; Sah et al., 1996), vascular smooth muscle cells (Chen et al., 1995; Hu et al., 1996), and fibroblasts (Allen et al., 1991). In addition, activation of α_1 -AR has also been implicated in uncontrolled cell growth and malignant transformation, as exemplified by focus formation and disordered growth of Rat-1 fibroblasts transfected with the α_{1B} -AR and enhanced tumor formation when the transfected cells were inoculated into nude mice (Allen et al., 1991). However, the mechanisms by which α_1 -ARs regulate diverse cellular functions remain to be further defined. Our findings in this study establish Stat3 as a novel α_1 -AR downstream target in hepatic cells. This conclusion is based on the following observations: (1) the α_1 -AR agonist phenylephrine induced Stat3 phosphorylation in primary and transformed hepatocytes; (2) phenylephrine treatment increased Stat3 binding to its DNA response element; (3) phenylephrine treatment increased Stat3 transcription activity; (4) transfection with human α_{1B} -AR expression vector enhanced Stat3 phosphorylation and reporter activity; and (5) the phenylephrine-induced Stat3 phosphorylation, DNA binding and reporter activity were blocked by the selective α_1 -AR antagonist, prazosin. In this context, it is worth mentioning that two other GPCRs, prostaglandin E_2 and angiotensin II receptors, have also been implicated in Stat3 phosphorylation in cells of bile duct origin (Han et al., 2006a) and vascular smooth muscle cells (Liang et al., 1999).

Although different α_1 -AR subtypes are expressed in the liver of different species (Deighan et al., 2004; Garcia-Sainz et al., 1995), they apparently serve the same functions. In this study we observed that overexpression of RGS2, which inhibits $G_{q/11}$ signaling, blocked α_1 -AR-induced Stat3 phosphorylation and reporter activity. These findings suggest the involvement of $G_{q/11}$

in α_1 -AR -induced Stat3 activation and are consistent with the documented coupling of all α_1 -AR subtypes to $G_{q/11}$. The specific effect of RGS2 in our system is further supported by the observations that RGS2 overexpression did not affect isoproterenol-induced CRE reporter activity (which is mediated by G_s activation) and that pertussis toxin (which inhibits G_i) had no effect on α_1 -AR-induced Stat3 activation.

We observed that the Hep3B cells transfected with α_{1B} -AR showed increased Stat3 activation in the absence of PE. The exact mechanism underlying this phenomenon is not clear and remains speculative at this time. In general, the function of transfected receptors including α_1 -AR depends on a number of factors, such as the cellular environment, association with other proteins (including G proteins), receptor level and localization, the internalization, recycling or degradation of the receptor, and the presence or absence of agonists/antagonists, among others (Hein and Michel, 2007; Perez, 2007). Thus, it is conceivable that some of the above factors might influence the activation of transfected receptors. Given that certain cellular proteins are known to bind α_{1B} -AR but not other α_1 -ARs (Hein and Michel, 2007; Hirasawa et al., 2001; Perez, 2007), it is possible that they might affect the activity of transfected α_{1B} -AR but not β_2 -AR. In addition, since oligomerization of α_{1B} -AR is known to affect its function (Lopez-Gimenez et al., 2007), cellular conditions affecting this process might also influence its activation.

c-Src is a member of a family of cytoplasmic tyrosine kinases that have several domains including a catalytic domain, a regulatory domain, and SH2 and SH3 binding domains (Thomas and Brugge, 1997). Activated c-Src is capable of interacting with and activating several substrates including Stat3 (Bromberg et al., 1999; Turkson et al., 1999; Yu et al., 1995). Our data in this study document the involvement of Src in α_1 -AR induced Stat3 activation in hepatic cells. This assertion is supported by the following observations: (1) activation of α_1 -AR induces the formation of Src-Stat3 binding complex; (2) inhibition of α_1 -AR prevents Src binding to Stat3; (3) Src inhibition blocks α_1 -AR-induced Stat3 phosphorylation; (4) Src inhibition prevents α_1 -AR-induced binding of Stat3 to its DNA response element; and (5) Src inhibition attenuates α_1 -AR-induced Stat3 reporter activity. These findings are consistent with the previous studies showing that α_1 -AR activates Src in other cells (Rybin et al., 2007; Yamauchi et al., 2002) and that G proteins are able to directly bind Src, leading to its activation (Ma et al., 2000). However, detailed mechanisms by which α_1 -AR or other G proteins activate Src remain to be further investigated.

