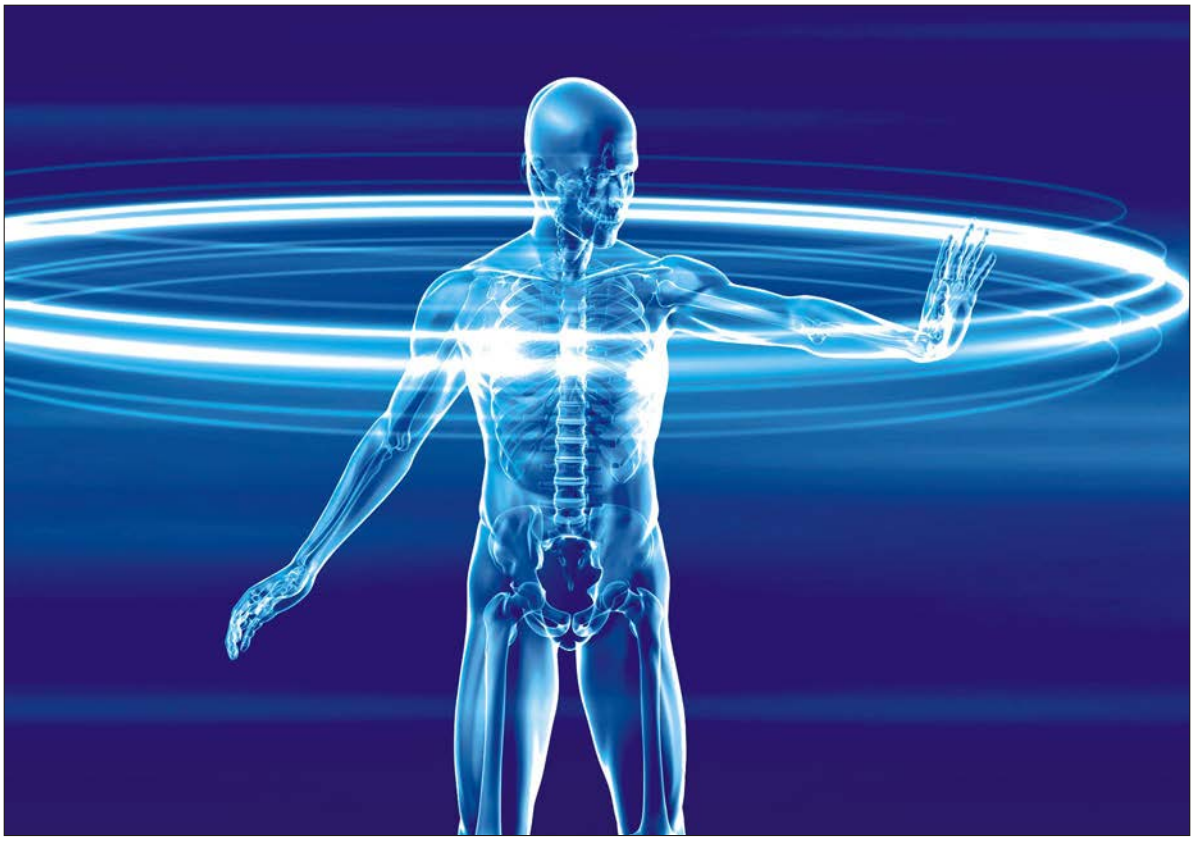

Research Journal of Immunology

ISSN 1994-7909

science alert

ANSInet
an open access publisher
http://ansinet.com

Research Journal of Immunology 1 (1): 21-28, 2008
ISSN 1994-7909

Expression of CXC Chemokine, IP-10/Mob-1 is Regulated by Specific p38, CAMK-II, NF-κB and Staurosporine Regulatory Pathways in Primary Hepatocytes

