Vasoactive intestinal peptide (VIP) induces proliferation of human hepatocytes

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Vasoactive intestinal peptide (VIP) induces proliferation of human hepatocytes

Running title: VIP stimulates proliferation of hepatocytes.

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MMS Khedr, AM Abdelmotelb, TA Bedwell an MN Alzoubi were responsible for data acquisition and analysis. AM Abdelmotelb and A Shtaya were concerned with ethical considerations. M Abu Hilal and SI Khakoo contributed to the conception, design of the work or of parts of it, and to its interpretation. MMS Khedr and SI Khakoo drafted and revised the manuscript, AM Abdelmotelb and M Abu Hilal revised it critically for intellectual content, and T Bedwell proofread the manuscript.

Abbreviations used in this article:

ERK, extracellular signal–regulated kinase; MAPK, mitogen-activated protein kinase; PH, Partial Hepatectomy; VPAC1, VIP and pituitary adenylate cyclase-activating polypeptide receptor-1.
Abstract: 225 words

Objectives: Proliferation of hepatocytes in vitro can be stimulated by growth factors such as epidermal growth factor (EGF), but the role of vasoactive intestinal peptide (VIP) remains unclear. We have investigated the effect of VIP on maintenance and proliferation of human hepatocytes.

Materials and Methods: Human hepatocytes were isolated from liver specimens obtained from patients undergoing liver surgery. Treatment with VIP or EGF was started 24 hours after plating and continued for three or five days. DNA replication was investigated by Bromodeoxyuridine (BrdU) incorporation and cell viability detected by MTT assay. Cell lysate was analysed by western blotting and RT–PCR. Urea and albumin secretion into the culture supernatants were measured.

Results: VIP increased DNA replication in hepatocytes in a dose dependant manner, with a peak response at day three of treatment. VIP treatment was associated with an increase in mRNA expression of antigen identified by monoclonal antibody Ki-67 (MKI-67) and Histone Cluster 3 (H3) genes. Western blotting analysis showed that VIP can induce a PKA/B-Raf dependant phosphorylation of extracellular signal-regulated kinases (ERK). Although EGF can maintain hepatocyte functions up to day five, no marked effect was found with VIP.

Conclusions: VIP induces proliferation of human hepatocytes with little or no effect on hepatocyte differentiation. Further investigation of the role of VIP is required to determine if it may ultimately support therapeutic approaches of liver disease.
Introduction:

Hepatocyte transfusions have shown promise as an alternative to conventional liver transplantation in treatment of some genetic disorders and acute liver failure\(^1\),\(^2\). These potential therapies are compromised by poor viability, rapid de-differentiation, the low proliferative capacity of primary hepatocytes \textit{in vitro}\(^3\) and the need for high numbers of hepatocytes. In addition, there is often poor liver cell viability after cryopreservation\(^4\).

Improving hepatocyte \textit{in vitro} viability and growth is crucial for progress in their use as a replacement therapy and in drug screening.

VIP is a 28-amino acid neuropeptide found largely in the brain, gastrointestinal tract and liver\(^5\). Moreover, VIP receptors have been characterised and purified from the liver\(^6\),\(^7\). Reports have shown that VIP can change the metabolic functions of rat hepatocytes, and can stimulate gluconeogenesis, ureagenesis, and inhibit glyconeogenesis\(^8\),\(^9\). VIP has been found to be involved in regulation of hepatic blood flow, and modulation of both innate and adaptive immune functions\(^10\)\(^-\)\(^12\). Interestingly, VIP mRNA expression is present in rat liver following partial hepatectomy (PH)\(^13\). Unlike Hepatic Growth Factor (HGF) and Epidermal Growth Factor (EGF), the role of VIP in liver regeneration is under-investigated. Previous reports have shown that VIP may exert bi-directional inhibitory or stimulatory effect on cell proliferation of a number of cell types. Kar \textit{et al.} (1996) described a stimulatory effect of VIP alone on hepatocytes obtained from regenerated liver of rats\(^13\). In addition, it has been reported that VIP may have a mitogenic effect on HT29 and H9 cell lines\(^14\),\(^15\), while it can cause an inhibition of proliferation of human HepG2 cells\(^16\).

The mitogen-activated protein kinase (MAPK) pathway has been reported to play a crucial role in hepatocyte replication\(^17\). Moreover, EGF induced proliferation of rat hepatocytes is
mainly dependant on the p44 and p42 isoenzymes (extracellular signal-regulated kinases, ERK1 and ERK2) of the MAPK pathway. VIP stimulates intracellular production of cyclic adenosine 3':5'-monophosphate (cAMP) in various cell types, including hepatocytes. Activation of cAMP-dependent Rap1 GTPase may be associated with either activation or inhibition of the (MAPK/ERK kinase) MEK/ERK cascade. This effect relies on the presence or absence of the serine/threonine-protein kinase B-Raf, respectively in cells. Of relevance is that B-Raf kinase has been detected in liver. These findings support the hypothesis that VIP may contribute in hepatocytes proliferation.

In the present study, we have investigated the effects of VIP on cell proliferation, gene expression, cell signalling and function in human hepatocytes.
Materials and Methods:

Isolation of human hepatocytes:

Tissue samples (2-10gm) were obtained from fresh surgical macroscopically normal liver tissue resections from patients undergoing hepatectomies with informed consent (Research Ethics Committee, REC North East - Newcastle & North Tyneside 2, REC ref. 13/NE/0070).

A total of 46 human liver cell preparations derived from the unaffected resection margins of the livers from 39 different donors with primary or metastatic liver tumors (24 men and 15 women) were used. Patients' ages ranged from 29 to 83 years. Hepatocytes were isolated using a two-step perfusion procedure as described previously\(^ {21} \) with some modifications. Cells were plated on mouse collagen type IV gel layer 1 - 2.5µg cm\(^{-2} \) (Corning Ltd., Flintshire, UK) in William’s E medium (Thermo Fisher, Inchinnan, UK) and incubated at 37°C in a humidified incubator with 5% CO\(_2\).

5-Bromo-2’-deoxyuridine (BrdU) DNA incorporation assay:

EGF (Sigma, Gillingham, UK) at 5, 10 and 20ng ml\(^{-1} \) or VIP (Sigma) at 10\(^{-8} \), 10\(^{-7} \) or 10\(^{-6} \)M was added 24 hours following cell seeding. Hepatocytes were incubated with BrdU (10µg ml\(^{-1} \), Sigma) for 2 hours at 37°C. DNA-integrated BrdU was detected by rat anti-BrdU antibody (Bio-Rad, Hertfordshire, UK) and subsequently donkey anti-rat IgG-Alexa 488 (Thermo Fisher). Nuclei were stained with 4’-6-diamidino-2-phenylindole, DAPI (Sigma). Using fluorescence microscopy, numbers of BrdU\(^+ \) and DAPI\(^+ \) cells were determined in 6 different high power fields per well.