Stat3 is normally activated in a regulated fashion when protein ligands bind their specific cell-surface receptors and activate tyrosine kinases (Calo et al., 2003; Levy and Darnell, 2002). It becomes activated by phosphorylation on a single tyrosine residue, dimerizes through reciprocal SH2-phosphotyrosine interaction and accumulates in the nucleus, where it binds DNA and direct transcription of target genes. Growth factor receptors with intrinsic tyrosine kinase activity, such as epidermal growth factor receptor (EGFR) and cMet (the receptor for hepatocyte growth factor) also have been shown to cause Stat3 activation in some cells (Boccaccio et al., 1998; Lo et al., 2005; Schaper et al., 1997). Our results in this study suggest that EGFR is involved in α_1 -AR-induced Stat3 activation in hepatic cells, which is based on the following observations: (1) activation of α_1 -AR induces a rapid phosphorylation of EGFR in primary and transformed hepatocytes, which coincides with Stat3 phosphorylation; (2) inhibition of EGFR prevents α_1 -AR-induced Stat3 phosphorylation; (3) inhibition of EGFR blocks α_1 -AR-mediated Stat3 DNA binding; and (4) inhibition of EGFR blocks α_1 -AR-induced Stat3 reporter activity. These findings are consistent with the observations that EGFR is transactivated by G_q -coupled (Gschwind et al., 2001) as well as G_s -coupled (Bertelsen et al., 2004) receptors. However, it remains to be further defined whether α_1 -AR transactivates EGFR through activation of Src or release of EGFR ligand in hepatic cells. Given that activation of α_1 -AR induces Src-Stat3 binding and that Stat3 is a downstream target of EGFR, it is possible

that Src may serve as EGFR downstream effector to mediate hepatic cell function, although the possibility of Src-induced EGFR activation cannot be excluded. Since the concentration of EGFR and Src inhibitors used in our system is relatively high (10–25 μM), further studies are needed to determine the precise role of EGFR and Src in α_1 -AR-mediated effects and the mechanisms for their actions.

Nguyen and Gao (Nguyen and Gao, 1999) showed that PE inhibits IL-6 activation of STAT3 in hepatic cells by a p42/44 mitogen-activated protein kinase-dependent mechanism, although the authors did not address whether α_1 -AR activation alone might alter Stat3 activity. The focus of our current study is to examine the direct effect of α_1 -AR on Stat3 activation in transformed and primary hepatocytes. Our data indicate that activation of α_1 -AR by PE or overexpression induces Stat3 phosphorylation, DNA binding and transcription activity. However, since the level of Stat3 activation induced by PE is approximately half of that induced by IL-6 (Figure 2), it is not surprising that combinational utilization of PE and IL-6 may cause less Stat3 activation than treatment with IL-6 alone, as described by Nguyen and Gao in their study. Thus, the effect of α_1 AR on Stat3 activation may be influenced by other growth-regulatory signaling pathways, especially the status of IL-6 activation.

In summary, our results in this study demonstrate a novel α_1 -AR-mediated Stat3 activation in hepatic cells which involves activation of $G_{q/11}$, Src and EGFR. These findings provide an insight into some of the mechanisms through which norepinephrine may be acting directly on hepatocytes to enhance hepatocyte proliferation. However, a limitation of this study is the relative high concentration of phenylephrine employed and the modest increase of Stat3 activation by α_1 -AR in cultured hepatic cells. Therefore, further studies are warranted to determine the potential impact of α_1 -AR-mediated EGFR/Stat3 activation in liver injury and regeneration *in vivo*.