[1]Gholamhossein Hassanshahiraviz, [1]Shokrollah Asar, [1]Mohammad Kazemi Arababadi, [1]Nazanin Zia Sheikholeslami and [2]Alan James Dickson
[1]Department of Hematology and Immunology, School of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[2]Department of Biochemistry, Faculty of Life Sciences, The University of Manchester, UK

Abstract: Hepatocytes were isolated from male Sprague Dawley rat liver and cultured on collagen Type-I matrix. We employed western blotting analysis to investigate the expression of a CXC chemokine IP-10/Mob-1 by isolated and cultured hepatocytes in the presence of inhibitors of specific protein kinases. The expression of the CXC chemokines IP-10/Mob-1 was inhibited in the presence of inhibitors of specific protein kinases and signal pathways SB203580, MG132, KN62 and Staurosporine. Hence, these *in vitro* data may aid to a better understanding of the pathways in chronic liver injuries and identify clinical studies that may aid in treatment or prevention of these conditions. Furthermore, there may be further potential to prevent changes to hepatocyte phenotype and allow isolation of hepatocytes with a greater physiological phenotype.

Key words: CXC chemokine, hepatocyte, SB203580, MG132, KN62, staurosporine

INTRODUCTION

Isolated, hepatocytes re-enter the cell cycle that is associated with extensive down-regulation of liver-specific gene expression (Rana *et al*., 1994). Mechanisms involved in control of these responses are not fully identified and to date several signalling cascades have been implicated, including the MAPK pathways (Keller *et al*., 2006). Use of the p38 specific inhibitor, SB203580, has shown that p38 is involved in regulating expression of several pro-inflammatory genes such as TNF-α and IL-1 (Lee and Maddrey, 1995), IL-6, GM-CSF and the chemokine, MCP-1 (Goebeler *et al*., 1999) in monocytes. MAPK isoforms (JNK, ERK, p38) are activated in the liver in response to cellular stresses such as osmolarity (Kim *et al*., 2000) and heat shock (JNK, p38) (Maroni *et al*., 2000). Increased activity of p38 kinase, c-Jun and N-terminal kinase (JNK-1 and 2) and MAPK is an event that occurs during hepatocyte isolation by collagenase (Paine and Andreakos, 2004). NF-κB has also a central role in integration of stress responses and cell survival pathways and participates in the transcription of several target genes. Nuclear extracts of liver contain almost no NF-κB binding activity but NF-κB binding activity undergoes dramatic increases (within minutes) after partial hepatectomy (Cressman *et al*., 1996), NF-κB binding activity in turn induces genes containing κB binding sites that are involved in liver regeneration and NF-κB is responsible for induction of cytokine expression and subsequent to hepatic ischaemia/reperfusion (Hur *et al*., 1999; Jeyabalan *et al*., 2006). More recently Paine and Andreakos (2004) demonstrated that trace amounts of LPS and Lipoteichoic Acid (LTA) in collagenase activate NF-κB pathways during hepatocyte isolation. Increased concentration of

Corresponding Author: Dr. Gholamhossein Hassanshahiraviz, Department of Hematology and Immunology, School of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
Tel: 00983915234003-5 Fax: 00983915225209

chemokines, a subgroup of cytokines, has been documented in conditions of liver transplantation, ischaemia/reperfusion, alcoholic hepatitis, stress hepatoma and fibrotic liver (Hassanshahiraviz *et al*., 2006). Chemokines are low molecular weight proteins (8-17 kDa) and are classified in four distinct groups as CXC, CC, CX3C and C. Depending on the presence or absence of a motif called ELR (Arg-Leu-Glu) before the first cysteine residue in their structure, CXC chemokines are also subdivided into ELR^+ and ELR^-(Hassanshahi *et al*., 2007a). Increasing evidence has indicated the existence of a chemokine network in the liver which is involved in both physiological responses and, under certain circumstances, pathological and repair processes following hepatic injury (Hassanshahi *et al*., 2007b). In this study we aimed to investigate the signalling events related to stress, such as MAPK (p38 and ERKs) and NFκB that are activated during hepatocyte isolation and early culture. Therefore we have chosen IP-10/Mob-1 as an ELR^- chemokine to study at the level of protein in response to p38 pathway inhibitor (SB203580), NFκB inhibitor (MG123), CAMK-II and Staurosporine.