Measurement of lactic dehydrogenase (LDH):

Equal volumes of 200mM Tris (hydroxymethyl) aminomethane (Tris) pH 8, 50mM Lithium lactate, freshly prepared substrate solution [100µl P-Iodonitrotetrazolium Violet, INT (33mg
ml\(^{-1}\) in dimethyl sulfoxide (DMSO) + 100µl, Phenazine methosulfate, PMS (9 mg ml\(^{-1}\)) + 2.3 ml β-nicotinamide adenine dinucleotide (NAD) hydrate (3.74 mg ml\(^{-1}\)) and samples or positive control (5µg ml\(^{-1}\) L-Lactic Dehydrogenase from bovine heart, Sigma) were loaded into an assay plate. The \(V_{max}\) was measured at 490nm for 10min in a SpectraMax® Plus 384 Microplate Reader (Molecular Devices, Wokingham, UK) and LDH activity (U ml\(^{-1}\)) was calculated.

Viability and Proliferation Assays:
Viability was determined using a colourimetric MTT assay (Sigma) and Quick Cell Proliferation Assay kit II (Abcam, Cambridge, UK) were used according to manufacturers’ instructions.

Polymerase Chain Reaction (PCR) and Real time PCR (rt-PCR):
RNA was extracted using a RNeasy® kit (Qiagen, Crawley, West Sussex, UK) following the manufacturer’s instructions. Complementary DNA (cDNA) was synthesised using a Primer Design Precision nanoScript 2 reverse transcriptase kit (Millbrook, Southampton, UK) according to the manufacturer’s instructions in a MasterCycler® 480 thermocycler (Eppendorf, Hamburg, Germany). The rt-PCR Primers were designed using the ProbeFinder software version 2.5 (Lifescience.roche.com) and oligonucleotide primers for albumin, Antigen Identified By Monoclonal Antibody Ki-67 (MKI-67), Histone Cluster 3 (H3) were obtained from Eurofins MWG/operon (Ebersberg, Germany) (Supplementary data 1). VIP and pituitary adenylate cyclase-activating polypeptide receptor-1 (VPAC1) and EGF receptor (EGFR) mRNA expression was assessed using GoTaq® Hot Start Polymerase (Promega UK Ltd, Southampton, UK) according to manufacturer’s instructions. PCR products were visualized on 2% agarose gel, band densities were measured and normalised to that of
Glycerinadehyde-3-Phosphate-Dehydrogenase, GAPDH using a ChemiDoc™ imaging system (Bio-Rad). The qPCR was performed using a SYBR green Mastermix buffer (Primer Design) in an A&B 7900HT Fast Real-Time PCR System thermocycler (Applied Biosystems, CA, USA). The Ct values were normalized to the GAPDH and calibrated to untreated cells. The fold change of mRNA expression was calculated according to the ∆∆Ct method.

Detection of Phospho-p44/42 MAPK (Erk1/2) and VPAC1 in hepatocytes using Western Blotting:

Hepatocytes were serum starved for 24 hours prior to incubation with EGF (20 ng ml⁻¹) or VIP (10⁻⁶ M). The B-RAF inhibitor, SB-590885 and the PKA inhibitor, Rp-cAMP triethylammonium salt (Rp-cAMPS) were used. Cells were lysed using TruPAGE™ LDS Sample Buffer (Sigma) with phosphatase and protease inhibitors. Protein concentrations were measured and separated in a TruPAGE® 10% precast gels (Sigma) under reducing conditions, then transferred to nitrocellulose membranes. The membranes were probed with rabbit anti-human Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody or rabbit anti-human p44/42 MAPK (Erk1/2) antibody (New England Biolabs, Hertfordshire, UK), followed by goat anti-rabbit-horseradish peroxidase (HRP) (DakoCytomation, Cambridgeshire, UK). Reactive bands were detected using the Luminata Forte Western HRP substrate (Millipore UK Ltd., Hertfordshire, UK). In another experiment, the level of VPAC1 protein expression in untreated or VIP (10⁻⁶M) treated hepatocytes, was investigated using a rabbit polyclonal anti VPAC1 (Abcam) and followed by goat anti-rabbit-horseradish peroxidase (HRP) (DakoCytomation).

cAMP Direct Immunoassay:
Levels of cAMP in hepatocytes 24 h following cell seeding and at day 3 or 5 following stimulation with $10^{-6}$M VIP treatment were detected using a cAMP direct immunoassay (Abcam) according to the manufacturer’s instructions. cAMP concentrations ($\mu$M) were determined and corrected to total proteins concentrations in samples ($\mu$g).

**Albumin ELISA and Urea concentration assay:**

Albumin and urea concentrations in the supernatant of hepatocytes cultures were determined using the ELISA DuoSET® kit for human albumin (R&D Systems, Oxfordshire, UK) and the QuantiChrom™ urea assay kit (QuantiChrom, BioAssay Systems, Hayward, CA) respectively, according to the manufacturer’s instructions.

**Statistics:**

Two-way analysis of variants (ANOVA) followed by Fisher's least significant difference (LSD) multiple comparisons tests were performed using GraphPad Prism version 7.7.1 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Data has been represented by Mean $\pm$ standard error of the mean (SEM) or standard deviation (SD) as indicated. $P < 0.05$ was taken as significant.

For further details regarding the materials and methods, please refer to the supplementary data 1.
Results:

Stimulation of DNA replication in hepatocytes by VIP:

EGF at high concentrations such as 50ng ml\(^{-1}\), has been reported to be responsible for an increase in [3H] methylthymidine incorporation in rat hepatocytes. The response to EGF maximised at 24 hour and continued with persistent exposure\(^{22}\). In the current work, proliferation of human hepatocytes was investigated by detecting BrdU incorporation (Figure 1A). Herein, EGF resulted in an increase of BrdU positive cells at concentrations of 10ng ml\(^{-1}\) (a mean of 1.3 ± SD 0.9 fold) and 20ng ml\(^{-1}\) (a mean of 1.8 ± SD 1.4 fold) at day 3 (Figure 1B), and this effect was continued at day 5 of treatment, 10 ng ml\(^{-1}\) (a mean of 1.5 ± SD 0.5 fold) and 20 ng ml\(^{-1}\) (a mean of 1.7 ± SD 0.5 fold) (Figure 1C). Interestingly, VIP stimulated proliferation of human hepatocytes in a dose dependant manner at day 3 up to a mean of 3.2 ± SD1.1 fold at 10\(^{-6}\)M (Figure 1D). However, a decline of hepatocyte response to VIP was observed at day 5 (a mean of 1.2 ± SD 0.6 fold up to 10\(^{-6}\)M) (Figure 1E). Similarly, EGF addition was associated with a rise in total cell numbers at day 3; 10ng ml\(^{-1}\) (a mean of 1.4 ± SD 1.1 fold) and 20ng ml\(^{-1}\) (a mean of 1.9 ± SD 1.7 fold) and day 5; 10ng ml\(^{-1}\) (a mean of 1.7 ± SD 0.4 fold) and 20 ng ml\(^{-1}\) (a mean of 1.7 ± SD 0.3 fold). VIP at day 3 resulted in an increase of total cells by a mean of 2.2 ± 0.9 fold at 10\(^{-7}\)M and 3.4 ± SD 1.4 fold at 10\(^{-6}\)M. The drastic decrease in hepatocyte response to VIP at day 5 raised a concern about changes in cell viability and status, and was investigated further.