Acknowledgments

Supported by the Cancer Research and Prevention Foundation grant (to CH) and the National Institutes of Health grants CA035373, CA103958 (to GKM) and CA102325 and CA106280 (to TW).

ABBREVIATIONS

AR	adrenergic receptor
CRE	cAMP response element
EGFR	epidermal growth factor receptor
GPCR	G protein-coupled receptor
RGS	regulator of G protein signaling
SH	src homology
Stat3	signal transducer and activator of transcription-3
PE	phenylephrine

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Figure 1. Phenylephrine, a selective α_1 -AR agonist, induces the phosphorylation of Stat3 in hepatocytes. This effect is blocked by prazosin, a selective α_1 -AR antagonist

A. Dose-dependent effect of PE on Stat3 phosphorylation in primary hepatocytes. Rat primary hepatocytes were treated with different doses of PE for 15 minutes. The cells were then harvested to obtain nuclear proteins. The obtained samples were subjected to SDS-PAGE and Western blotting to detect Stat3 phosphorylation as described in the Experimental Procedures. PARP level was determined by reprobing the same blot with anti-PARP antibody. Quantitative analysis of Stat3 phosphorylation was performed by determining the ratio between Stat3 phosphorylation and PARP protein levels from three different experiments with densitometry (* $p < 0.01$ compared with control cells).

B. Time-course effect of PE on Stat3 phosphorylation in primary hepatocytes. Rat primary hepatocytes were treated with 50 μ M PE for different times. The cells were then harvested to isolate nuclear proteins. The obtained samples were subjected to SDS-PAGE and Western blot to detect Stat3 phosphorylation and PARP level. Quantitative analysis of Stat3 phosphorylation was performed by determining the ratio between Stat3 phosphorylation and PARP protein levels from three different experiments with densitometry (* $p < 0.01$ compared with control cells).

C. Dose-dependent effect of PE on Stat3 phosphorylation in Hep3B cells. Hep3B cells were treated with different doses of PE for 15 minutes. The cells were then harvested to isolate nuclear proteins. The obtained samples were subjected to SDS-PAGE and Western blot to

detect Stat3 phosphorylation and PARP level. Quantitative analysis of Stat3 phosphorylation was performed by determining the ratio between Stat3 phosphorylation and PARP protein levels from three different experiments with densitometry (* $p < 0.05$ compared with control cells).

D. Time-course effect of PE on Stat3 phosphorylation in Hep3B cells. Hep3B cells were treated with 50 μ M PE for different times. The cells were then harvested to isolate nuclear proteins. The samples were subjected to SDS-PAGE and Western blot to detect Stat3 phosphorylation and PARP level. Quantitative analysis of Stat3 phosphorylation was performed by determining the ratio between Stat3 phosphorylation and PARP protein levels from three different experiments with densitometry (* $p < 0.05$ compared with control cells).

E. Prazosin blocks PE-induced Stat3 phosphorylation in primary hepatocytes. Rat primary hepatocytes were treated with 50 μ M PE for 15 minutes with or without prazosin pretreatment (10 μ M, 30 minutes). The cells were then harvested to isolate nuclear proteins. The obtained samples were subjected to SDS-PAGE and Western blot to detect Stat3 phosphorylation and PARP level. Quantitative analysis of Stat3 phosphorylation was performed by determining the ratio between Stat3 phosphorylation and PARP protein levels from three different experiments with densitometry (* $p < 0.01$ compared with control cells; ** $p < 0.01$ compared with PE treatment).