MATERIALS AND METHODS

This project has been performed during 2001-2005 in Faculty of Life Sciences, University of Manchester, UK. Perfusion, isolation and maintenance of hepatocytes in culture. Hepatocytes were obtained from fed male Sprague-Dawley rats (BSU, University of Manchester) weighing approximately 200 g. Hepatocytes were isolated from rats by perfusion of the liver with Krebs-Henseleit bicarbonate (128 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$)/collagenase (Sigma, Poole, UK) under aseptic conditions. After 10 min, the liver was removed and under sterile conditions, the liver was gently broken down and filtered through sterile gauze with Krebs-Henseleit bicarbonate. The cells were washed 3 times by differential centrifugation to harvest a population of large parenchymal cells and each time gently resuspending the pellet in Krebs- Henseleit bicarbonate. The final pellet was resuspended in inoculation medium (serum-free Waymouths MB/721 media; Invitrogen Ltd, Paisley, Scotland, UK) and the viability of the cells was assessed using trypan blue. The hepatocytes were used only if they were >85% viable and were generally 90-95% viable. The hepatocytes were of higher purity and under the light microscope, endothelial cells were rare, (never more than 1% of the population). Random batches of cells were checked for endothelial cell contamination using specific antibody immunofluorescence with antibodies to von Willebrands factor (Santa Cruz Biotechnology, California, USA). The hepatocytes were seeded (2×106 cell mL^{-1}) on to collagen type 1-coated plates (3 cm plates for ribonucleic acid (RNA) and 6 cm plates for protein) and cultured in inoculation medium at 37°C under an atmosphere of 5% CO_2 in O_2. After 3 h in culture, the media on the cells was replaced with maintenance medium (Waymouths MB/721 media supplemented with Bovine Serum Albumin (BSA) [0.2% w/v] and sodium oleate [0.0005% w/v]). The cells were treated as described in the figure legends.

Western Blot Analysis

At indicated time points, medium was removed from hepatocyte cultures and centrifuged. Clarified supernatants from 0 h and incubated samples were used for Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDSPAGE). Immunoblotting and densitometry was performed to quantify the expression of IP-10/Mob-1. Equal amounts of protein (35 g) were loaded and resolved on a 10% SDS-PAGE and then transferred to a nitrocellulose membrane. After blocking with 3% (w/v) milk in phosphate-buffered saline (PBS)/Tween (10 mM Tris, pH 7.4 containing 140 mM NaCl, 0.1% [v/v] Tween 20), the nitrocellulose membrane was incubated overnight at 4°C in PBS/Tween containing 3% (w/v) milk including anti-rat IP-10 (Chemokine. com, Houston, USA supplied by AMS Biotechnology, Abingdon, UK). Subsequently, anti-rabbit horseradish peroxidase-conjugated antibodies (diluted, 1:1000) were used accordingly and the Enhanced Chemiluminescence (ECL) detection system (Amersham International) were used to define protein localization and amount.

Statistical Analysis

All data are expressed as mean±SEM. Comparisons of variables between 2 groups were performed using an unpaired Student's t-test. Differences were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