VIP treatment has a limited effect on hepatocyte survival \textit{in vitro}:

Effects of EGF or VIP on hepatocyte integrity was tested by measuring LDH release in the cell culture supernatants. In the first 24 hours following cell extraction, LDH activity was high (a mean of 0.90 ± SD 0.29 U ml\(^{-1}\)) (Figure 2A), which may be a result of the isolation process or spontaneous activation of hepatocyte apoptosis\(^{23,24}\). A dramatic decrease in LDH
levels was observed in the following 24 hours (a mean of $0.14 \pm 0.16$ U/ml). This may have been caused by washout of old medium containing dead and apoptotic cells. No further change in LDH activity was observed up to day 5. Treatment of hepatocytes with EGF resulted in a minimal change in LDH activity in the supernatants at day 1 compared to untreated cells. A decrease in LDH activity was observed at days 3 and 5 at various concentrations of EGF (Figure 2B). When VIP was added to the medium, no change in LDH activity was observed at day 1 or 3 of treatment (Figure 2C). At day 5, cells treated with VIP showed a marked increase in LDH levels, with approximately 2, 8 and 10 fold changes at $10^{-8}$, $10^{-7}$ and $10^{-6}$M of VIP respectively. There was also a rise in LDH activity when both agents were added together to the hepatocyte culture medium (data not shown).

The metabolic activity of the cell was assessed using the MTT assay. At day 3, EGF showed a marked improvement in cell viability (Figure 2D) and VIP treatment was associated with a concentration dependant increase in hepatocyte metabolic activity, peaking at a concentration of $10^{-6}$M (Figure 2E). Results showed low metabolic activity of primary human hepatocytes after day 5 of cell seeding, irrespective of the addition of EGF or VIP. This result may reflect cell loss.

Previous results have shown that the support of hepatocyte survival was lacking when VIP was used alone and cells have entered a late phase of death or apoptosis. In order to address this, we have tested DMSO as an agent which may prevent this deterioration of cell viability and as reported, can maintain hepatocyte differentiation and improve liver-specific functionality. DMSO alone induced cell death as compared to medium alone, however addition of 2% DMSO to culture medium was associated with the restoration of the hepatocyte response to EGF and the VIP mitogenic effect at day 5 of treatment (Figure 3A).
and 3B). In addition to hepatocytes loss, the noticeable decrease in the effect of VIP by day 5 and a change in expression of VIP receptors may contribute to hepatocyte resistance VIP. To test this possibility mRNA expression of VPAC1, the most abundant VIP receptor in the liver, was investigated using a semi-quantitative RT-PCR technique. In untreated hepatocytes, level of mRNA expression of VPAC1 or EGFR did not change significantly at day 3 (Figure 3C). However, at day 5 cells expressed lower levels of EGFR mRNA which is a phenomenon that has been reported previously but VPAC1 mRNA expression did not show any change. Western blotting revealed several forms of VPAC1 in human hepatocytes at molecular weights of ~250, ~100 and ~52 kDa (Figure 3D), as described previously. During hepatocytes culture, VPAC1 protein expression did not show marked changes, but VIP treatment was associated with a marked decrease in VPAC1 gene mRNA expression at day 5 of cell culture (Supplementary Figure 1). The level of VPAC1 activation has previously been assessed by measuring intracellular cAMP concentrations. Interestingly, exposure of VPAC1 to VIP at a concentration of $10^{-6}$ M at 24 hours following cell seeding was found to stimulate production of cAMP by hepatocytes as compared to untreated cells (mean concentration 5.96 µM/µg of protein ± SEM 0.64 versus 4.18 ± 0.60 respectively, $P = 0.0029$) (Figure 3E). Production of cAMP as a response to VIP continued but to a lesser extent until day 3 of hepatocyte culture (mean of 5.90 µM/µg of protein ± 0.77 and 4.95 ± 0.97 respectively, $P = 0.0761$). Notably, constitutive cAMP showed a lower concentration at day 5 of cell culture in untreated cells (a mean of 3.85 ± SEM 0.84 µM/µg of protein) and VPAC1 receptors did not show as clear a response to VIP as that seen at early time points (4.52 ± 0.69 µM/µg of protein). Taken together, these finding may suggest a change in receptor functionality over time.
Expression of proliferation-associated genes was induced by VIP treatment:

Expression of the active cell cycle marker, MKI-67\textsuperscript{29} and the mitotic marker, H3\textsuperscript{30} genes were studied using quantitative rt-PCR. EGF alone induced a six-fold increase in mRNA expression of MKI-67, most significantly at day 3 of treatment at concentrations up to 10ng ml\textsuperscript{-1} (Figure 4A). In addition, EGF treatment resulted in up to a four-fold increase in expression of H3 mRNA by day 3 of treatment, most noticeably at 20ng ml\textsuperscript{-1} EGF (Figure 4B). Addition of VIP to cultured hepatocytes were associated with a two-fold increase in MKI-67 gene expression at day 3, rising to four-fold at day 5 of treatment at a concentration of 10\textsuperscript{-6}M (Figure 4C). Similarly, VIP induced a concentration dependant increase in expression of H3 at days 3 and 5 (Figure 4D). Although the combination of EGF and VIP was associated with a considerable increase in expression of MKI-67 at day 5, there was no difference compared to either EGF or VIP alone (Figure 4E). The presence of EGF and VIP together in the culture medium had little effect on expression of H3 at day 3 (Figure 4F).