F. Prazosin blocks PE-induced Stat3 phosphorylation in Hep3B cells. Hep3B cells were treated with 50 μ M PE for 15 minutes with or without prazosin pretreatment (10 μ M, 30 minutes). The cells were then harvested to isolate nuclear proteins. The samples were subjected to SDS-PAGE and western blot to detect Stat3 phosphorylation and PARP level. Quantitative analysis of Stat3 phosphorylation was performed by determining the ratio between Stat3 phosphorylation and PARP protein levels from three different experiments with densitometry (* $p < 0.05$ compared with control cells; ** $p < 0.05$ compared with PE treatment).

Figure 2. The effect of phenylephrine versus IL-6 on Stat3 phosphorylation

A. The effect of phenylephrine versus IL-6 on Stat3 phosphorylation in rat primary hepatocytes. Rat primary hepatocytes were treated with either 10 ng/ml IL-6 or 50 μ M PE and vehicle as control for 15 minutes and then isolated either nuclear proteins to determine Stat3 phosphorylation (top panel) or cellular proteins to determine Stat3 protein level (middle panel) by immunoblotting (* p <0.01 compared with control cells).

B. The effect of phenylephrine versus IL-6 on Stat3 phosphorylation in Hep 3B cells. The serum-starved Hep 3B cells were treated with either 10 ng/ml IL-6 or 50 μ M PE and vehicle as control for 15 minutes and then isolated either nuclear proteins to determine Stat3 phosphorylation (top panel) or cellular proteins to determine Stat3 protein level (middle panel) by immunoblotting (* p <0.01 compared with control cells).

Figure 3. Phenylephrine increases Stat3 DNA binding and transcription activity; the effect is blocked by prazosin

A. PE increases the binding of Stat3 to its DNA response element; the effect is blocked by prazosin. Hep3B cells were treated with vehicle or 50μM PE for 15 minutes with or without prazosin pre-treatment (10μM prazosin for 30 minutes). The cells were harvested to isolate cellular proteins. The samples were precipitated with the biotinylated oligonucleotides (containing Stat3 binding site) followed by immunoblotting with anti-Stat3 antibody (top panel). Equal amounts of the cell lysates were used to detect Stat3 by direct western blot (mid panel). Quantitative analysis of Stat3 binding ability to its response element was performed by determining the ratio between bound and input Stat3 levels with densitometry (bottom panel) (* $p < 0.01$ compared with control cells; ** $p < 0.05$ compared with PE treatment).

B. PE increases Stat3 transcription activity; the effect is blocked by prazosin. Hep3B cells with eighty percent confluence were transiently transfected with pStat3-Luc reporter vector. After transfection the cells were treated with vehicle as control, or 50μM PE in the absence or presence of 10μM prazosin overnight. The cell lysates were obtained to determine the luciferase activity as described in the Experimental Procedures. The relative luciferase activity was normalized to cellular proteins. The data are presented as mean $\pm$ S.D. of six independent experiments. PE significantly increased Stat3 luciferase reporter activity when compared with

control (* $p < 0.01$). The PE-induced Stat3 luciferase reporter activity is blocked by prazosin (** $p < 0.05$).

Figure 4. Overexpression of α_{1B} -adrenergic receptor, but not β_2 -adrenergic receptor, increases Stat3 phosphorylation

A. Overexpression of α_{1B} -AR increases Stat3 transcription activity. Hep3B cells with eighty percent confluence were transiently co-transfected with the α_{1B} -AR expression plasmid or pcDNA control plasmid together with pStat3-Luciferase vector and pSV--galactosidase. After transfection the cells were cultured in the absence or presence of 50 μ M PE in serum-free medium overnight. The cell lysates were then obtained to determine the luciferase activity as described in the Experimental Procedures. The relative luciferase activity was normalized to β -galactosidase activity. The data are presented as mean $\pm$ S.D. of six independent experiments (* p <0.05 compared with control vector cells without PE treatment; ** p <0.01 compared with control vector cells without PE treatment; *** p <0.01 compared with control vector cells plus PE treatment).