This study has been designed to investigate the involvement of protein kinase pathways in chemokine expression. As other investigators have implicated the involvement of MAPK (Varley *et al*., 2003; Matthias *et al*., 2003) and NF-κB (Wang *et al*., 1999) (by using SB203580 and MG132, respectively) in regulation of chemokines, we have also selected inhibitor, KN62 for the CAMKII pathway (Rovin *et al*., 1999). Staurosporine, as a wide variety inhibitor for several protein kinases and serine/threonine kinases (Cho *et al*., 2003) such as cAMP-protein kinase, PKC and CAMK II (Johnson *et al*., 2002). Statistical analysis of results showed that there is a significant difference in expression of IP-10/Mob-1 in the presence of 20 and 50 μM of SB203580 and MG132 but not at lower concentrations (Fig. 1, 2). KN62 inhibited expression of IP-10/Mob-1 at higher concentrations. There is a significant decrease in expression of IP-10/Mob-1 in the presence of 10 and 20 μM but not at lower concentrations of KN62 (Fig. 3). Analysis of data showed that Staurosporine produce a significant decrease in expression of IP-10/Mob-1 at 5 and 10 μM but not at lower concentrations (Fig. 4).

MAPKs are part of an important pathways by which signals from endogenous stimuli are transmitted to the nucleus of cells. MAPK family members include ERK1, ERK2, c-Jun NH_2-terminal kinase (JNK) and p38 (Goh *et al*., 1999). SB203580 is a pyrinidyl-imidazole, which has been widely

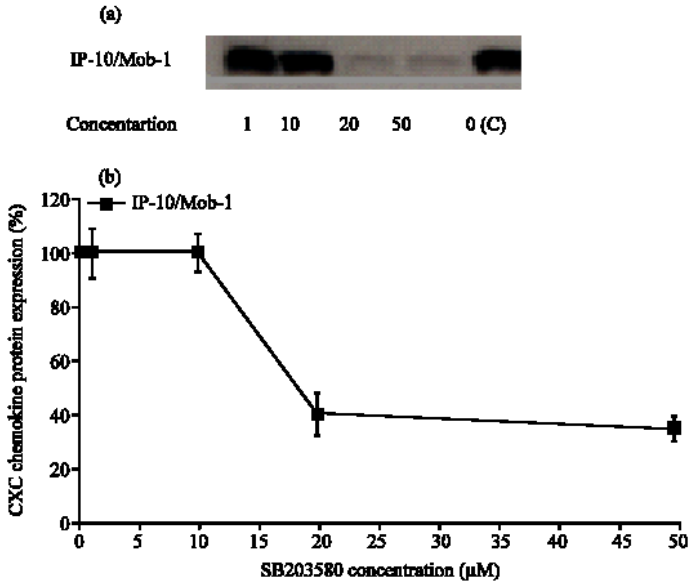

Fig. 1: Expression of IP-10/Mob-1 at protein level by hepatocytes in presence and absence of different concentration of SB203580, (a) Representative profile of protein bands from western blotting of IP-10/Mob-1 in presence and absence of different concentration of SB203580 and (b) Concentration dependent variation of IP-10/Mob-1 protein expression in presence and absence of SB203580. As it is clear, the expression of chemokines is decreased in higher concentration of SB203580. [($p<0.05$ V 100% value)]