Production of Phospho-p44/42 MAPK (Erk1/2) in VIP treated hepatocytes:

Binding of VIP to its receptors initiates cAMP production and subsequent protein kinase A (PKA)\textsuperscript{31}. A PKA-dependent phosphorylation of the GTPase Rap1 resulted in stimulation of ERKs in the presence of B-Raf in cells such as hepatocytes\textsuperscript{19}. EGF at 20ng ml\textsuperscript{-1} stimulated phosphorylation of ERK as early as 10 minutes, after which activation declined with time (Figure 5A). Interestingly, VIP was found to increase pERK following 10 minutes incubation with hepatocytes. However, ERK activation increased further up to 60 minutes (Figure 5B). In addition, VIP stimulation of freshly isolated hepatocytes failed to elicit phosphorylation of ERK (data not shown). Both agents did not preferentially activate either pERK 1 or 2. Pre-incubation of human hepatocytes with 5\mu M of SB-59085 (SB), a B-RAF inhibitor prior to treatment or Rp-cAMPS (cAMP inhibitor) at 500\mu M was associated with inhibition of VIP
induced pERK (Figure 5C and 5D). Interestingly, SB was found to preferentially block ERK2 phosphorylation to a greater extent than ERK1. Whereas inhibition of cAMP mobilization with Rp-CAMP inhibitor blocked both ERK1 and ERK2.

VIP treatment does not support human hepatocytes specific functions:

Albumin gene expression was suppressed initially, but recovered by day 3 of incubation with EGF at a concentration of 5 ng ml$^{-1}$ and markedly increased at day 5 with concentrations up to 5 to 20 ng ml$^{-1}$ (Figure 6A). Conversely, VIP had no marked effect on albumin gene expression in human hepatocytes in this model (Figure 6B). When EGF and VIP were combined together, the stimulatory effect of EGF on albumin gene expression was significantly lower than that of EGF alone (Figure 6C).

Albumin levels in the supernatants dropped from a mean of 75.14 ± SD 22.13ng ml$^{-1}$ in the first 24 hours following hepatocyte seeding to a mean of a mean of 40.24 ± SD 16.82 ng ml$^{-1}$ at day 2 and no marked change was observed subsequently. EGF stimulated production of albumin from liver cells in a concentration dependent manner as compared to the untreated control at day 1 of treatment yielded a mean of 120.91± SD 79.91 ng ml$^{-1}$ which continued up to day 5 of treatment to reach a mean of 152.80 ± SD 87.20 ng ml$^{-1}$ with 20ng ml$^{-1}$ EGF (Figure 6D). At day 3, there was an increase in albumin production up to a mean of 66.9 ± SD 76.83 ng ml$^{-1}$ from hepatocytes cultured in the presence of $10^{-6}$M VIP (Figure 6E). When both agents were added together, the stimulatory effect of EGF was inhibited (Figure 6F). When both agents were added sequentially, an inhibitory effect of VIP on EGF stimulated albumin production was observed (Supplementary Figure 2). Urea production from hepatocytes was dramatically decreased during the 24 hours following cell plating from a mean of 3.01 ± SD 0.38 mg dL$^{-1}$ to a mean of 0.80 ± SD 0.98 mg dL$^{-1}$, but partial recovery
was observed at day 3 and 5 (a mean of $1.26 \pm SD 0.37 \text{ mg dL}^{-1}$ and $1.10 \pm SD 0.36 \text{ mg dL}^{-1}$ respectively). EGF increased urea production on the first day of hepatocyte culture compared to untreated cells (a mean of $1.31 \pm SD 0.23 \text{ mg dL}^{-1}$ at 10 ng ml$^{-1}$ EGF), but this effect disappeared with time (Figure 6G). However, $10^{-7}\text{M VIP}$ resulted in a limited increase (a mean of $1.53 \pm SD 0.51 \text{ mg dL}^{-1}$) in urea production at day 3 as compared to control (Figure 6H) and adding VIP to EGF abolished the effect of EGF on urea production in cultures hepatocytes (Figure 6I).
Discussion:

Our findings have shown that EGF or VIP alone has the ability to induce DNA synthesis in cultured human hepatocytes and to stimulate expression of genes that may be involved in cell proliferation. Interestingly, EGF was able to maintain hepatocyte proliferation further up to day 5 whilst VIP did not. In addition, VIP was found to stimulate phosphorylation of ERK1 and 2 protein kinases. However, unlike EGF, VIP has a limited effect on hepatocyte function in vitro.

Hepatocytes move from G0 to G1 phase of cell cycle spontaneously during isolation process and progress further towards and stop at a restriction point in mid-late G1 phase usually 24 and 48 h after plating. Onward movement to S phase is dependent on growth factors such as EGF. In agreement with that, we have demonstrated that EGF stimulated DNA synthesis when added 24 h following hepatocytes seeding. Strikingly, we observed a comparable effect with VIP which disagree to that previously reported by Kar et al. The outcome of proliferative stimuli is related to the cell cycle. A few hours following isolation, VIP can facilitate entry of cells into G1 phase but it did not encourage them to pass the restriction point. This effect could increase the number of cells at susceptible to the mitogenic effect of EGF. These findings might explain why VIP alone failed to stimulate DNA synthesis in hepatocytes but may potentiate the effect of EGF on cell proliferation at this early time point. We found that VIP did activate MAPK at this early time which consistent with that has been reported. The underling mechanism could involve activation of p70 ribosomal S6 protein kinase (p70S6k) activity, cyclin D3-cyclin-dependent kinase (CDK)-4 assembly or a CDK2/cyclin C-dependent inhibitory phosphorylation of the transcription factor LSF (late simian virus 40 factor) at serine.
As we have shown, later in culture VIP or EGF stimulated formation of pERK which has been described previously\textsuperscript{18,39}. This effect was found to be closely related to induction of hepatocyte proliferation\textsuperscript{17} and may involve an MAPK-dependent reactivation phosphorylation of LSF at serine 291 which could be essential for cell cycle progression to S phase\textsuperscript{40}. Dependence of VIP induced ERK activation on B-Raf kinase could support our hypothesis that VIP alone is able to induce hepatocyte proliferation, but VIP exerted an inhibitory effect on EGF (Figure 7). In accordance with these results, it has been reported that high levels of cAMP could result in a decrease in EGF-dependent MAPK production and loss of its DNA stimulatory effect\textsuperscript{35}. In addition, several reports have shown that cAMP-dependent PKA is able to phosphorylate EGFR on serine residues which results in decrease in tyrosine kinase activity and EGFR auto-phosphorylation induced by EGF\textsuperscript{41,42}. Moreover, cAMP-GEFs can directly inhibit Raf-1 by phosphorylation at ser259 or indirectly by a PKA-dependent activation of the Raf-1 inhibitor, Akt (protein kinase B, PKB)\textsuperscript{43,44}. This interaction could explain the reported VIP inhibitory effect on HepG2 proliferation. HepG2 survival and proliferation is depending on the presence of FBS in medium\textsuperscript{45,46}. VIP has been shown to inhibit HepG2 proliferation through a cAMP – dependent signal transducers and activators of transcription-3 (STAT-3) pathway inhibition\textsuperscript{16}, the pathway that can be stimulated by growth factors which present in FBS.