B. Overexpression of β_2 -AR increases CRE reporter activity. Hep3B cells with eighty percent confluence were transiently transfected with the β_2 -AR expression plasmid or pcDNA control plasmid with co-transfection of pCRE-Luc reporter vector. After transfection the cells were treated with vehicle as control, or 100 μ M isoproterenol (Iso) in serum-free medium overnight. The cell lysates were obtained to determine the luciferase activity as described in the Experimental Procedures. The relative luciferase activity was normalized to cellular proteins. The data are presented as mean $\pm$ S.D. of six independent experiments. Isoproterenol

significantly increased CRE-luciferase reporter activity in β_2 -AR overexpressed cells when compared with control vector cells (* $p < 0.01$).

C. Overexpression of β_2 -AR has no effect on Stat3 transcription activity. Hep3B cells with eighty percent confluence were transiently transfected with the β_2 -AR expression plasmid or pcDNA control plasmid with co-transfection of pStat3-Luc reporter vector. After transfection the cells were treated with vehicle as control, or 100 μ M isoproterenol (Iso) in serum-free medium overnight and the cell lysates were obtained to determine the luciferase activity. The relative luciferase activity was normalized to cellular proteins. The data are presented as mean $\pm$ S.D. of six independent experiments. A similar level of Stat3 reporter activity was observed in β_2 -AR overexpressed cells and control vector cells, with or without isoproterenol treatment.

D. Overexpression of α_{1B} -AR increases Stat3 phosphorylation. Hep 3B cells with eighty percent confluence were transiently transfected with the α_{1B} -AR expression plasmid or pcDNA control plasmid. After transfection the cells were cultured in serum-free medium for 24 hours. The cells were then harvested and nuclear proteins were obtained to determine Stat3 phosphorylation (top panel) and PARP level (mid panel) by immunoblotting. Quantitative analysis of Stat3 phosphorylation was performed by determining the ratio between Stat3 phosphorylation and PARP protein levels from three different experiments with densitometry (bottom panel; * $p < 0.05$ compared with control vector cells).

Figure 5. RGS2 overexpression blocks PE-induced Stat3 phosphorylation and transcription activity

A. RGS2 overexpression blocks PE-induced Stat3 phosphorylation. Hep3B cells with eighty percent confluence were transiently transfected with either the pcDNA control plasmid or RGS2 expression plasmid. After transfection the cells were cultured in serum-free medium overnight and then treated with either vehicle or 50 μ M PE for 15 minutes. The cells were harvested and nuclear proteins were obtained to determine the phosphorylation of Stat3 (top panel) and the level of PARP (mid panel) by immunoblotting. Quantitative analysis of Stat3 phosphorylation was performed by determining the ratio between the Stat3 phosphorylation and PARP protein levels from three different experiments with densitometry (bottom panel;

* $p < 0.05$ compared with control vector cells without PE treatment; ** $p < 0.05$ compared with control vector cells with phenylephrine treatment).

B. RGS2 overexpression blocks PE-induced Stat3 transcription activity. Hep3B cells with eighty percent confluence were transiently transfected with pcDNA control plasmid or the RGS2 expression plasmid with co-transfection of pStat3-Luc reporter vector. After transfection the cells were cultured in the absence or presence of 50 μ M PE in serum-free medium overnight. The cell lysates were then obtained to determine the luciferase reporter activity as described in the Experimental Procedures. The relative luciferase activity was normalized to cellular proteins. The data are presented as mean $\pm$ S.D. of six independent experiments (* $p < 0.01$ compared with phenylephrine treatment of control vector cells).

C. RGS2 overexpression has no effect on isoproterenol-induced CRE reporter activity. Hep3B cells with eighty percent confluence were transiently co-transfected with pcDNA control plasmid or the RGS2 expression plasmid together with β_2 -AR expression plasmid and pCRE-Luc reporter vector. After transfection the cells were treated overnight with vehicle as control or 100 μ M isoproterenol (Iso) in serum-free medium. The cell lysates were then obtained to determine the luciferase reporter activity. The relative luciferase activity was normalized to cellular proteins. The data are presented as mean $\pm$ S.D. of six independent experiments.