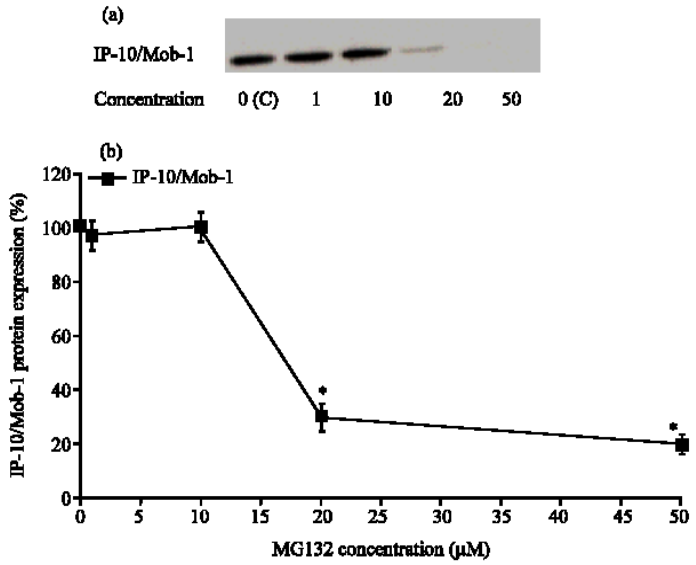

Fig. 2: Expression of IP-10/Mob-1 at protein level by hepatocytes in presence and absence of different concentration of MG132, (a) Representative profile of protein bands from western blotting of IP-10/Mob-1 in presence and absence of different concentration of MG132 and (b) Concentration dependent variation of IP-10/Mob-1 protein expression in presence and absence of MG132. As it is clear, the expression of IP-10/Mob-1 is decreased in higher concentration of MG132. [(p<0.05 V 100% value)]

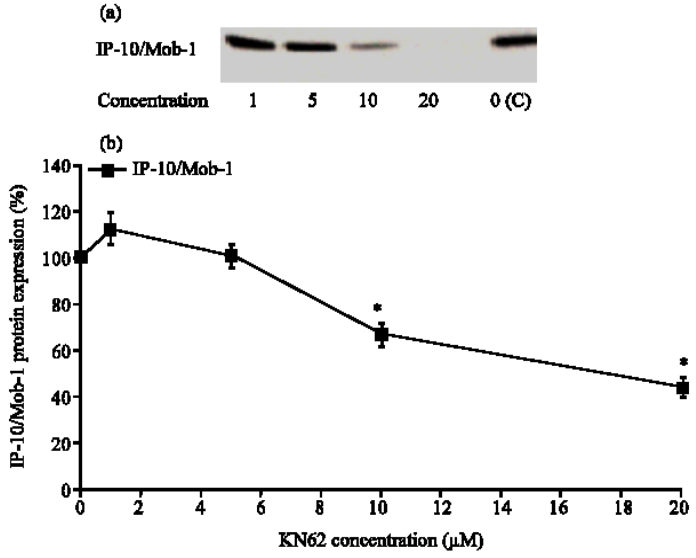

Fig. 3: Expression of IP-10/Mob-1 at protein level by hepatocytes in presence and absence of different concentration of KN62, (a) Representative profile of protein bands from western blotting of IP-10/Mob-1 in presence and absence of different concentration of KN62 and (b) Concentration dependent variation of IP-10/Mob-1 protein expression in presence and absence of MG132. As it is clear, the expression of IP-10/Mob-1 is decreased in higher concentration of KN62. [(p<0.05 V 100% value)]

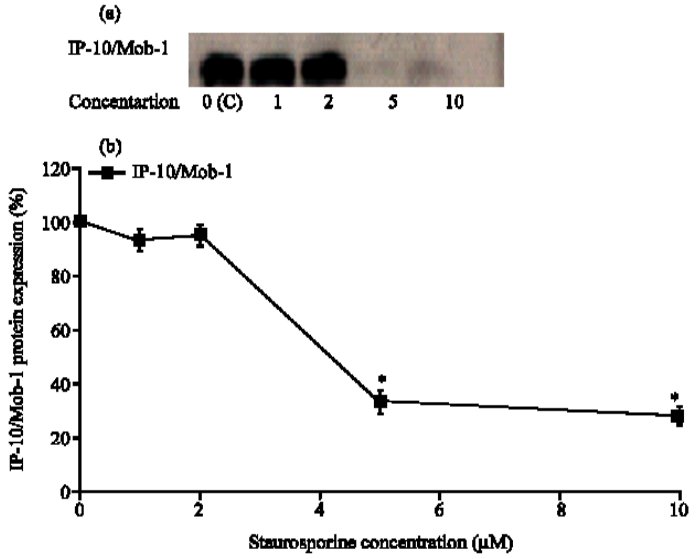

Fig. 4: Expression of IP-10/Mob-1 at protein level by hepatocytes in presence and absence of different concentration of Staurosporine, (a) Representative profile of protein bands from western blotting of IP-10/Mob-1 in presence and absence of different concentration of Staurosporine and (b) Concentration dependent variation of IP-10/Mob-1 protein expression in presence and absence of Staurosporine. As it is clear, the expression of IP-10/Mob-1 is decreased in higher concentration of Staurosporine. [($p<0.05$ V 100% value)]