The DNA synthesis in primary hepatocytes started early in culture and maximised at day 3, with expression of activated transcriptional regulators for EGF and ERK pathway\textsuperscript{47}, but decreased afterwards even in the presence of EGF\textsuperscript{13,48}. Following day 3 of culture, substantial hepatocyte death has been reported and the remaining cells may become flattened and polykaryotic or smaller and apoptotic\textsuperscript{3}. We have noticed that, at day 5 of EGF treatment, there was a lower number of living hepatocytes, and that the remaining cells replicated, but to
a lower extent. This is in agreement with previous findings to that has been reported before\textsuperscript{33,49}.

In our model, VIP did not show any change in hepatocyte proliferation, consistent with previous work\textsuperscript{13}. Notably, the cells which proliferated under the effect of VIP mostly died by day 5 of treatment and VIP did not markedly increase DNA synthesis in the remaining cells. The lack of support of the differentiated state of hepatocytes with VIP treatment was observed from the albumin production and urea secretion at day 5, a finding that has been previously reported\textsuperscript{9}. Interestingly, MKI-67 and H3 mRNA expression in hepatocytes showed a tendency to increase at day 5 of treatment while albumin expression decreased with time, which may be an indication of a loss of differentiation.

The dramatic change in hepatocyte response to VIP could be a consequence of changes in VIP receptors expression. We found that hepatocytes did not show such a change in expression of VPAC1 during culture time course. However, VIP failed to induce cAMP production in hepatocytes at day 5 of cell culture, which suggests an alteration of receptor signaling response. Indeed, the interaction between VIP and its receptors in proliferating hepatocytes is not completely understood. In rat liver 3 days after PH, the maximal response of VIP was reduced as a result of low number of receptors and changes in the receptor structure\textsuperscript{50}. In addition, the decrease in VIP receptors sensitivity could be a result of high expression of VIP in proliferating liver\textsuperscript{13}. Moreover, VPAC1 harbours several potential N-glycosylation sites which are critical for VIP binding\textsuperscript{51} and receptor delivery to plasma membrane\textsuperscript{52}. An alteration in N-glycosylation of proteins has been reported in dedifferentiated rat hepatocytes\textsuperscript{53}, and could explain the decreased in VPAC1 response to VIP, but this possibility needs further investigations. In addition, we have demonstrated that...
addition of high concentration of VIP was associated with downregulation of VPAC1, the phenomenon that has been reported with VIP with other cell types\textsuperscript{54,55}. Our findings have demonstrated that VIP alone was able to induce proliferation of adult human hepatocytes when added 24 hours following hepatocyte platting and this effect may be PKA/B-Raf–ERK dependent. VIP exerts an inhibitory effect on EGF signaling pathway at this time point of cell cycle. Stimulation of the VIP pathway may aid hepatocyte proliferation 
\textit{in vitro}.

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Conflict of interest:

All authors declare no conflict of interest.
References:


Figures’ Legends:

Figure 1 Hepatocyte proliferation was stimulated by EGF or VIP. (A) Representative images of hepatocytes treated with either EGF (20 ng ml$^{-1}$) or VIP (10$^{-6}$ M) for 3 days. DNA incorporation of BrdU was determined (Green) and DAPI (Blue) was used as a nuclear counter stain. (B - E) The effects of EGF or VIP were demonstrated on total and proliferating cell numbers. n = 3 different donors per condition. $P$ values shown in the graph are for comparison to hepatocytes maintained on medium alone. * $P<0.05$, ** $P<0.005$, *** $P<0.0005$, **** $P<0.0001$. Mean ± SEM. Two-way ANOVA followed by Fisher's least significant difference (LSD).

Figure 2 Hepatocyte viability with EGF or VIP. (A) LDH activity (U ml$^{-1}$) in supernatants of untreated human hepatocytes with time course. (B) LDH release (expressed as percentage of total LDH activity) in supernatants of human hepatocytes treated with EGF or (C) VIP at previous concentrations following day 1, 3 and 5 of treatments. n = 3 different donors per condition. $P$ values shown in the graph are for overall comparison with hepatocytes at day 0 (A) or untreated control (B and C). * $P<0.05$, ** $P<0.005$, *** $P<0.0005$, **** $P<0.0001$. (D) Viable cells were detected following addition of EGF (5, 10 or 20 ng ml$^{-1}$) or (E) VIP (10$^{-8}$, 10$^{-7}$ or 10$^{-6}$ M) treatment for 3 or 5 days by MTT assay. $A$, Absorbance. n = 3 different donors per condition. $P$ values shown in the graph are for overall comparison between hepatocytes at day 3 and 5. Mean ± SEM. Two-way ANOVA followed by Fisher's LSD.

Figure 3 The effect of DMSO on cell response to VIP and VIP and pituitary adenylate cyclase-activating polypeptide receptor-1 (VPAC1) expression and activation in hepatocytes. (A and B) Hepatocytes were treated with either EGF (5, 10 or 20 ng ml$^{-1}$) or VIP (10$^{-8}$, 10$^{-7}$ or 10$^{-6}$ M), 2 % DMSO was added at day 3 and cell proliferation was...
investigated at day 5 using the WST-1 Quick Cell Proliferation Assay kit II (Abcam). n = 3 different donors per condition. P values shown in the graph are for comparison at individual concentrations and overall comparison with hepatocytes maintained in medium without DMSO. (C) Band density analysis (fold change) of VPAC1 and Epidermal Growth Factor Receptor (EGFR) mRNA of gene expression on 2% agarose gel in non-treated cells following 1, 3 or 5 days of hepatocyte culture (6 donors), (D) VPAC1 protein expression as detected in hepatocytes by western blotting techniques at day 3 and 5 of hepatocyte culture, a representative blot of 3 independent experiments. Molecular weights were indicated for VPAC1 isoforms. (E) Effect of VIP (10⁻⁶ M) on cAMP concentrations (µM µg⁻¹ of protein) in hepatocytes with time course control (ctrl.). n = 3. * P<0.05, ** P<0.005. Mean ± SEM. Two-way ANOVA followed by Fisher's LSD.

**Figure 4 Expression of Monoclonal Antibody Ki-67 (MKI-67) and Histone Cluster 3 (H3) genes in human hepatocytes cultured in the presence of EGF or VIP.** Expression of mRNA was quantified by qPCR at days 1, 3 and 5 of EGF (5, 10 or 20 ng ml⁻¹) or VIP (10⁻⁸, 10⁻⁷ or 10⁻⁶ M). (A – D) Concentration dependant effects of EGF or VIP, and (E and F) the effect of EGF (20 ng ml⁻¹) or VIP (10⁻⁶ M) or a combination of both. n = 3 different donors per condition. P values shown in the graph are for comparison at individual concentrations and overall comparison with hepatocytes at day 1. * P<0.05, ** P<0.005, *** P<0.0005. Mean ± SEM. Two-way ANOVA followed by Fisher's LSD.