D. Pertussis toxin has no effect on PE-induced Stat3 transcription activity. Hep3B cells with eighty percent confluence were transiently transfected with pStat3-Luc reporter vector. After transfection the cells were cultured with vehicle as control or 50 μ M PE in the absence or presence of 10 μ M prazosin or 200 ng/ml pertussis toxin (PTX) in serum-free medium overnight. The cell lysates were then obtained to determine the luciferase reporter activity. The relative luciferase activity was normalized to cellular proteins. The data are presented as mean $\pm$ S.D. of six independent experiments. The Stat3 luciferase reporter activity induced by PE is blocked by prazosin (* $p < 0.01$ compared with PE treatment alone), but not by pertussis toxin.

Figure 6. PE increases the binding of c-Src to Stat3 and the effect is blocked by prazosin
Hep3B cells were treated with 50 μ M PE for 15 minutes with or without prazosin pretreatment (10 μ M for 30 minutes). The cells were harvested and cellular proteins were obtained for immunoprecipitation with anti-c-Src antibody and subsequent immunoblotting with anti-Stat3 antibody. The precipitated Stat3 (92 kDa) is shown in the top panel. For negative control, the cellular proteins from PE-treated cells were immunoprecipitated with IgG. Equal amounts of the same cell lysates were used for regular western blot to detect the protein levels of Stat3 (mid panel) and c-Src (bottom panel). Quantitative analysis of bound Stat3 was performed by determining the ratio between immunoprecipitated and input Stat3 levels with densitometry (* p <0.01 compared with vehicle treatment; ** p <0.05 compared with PE treatment).

Figure 7. PE induced the phosphorylation of EGFR in hepatocytes

A. Dose-dependent effect of PE on EGFR phosphorylation in primary hepatocytes. Rat primary hepatocytes were treated with increasing concentrations of PE for 15 minutes. The cells were harvested and nuclear proteins were obtained. EGFR phosphorylation was determined by immunoblotting as described in the Experimental Procedures. PARP was determined by reprobing the same blot with anti-PARP antibody. Quantitative analysis of EGFR phosphorylation was performed by determining the ratio between EGFR phosphorylation and PARP protein levels from three different experiments with densitometry (* $p < 0.01$ compared with control cells).

B. Time-course of PE on EGFR phosphorylation in primary hepatocytes. Rat primary hepatocytes were treated with 50 μ M PE for different times. The cells were harvested to obtain nuclear proteins. EGFR phosphorylation and PARP level were determined by immunoblotting. PE-induced EGFR phosphorylation was observed within 15 minutes after treatment. Quantitative analysis of EGFR phosphorylation was performed by determining the ratio between EGFR phosphorylation and PARP protein levels from three different experiments with densitometry (* $p < 0.01$ compared with control cells).

C. Dose-dependent effect of PE on EGFR phosphorylation in Hep3B cells. The cells were treated with different doses of PE for 15 minutes. The treated cells were harvested to obtain nuclear proteins. EGFR phosphorylation was determined by immunoblotting with anti-phospho-EGFR antibody. PARP was determined by reprobing the same blot with anti-PARP antibody. Quantitative analysis of EGFR phosphorylation was performed by determining the

ratio between EGFR phosphorylation and PARP protein levels from three different experiments with densitometry (* $p < 0.05$ compared with control cells).

D. Time-course effect of PE on EGFR phosphorylation in Hep3B cells. The cells were treated with 50 μ M PE for different times. The treated cells were harvested to obtain nuclear proteins. EGFR phosphorylation was determined by immunoblotting with anti-phospho-EGFR antibody. PARP was determined by reprobing the same blot with anti-PARP antibody. PE-induced EGFR phosphorylation was observed within 15 minutes after treatment. Quantitative analysis of EGFR phosphorylation was performed by determining the ratio between EGFR phosphorylation and PARP protein levels from three different experiments with densitometry (* $p < 0.05$ compared with control cells).