used as an inhibitor of p38 (Keller *et al*., 2006; Lee and Maddrey, 1995). SB203580 inhibited MAPK kinases pathways in several cell types including mesangial cells (Rovin *et al*., 1999), endothelial cells (Goh *et al*., 1999), HeLa cells (Dillman *et al*., 2004) and hepatocytes (Varley *et al*., 2003). In this study, SB203580 was able to inhibit IP-10/Mob-1 expression at concentration greater that 20 µM. SB203580 has been shown to produce biological effects in several cell types in concentrations that range from 10 µM in epidermal keratinocytes (Dillman *et al*., 2004) to 30 µM in human myeloid leukemia cells and mesangial cells (Rovin *et al*., 1999; Blinman *et al*., 2000). In particular, it is relevant that 20 µM SB203580 was used to inhibit the expression of Gro/KC and MCP-1 that occurred with isolation of acinar pancreatic cells (Ohmori and Hamilton, 1993). Given the above information the effects we have observed for SB203580 appear to occur within the acceptable concentration range reported by others and hence reflect a degree of sensitivity for inhibitory action. The inability to totally inhibit IP-10/Mob-1 expression could be due to the involvement of other pathways in the control of expression of this chemokine. For example, at transcriptional level response elements for NF-κB are present in the promoters of this gene and SB203580 may not affect the NF-κB pathway (Wang *et al*., 1999; Joshi-Brave *et al*., 2003). In another epithelial cell system (pancreatic cells), it has been demonstrated that expression of the chemokines Gro/KC, MIP-2 and a CC chemokine MCP-1 requires p38 and that SB203580 blocked the stimulation of production of these chemokines in response to isolation of acinar pancreatic cells (Ohmori and Hamilton, 1993). Intracellular protein degradation by the ubiquitin-proteosome system is involved in regulation of a variety of important biological processes. In this study the expression of IP-10/Mob-1 in early hepatocyte culture was inhibited by MG132 at concentrations greater than 20 µM. IP-10/Mob-1 contains NF-κB consensus elements in their promoter and this appears to explain the inhibition of the IP-10/Mob-1 and Gro/KC expression in presence of MG132. In HepG2 cells MG132 inhibited other cytokines such as, TNF-α and IL-1 induced IL-8 expression at a concentration of 25 µM and in acinar pancreatic cells the

expression of Gro/KC and MCP-1 that occurred due to cell isolation was inhibited by 10 μM MG132 (Ohmori and Hamilton, 1993; Joshi-Brave *et al*., 2003). By comparison to the concentration-dependency of our results it would appear that the effects of MG132 are operating at concentrations that reflect an action on proteosome function. Other studies in agreement with my results in hepatocytes showed the involvement of NF-κB pathway in expression of IP-10/Mob-1 in response to isolation (Wang *et al*., 1999). To date there has been no other studies to show the effects of MG132 in chemokine expression by hepatocytes, but the inhibitory effects of MG132 have been shown for NF-κB and chemokine expression in acinar pancreatic cells (Ohmori and Hamilton, 1993). KN62 is a specific inhibitor for Ca^{2+}/calmodulin-dependent protein kinase-II (Ca^{2+}/CAMK-II) pathway. In this study IP-10/Mob-1 was inhibited at the higher concentrations of KN62 tested. A role for Ca^{2+}/CAMK-II in regulation of chemokine expression has not been proposed and there are no reports for effects of KN62 on chemokine expression. Although the exact mechanism of regulation of IP-10/Mob-1 expression in relation to the inhibitory effects of KN62 is not clear, it probably involves a more common and general pathway of inhibition and may involve in reduction of other genes such as pro-inflammatory cytokines that may regulate the expression of these chemokines in hepatocytes. Staurosporine has a wide variety of inhibitory and stimulatory effects. It has relatively non-specific inhibitory functions against a variety of Serine/Threonine protein kinases (Cho *et al*., 2003) including cAMP-dependent protein kinase and PKC (Johnson *et al*., 2002), cGMP-dependent protein kinase and Ca^{2+}/CAMK-II, MAPK (Johnson *et al*., 2002) and insulin receptor tyrosine kinase activity. In the present study, we found that staurosporine in higher concentrations inhibited expression of IP-10/Mob-1. It is possible that due to its very non-specificity, staurosporine affects some other genes that may be indirectly involved in IP-10/Mob-1 expression. Staurosporine has been reported to inhibit NF-κB activation (probably by inhibition of I-κB kinases) and has been shown to inhibit pro-inflammatory cytokines (TNF-α, IL-6 and IL-1) signalling outcomes and control the expression of chemokines (Feng and Kaplowitz, 2002).