**Figure 5 Phosphorylation of ERK in EGF or VIP-treated human hepatocytes analysis using western blotting.** (A) Hepatocytes treated with either EGF (20 ng ml⁻¹) or (B) VIP (10⁻⁶ M) and analysed by western blotting at indicated time points. (C) The effects of downstream pathway inhibitors was investigated using 2.5 - 10 µM of SB-590885 (SB) or (D) 500 µM of
Hepatocytes were incubated with inhibitors for 1 h prior to addition of EGF or VIP for another 1 h. n = 4 with different donors.

**Figure 6 Expression and production of albumin, and urea from human hepatocytes cultured with EGF and VIP.** Albumin gene mRNA expression at days 0, 1, 3 and 5 of (A) EGF (5, 10 or 20 ng ml⁻¹) or (B) VIP (10⁻⁸, 10⁻⁷ or 10⁻⁶ M) treatments, and (C) the effects of either EGF (20 ng ml⁻¹), VIP (10⁻⁶ M) or combination of both were determined. P values shown in the graph are for comparison at individual concentrations and overall comparison with hepatocytes at day 1. (D – F) Albumin (ng ml⁻¹) and (G – I) urea (mg dL⁻¹) concentrations in supernatants of cultured hepatocytes with EGF, VIP or both were determined. n = 3 different donors per condition. P values shown in the graph are for comparison with hepatocytes at day 0 or with untreated cells. * P<0.05, ** P<0.005, *** P<0.0005, **** P<0.0001. Mean ± SEM. Two-way ANOVA followed by Fisher's LSD.

**Figure 7 A schematic diagram for VIP and EGF signaling in hepatocytes.** Late in culture, binding of VIP with the G-protein coupled VIP receptor type 1 (VPAC1) activates intracellular adenylyl cyclase (AC) resulting in cAMP production and the following protein kinase A (PKA) activation. Subsequently, phosphorylated Rap-1 can activate B-Raf and thereby, stimulate the mitogen-activated protein kinase (MAPK) / extracellular signal-regulated kinase (ERK) kinase, MEK/ERK cascade. Phosphorylation of ERK1/2 results in stimulation of cell proliferation and induces mRNA expression the proliferation-associated genes, the monoclonal antibody Ki-67 (MKI-67) and Histone Cluster-3 (H3) genes. EGF interaction with its receptors, EGFR results in a Ras/Raf dependent activation of MEK, induction of cell proliferation and improvement of cell functions. VIP-activated Rap-1 may block EGF signaling through inhibition of Ras/Raf activation. VIP signaling can be inhibited...
by the B-RAF inhibitor, SB-590885 and PKA inhibitor, Rp-cAMP triethylammonium salt (Rp-cAMPS).
Figure 1

A

VIP

DAPI⁺

5x

BrdU⁺

5x

DAPI⁺BrdU⁺

5x

EGF

5x

5x

5x

B

EGF (d3)

Cell numbers (fold change)

Concentration (ng ml⁻¹)

0 5 10 20

1 2 3 4

0 1 2 3

0 1 2 3

D

VIP (d3)

Cell numbers (fold change)

Concentration (M)

0 10⁻⁸ 10⁻⁷ 10⁻⁶

0 1 2 3

0 1 2 3

C

EGF (d5)

Cell numbers (fold change)

Concentration (ng ml⁻¹)

0 5 10 20

0 1 2 3

0 1 2 3

E

VIP (d5)

Cell numbers (fold change)

Concentration (M)

0 10⁻⁸ 10⁻⁷ 10⁻⁶

0 1 2 3

0 1 2 3
Figure 2

A

Hepatocytes used with EGF
Hepatocytes used with VIP

Time (days)

0 1 3 5

0.0 0.5 1.0 1.5

LDH activity (U ml⁻¹)

B

EGF

d1
d3
d5

Concentration (ng ml⁻¹)

0 5 10 20

LDH release (% untreated)

1,500 1,000 500 0

C

VIP

d1
d3
d5

Concentration (M)

0 10⁻⁶ 10⁻⁷ 10⁻⁸

LDH release (% untreated)

2,000 1,500 1,000 500 0

D

EGF

d3
d5

Concentrations (ng ml⁻¹)

0 5 10 20

MTT (A 570 nm)

0.8 0.6 0.4 0.2 0.0

E

VIP

d3
d5

Concentrations (M)

0 10⁻⁶ 10⁻⁷ 10⁻⁸

MTT (A 570 nm)

0.8 0.6 0.4 0.2 0.0
Figure 3

A) EGF

B) VIP

C) mRNA expression

D) Western blot

E) cAMP levels
Figure 4

A. MKI-67 mRNA expression (fold change) over Concentration (ng ml⁻¹)

B. H3 mRNA expression (fold change) over Concentration (ng ml⁻¹)

C. MKI-67 mRNA expression (fold change) over Concentration (M)

D. H3 mRNA expression (fold change) over Concentration (M)

E. MKI-67 mRNA expression (fold change) over Medium, EGF, VIP, EGF+VIP

F. H3 mRNA expression (fold change) over Medium, EGF, VIP, EGF+VIP
Figure 5

A  EGF

\[ \text{pERK1, pERK2, ERK1, ERK2} \]

\[ 0 \ 10 \ 20 \ 30 \ 60 \text{ m} \]

B  VIP

\[ \text{pERK1, pERK2, ERK1, ERK2} \]

\[ 0 \ 10 \ 20 \ 30 \ 60 \text{ m} \]

C  

\[ \text{pERK1, pERK2, ERK1, ERK2} \]

\[ \text{VIP: + + + + -} \]

\[ \text{SB-590885: 0 2.5 5 10 -} \]

D  

\[ \text{pERK1, pERK2, ERK1, ERK2} \]

\[ \text{VIP: - + - +} \]

\[ \text{Rp-CAMPS: - - + +} \]
Figure 6

A. EGF

B. VIP

C. Time (days)

D. EGF

E. VIP

F. Time (days)

G. EGF

H. VIP

I. Time (days)

Cell Proliferation
Figure 7
Vasoactive intestinal peptide (VIP) induces proliferation of human hepatocytes


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Materials and Methods:

Isolation of human hepatocytes:

Tissue samples (2-10gm) were obtained from fresh surgical macroscopically normal liver tissue derived from resections from patients undergoing hepatectomies. Experimental procedures were performed according to the Health Research Authority (HRA), Research Ethics Committee (REC) North East - Newcastle & North Tyneside 2 (REC ref. 13/NE/0070) with informed consent. A total of 46 human liver cell preparations from 39 different donors have liver primary or secondary metastatic tumors (24 men and 15 women) were used. Patients' ages ranged from 29 to 83 years. Hepatocytes were isolated using a two-step perfusion procedure as described previously with some modifications. Liver tissue was washed for 10 minutes with a calcium chelating buffer [1x Hanks' balanced salt solution (HBSS), 25 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) and 0.5mM Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetra acetic acid (EGTA)]. Specimens were digested using 0.05% collagenase in Dulbecco's Modified Eagle Medium (DMEM) with 1 mM CaCl$_2$ for 10 to 15 minutes and collagenase activity was stopped by adding an equal volume of cold medium containing 10% fetal bovine serum (FBS). Following mechanical disruption, the cell suspension was filtered through a 70µm pore nylon mesh and then spun at 50g/5 minutes 3 times at 4°C before the cell pellets were collected. Cell number and viability were determined by trypan blue exclusion using a Leica DMIL inverted microscope (Leica Microsystems, Wetzlar, Germany). Average cell yield was 1.29 x 10$^6$ ± SEM 193,540 cell per gm of liver tissue. Specimens with cell viability >85% by were chosen for subsequent experiments. Cells were plated at density of 1.5 – 2.5 x10$^5$ cell cm$^{-2}$ on mouse collagen type IV gel layer 1 - 2.5µg cm$^{-2}$ (Corning Ltd., Flintshire, UK) in William’s E medium supplemented with 5% FBS (to facilitate cell adhesion) in Plating Supplement Pack (Thermo Fisher, Inchinnan, UK) and incubated at 37°C in a humidified incubator with 5% CO$_2$. 6 – 12
hours later, medium (containing non-attached hepatocytes) was aspirated and cells were maintained in William’s E medium supplemented with serum free Maintenance Supplement Pack (Thermo Fisher). Medium has been changed every 3 days.

5-Bromo-2’-deoxyuridine (BrdU) DNA incorporation assay:

EGF (Sigma, Gillingham, UK) at 5, 10 and 20ng ml\(^{-1}\) or VIP (Sigma) at 10\(^{-8}\), 10\(^{-7}\) or 10\(^{-6}\)M was added to the medium 24 hours following cell seeding. Assays were performed in quadruplicates. Hepatocytes were incubated with BrdU (10µg ml\(^{-1}\), Sigma) for 2 hours at 37°C. Cells were washed for 5 minutes with Phosphate buffered saline (PBS) 3 times and fixed with ice-cold methanol for 30 minutes at 4°C. The nuclear membrane was permeabilised by using 2M HCl for 30 min at 37°C and acid was neutralised using an equal volume of 0.1M sodium borate for 2 minutes. Non-specific reactivity was blocked with 5% Donkey Serum in PBS with 0.1% Triton-X (PBS-T) for 30 minutes at room temperature and then washed as before with 1 x PBS-T. Rat anti-BrdU antibody (Bio-Rad, Hertfordshire, UK) was used to detect DNA-integrated BrdU, 1µg ml\(^{-1}\) in PBS-T for 1 hour at room temperature and subsequently donkey anti-rat IgG-Alexa 488 (Thermo Fisher) 4µg ml\(^{-1}\) in PBS-T. Cells were incubated with 1µg ml\(^{-1}\) 4'-6-diamidino-2-phenylindole, DAPI (Sigma) in water for 5 minutes in the dark and washed with PBS, then analysed by fluorescence microscopy. The numbers of BrdU\(^{+}\) cells were determined in 6 different high power fields per well against DAPI\(^{+}\) cells using an inverted Olympus IX81 fluorescent microscope with Olympus xcellence software version 01.2 (Olympus Life Science Solutions, Tokyo, Japan).

Measurement of lactic dehydrogenase (LDH):

Aliquots of substrate solution [100µl INT + P-Iodonitrotetrazolium Violet (33mg ml\(^{-1}\) in DMSO) + 100µl PMS, Phenazine methosulfate (9 mg ml\(^{-1}\)) + 2.3 ml β-nicotinamide adenine...
dinucleotide (NAD) hydrate (3.74 mg ml\(^{-1}\)) were freshly prepared. Equal volumes of 200mM Tris (hydroxymethyl)aminomethane (Tris) pH 8, 50mM Lithium lactate, substrate solution and samples or positive control (5µg ml\(^{-1}\) L-Lactic Dehydrogenase from bovine heart) were loaded into an assay plate. The \(V_{max}\) was measured at 490nm for 10min. LDH activity (U ml\(^{-1}\)) and LDH release in the supernatants (expressed as percentage of total cellular LDH activity) were calculated.

**Viability assay:**

Viability was determined using a colourimetric MTT assay (Sigma) according to the manufacturers instruction. Cells (2x10^5 cell/well) were seeded in duplicate into 96-well plates and treated with various concentrations of EGF or VIP for 3 or 5 days. Viable cells were detected by measuring the absorbance at 570nm in a SpectraMax® Plus 384 Microplate Reader (Molecular Devices, Wokingham, UK). The water-soluble tetrazolium salts (WST-1) Quick Cell Proliferation Assay kit II (Abcam, Cambridge, UK) were used according to manufacturer instructions. Absorbance was detected at 440nm.

**Total RNA extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR):**

RNA extraction was performed using a microspin column extraction kit (RNase\(^{\text{TM}}\) mini kit) (Qiagen, Crawley, West Sussex, UK) following the manufacturer’s instructions. RNA quantity and purity was assessed using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific). The ratio of 260nm and 280nm absorbance readings (A260/A280) of 1.8 to 2.0 was considered as an acceptable indicator of nucleic acid purity. Complementary DNA (cDNA) was synthesised using a Primer Design Precision nanoScript 2 reverse transcriptase kit (Millbrook, Southampton, UK) according to the manufacturer’s instructions in a
MasterCycler® 480 thermocycler (Eppendorf, Hamburg, Germany). The cDNA was stored at -20°C until use.