Figure 8. The phenylephrine-induced Stat3 phosphorylation, DNA binding and transcription activity are inhibited by the Src inhibitor, PP2, and the EGFR kinase inhibitor, AG1478

A. PP2 and AG1478 block PE-induced Stat3 phosphorylation in primary hepatocytes.

Rat primary hepatocytes were treated with 50μM PE for 15 minutes with or without 30 minute pretreatment of prazosin (10μM), PP2 (10μM), or AG1478 (25μM). The cells were harvested to obtain nuclear proteins. Stat3 phosphorylation and PARP level were determined by immunoblotting. Quantitative analysis of Stat3 phosphorylation was performed by determining the ratio between Stat3 phosphorylation and PARP protein levels from three different experiments with densitometry (*p<0.01 compared with control cells; **p<0.01 compared with PE treatment).

B. PP2 and AG1478 block PE-induced Stat3 phosphorylation in Hep3B cells. The cells were treated with 50μM PE for 15 minutes with or without 30 minute pretreatment of prazosin (10μM), PP2 (10μM), or AG1478 (25μM). The cells were harvested to obtain nuclear proteins. Stat3 phosphorylation and PARP level were determined by immunoblotting. Quantitative

analysis of Stat3 phosphorylation was performed by determining the ratio between Stat3 phosphorylation and PARP protein levels from three different experiments with densitometry (* $p < 0.01$ compared with control cells; ** $p < 0.01$ compared with PE treatment).

C. PP2 and AG1478 block PE-induced Stat3 transcription activity in Hep3B cells. The cells with eighty percent confluence were transiently transfected with pStat3-Luc reporter vector. After transfection the cells were treated with vehicle as control or 50 μ M PE in the absence or presence of 10 μ M prazosin, 10 μ M PP2 or 25 μ M AG1478 overnight. The cell lysates were obtained to determine the luciferase reporter activity. The relative luciferase activity was normalized to cellular proteins. The data are presented as mean $\pm$ S.D. of six independent experiments (* $p < 0.01$ compared with control; ** $p < 0.05$ compared with PE treatment).

D. PP2 and AG1478 block PE-induced Stat3 binding to its response element in Hep3B cells. The cells were treated with 50 μ M PE for 15 minutes with or without 30 minute pretreatment of prazosin (10 μ M), PP2 (10 μ M), or AG1478 (25 μ M). The cells were harvested to obtain cellular proteins. The samples were then precipitated with biotinylated oligonucleotides containing Stat3 binding site followed by immunoblotting with anti-Stat3 antibody (top panel). Equal amounts of the cell lysates were used to detect Stat3 by direct western blot (mid panel). Quantitative analysis of Stat3 binding ability to its response element was performed by determining the ratio between bound and input Stat3 levels with densitometry (bottom panel) (* $p < 0.01$ compared with control cells; ** $p < 0.05$ compared with PE treatment).

Figure 9. Rat hepatocyte DNA synthesis was inhibited by prazosin, PP2 and AG1478

Following isolation of rat primary hepatocytes, the cells were incubated and checked for adherence of monolayers after 2 to 4 hours. Once adhered, the cells were subjected to treatment with 10μM phenylephrine without or with 10μM prazosin, 10μM PP2, or 10 μM AG1478 together with the addition of [³H]thymidine. The cells were subsequently cultured for overnight. [³H]thymidine incorporation into DNA was determined by liquid scintillation and the values are relative to control. The results are expressed as mean ± S.D of three experiments. The cells treated with prazosin, or PP2 or AG1478 showed significantly decreased DNA synthesis when compared with vehicle or PE treated cells(* p<0.01).