Polymerase chain reaction (PCR) and Real time PCR (rt-PCR):

The rt-PCR Primers were designed using the ProbeFinder software v2.5 (Lifescience.roche.com). Oligonucleotide primers were obtained from Eurofins MWG/operon (Ebersberg, Germany) and their sequences as following: Human Albumin (NM_000477.5) F: 5’-GTGAGGTTGCTCATCGGTTT-3’ and R: 5’-GAGCAAAGGCAATCAACACC-3’), Antigen Identified By Monoclonal Antibody Ki-67, MKI-67 (NM_002417.4) F: 5’-TCAAGGAACTGATTCAGGAGAAG-3’ and R: 5’-GTGCACTGAAGAACAACATTTCC-3’), Histone Cluster 3, H3 (NM_003493.2) F: 5’-GAGCTGCTAATCCGCAAGTT-3’ and R: 5’-GCGCAGGTCGGTCTTAAA-3’), Vasoactive Intestinal Peptide Receptor 1, VIP and pituitary adenylate cyclase-activating polypeptide receptor-1 (VPAC1) (NM_004624) F: 5’-CTTCTGGTCGCCACAGCTATCCTG-3’ and R: 5’-ACTGCTGTCACTCCTCCTGATATC-3’), Epidermal Growth Factor Receptor, Epidermal Growth Factor Receptor (EGFR) (NM_005228.3) F: 5’-TTTCCTCCACGTGCTGAA-3’ and R: 5’-GGGTTCAAGGCTGATTGTTTATGCT-3’), and Glycerinadehyde-3-Phosphate-Dehydrogenase, GAPDH (NM_002046) F: 5’-GATGACATCAAGAAGGTGGTG-3’ and R: 5’-GCTGTAGCCAAATTCGTTGTC-3’). Level of VPAC1 and EGFR mRNA expression was assessed using GoTaq® Hot Start Polymerase (Promega UK Ltd, Southampton, UK) according to manufacturer instructions. PCR conditions were 2 minutes at 95°C, and followed by 35 PCR cycles of 60 seconds at 95°C, 60 seconds at 60°C and 60 seconds at 72°C. PCR products were visualized on 2% agarose gel, band density were measured and normalised to that of GAPDH using a ChemiDoc™ imaging system (Bio-Rad).
reaction volume was 10µl of 2x qPCR SYBR green Mastermix buffer (Primer Design),

primer pairs and cDNA template were mixed and PCR products were detected in an A&B
7900HT Fast Real-Time PCR System thermocycler (Applied Biosystems, CA, USA). PCR
conditions were 10 min at 95°C, and followed by 40 PCR cycles of 15 seconds at 95°C and
60 seconds at 60°C. All assays were performed in triplicates. The melting curve was
generated with a stepped temperature transition from 60 to 95°C with a rise of 1°C/5 sec for
each step. The Ct values were normalized to the GAPDH housekeeping gene and calibrated
to untreated cells. The relative quantification (RQ), expressed as fold change, was calculated
according to the ΔΔCt method.

Detection of Phospho-p44/42 MAPK (Erk1/2) and VPAC1 in hepatocytes:

Gel electrophoresis:

Hepatocytes were serum starved for 24 hour following 12 to 16 hours of attachment. Then
incubated with EGF (20 ng ml⁻¹) or VIP (10⁻⁶ M). To block B-RAF protein or cAMP cells
were incubated with SB-590885 or Rp-Adenosine 3’, 5’-cyclic monophosphorothioate
triethylammonium salt (Rp-cAMPS) inhibitors. Cells were lysed using 1x TruPAGE™ LDS
Sample Buffer [with 2mM Sodium orthovanadate (Na₂VO₃), 20mM Sodium Pyrophosphate
(Na₄P₂O₇), 1mM ethylenediaminetetraacetate (EDTA), 1mM EGTA and 0.5µg ml⁻¹
Leupeptin] and run a TruPAGE® 10% precast gels (Sigma) under reducing conditions. Total
protein concentrations were measured using bicinchoninic acid (BCA) colorimetric protein
assay kit (Sigma) in accordance with the manufacturer’s instructions using a BSA standard.

Western blotting:

Proteins were transferred to nitrocellulose membranes by a wet transfer method. Membranes
were blocked for 1 hour in 5% non-fat blotting grade cow’s milk (Bio-Rad) in 0.05 % Tris-
buffered saline (TBS)–Tween® 20 solution. The membranes were then probed with 1:1000
rabbit anti-human Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (New England Biolabs, Hertfordshire, UK) or 1:000 rabbit anti-human p44/42 MAPK (Erk1/2) antibody (New England Biolabs) overnight at 4°C, followed by goat anti-rabbit-horseradish peroxidase (HRP) (DakoCytomation, Cambridgeshire, UK) at a dilution of 1:2000 for 45 minutes. Reactive bands were visualised using the Luminata Forte Western HRP substrate chemiluminescent substrate (Millipore UK Ltd., Hertfordshire, UK) in a ChemiDoc™ imaging system (Bio-Rad). In another experiment, level of VPAC1 protein expression in untreated or VIP (10⁻⁶ M) treated hepatocytes, was investigated using a rabbit polyclonal anti VPAC1 (Abcam) and followed by goat anti-rabbit-horseradish peroxidase (HRP) (DakoCytomation) as before.

cAMP Direct Immunoassay:
Levels of cAMP in hepatocytes at 24 h following cell seeding (day 0) and at day 3 or 5 following stimulation with 10⁻⁶ M VIP were detected using a cAMP direct immunoassay (Abcam) according to the manufacturer’s instructions. Absorbance reading were determined at 450 nm and cAMP concentrations (µM) were calculated by plotting values against cAMP standard (0-0.25 µM) following background subtraction. cAMP concentrations (µM) were determined and corrected to total proteins concentrations in samples (µg).

Albumin ELISA:
Albumin concentrations in the supernatant of hepatocytes cultures was determined using the ELISA DuoSET® kit for human albumin (R&D Systems, Oxfordshire, UK) according to manufacturer’s instructions. Absorbance values were detected at 450nm with subtraction of readings at 570nm to compensate for optical interference on a microplate Reader. The detection range was from 2.5 to 160ng ml⁻¹.
Urea concentration assay:

The QuantiChrom™ urea assay kit (QuantiChrom, BioAssay Systems, Hayward, CA) was used according to the manufacturer’s instructions. Following 20 minutes incubation at room temperature, Absorbance at 430nm was measured and concentration of urea of the sample against 5mg dl⁻¹ standard was calculated in mg dl⁻¹.
References:


Vasoactive intestinal peptide (VIP) induces proliferation of human hepatocytes

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Supplementary Figure 1 ———————————————————————————————————————————— 1
Supplementary Figure 1 The effect of VIP on VPAC1 mRNA gene expression in hepatocytes. n = 3 for each condition. P values shown in the graph are for overall comparison with hepatocytes at day 1 of treatment. * P<0.05. Mean ± SEM. Two-way ANOVA followed by Fisher's least significant difference (LSD).
Vasoactive intestinal peptide (VIP) induces proliferation of human hepatocytes

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Supplementary Figure 2

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Supplementary Figure 2 Albumin production from human hepatocytes cultured with EGF and VIP. Hepatocytes were cultured in William’s E maintenance medium and EGF (5, 10 or 20 ng ml⁻¹) and VIP (10⁻⁶ M) was added one day later. n = 3 for each condition. P values shown in the graph are for comparison at individual concentrations. * P<0.05, ** P<0.005, *** P<0.0005, **** P<0.0001. Mean ± SEM. Two-way ANOVA followed by Fisher's least significant difference (LSD).