CONCLUSION

For a future study it is possible to investigate and look at the mechanisms are involved in the expression of IP-10/Mob-1 and the other CXC chemokines at the level of mRNA in the liver cells.

ACKNOWLEDGMENTS

We would like to thank members of Lab 2.300, both past and present, for their help and patience and for making life in the lab a very enjoyable experience. Dr. Gholamhossein Hassanshahiraviz would also like to thank from the bottom of his hearth from Ministry of Health and Medical Education of Islamic Republic of Iran for financial supports.

REFERENCES

Blinman, T.A., I. Gukovsky, M. Mouria, V. Zaninovic, E. Livingston, S.J. Pandol and A.S. Gukovskaya, 2000. Activation of pancreatic acinar cells on isolation from tissue: Cytokine upregulation via p38 MAP kinase. Am. J. Physiol. Cell Physiol., 279 (6): 1993-2003.

Cho, J.Y., D.R. Katz and B.M. Chain, 2003. Staurosporine induces rapid homotypic intercellular adhesion of U937 cells via multiple kinase activation. Br. J. Pharmacol., 140 (2): 269-276.

Cressman, D.E., L.E. Greenbaum, R.A. DeAngelis, G. Ciliberto, E.E. Furth, V. Poli and R. Taub, 1996. Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. Science, 274 (5291): 1379-1383.

Dillman, J.F., K.L. McGary and J.J. Schlager, 2004. An inhibitor of p38 MAP kinase downregulates cytokine release induced by sulfur mustard exposure in human epidermal keratinocytes. Toxicol. *In vitro*., 18 (5): 593-599.

Feng, G. and N. Kaplowitz, 2002. Mechanism of staurosporine-induced apoptosis in murine hepatocytes. Am. J. Physiol. Gastrointest. Liver Physiol., 282 (5): 825-834.

Goebeler, M., K. Kilian, R. Gillitzer, M. Kunz, T. Yoshimura, E.B. Bröcker, U.R. Rapp and S. Ludwig, 1999. The MKK6/p38 stress kinase cascade is critical for tumor necrosis factor-alpha-induced expression of monocyte-chemoattractant protein-1 in endothelial cells. Blood, 93 (3): 857-865.

Goh, K.C., S.J. Haque and B.R.G. Williams, 1999. p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons. EMBO. J., 18 (20): 5601-5608.

Hassanshahiraviz, G., A.M. Kazemi and A.J. Dikson, 2006. Expression of CXC chemokine Gro/Kc and SDF-1α in rat H4 hepatoma cells in respond to different stimuli. IJI., 3 (2): 55-60.

Hassanshahi, G., S.P. Sanjai, A. Jafarzadeh and J.D. Alan, 2007a. Expression of CXC chemokine IP-10/Mob-1 by primary hepatocytes following heat shock. Saudi Med. J., 28 (4): 181-186.

Hassanshahi, G., A. Jafarzadeh, Z. Ghorashi, S.N. Zia and D.A. James, 2007b. Expression of IP-10 chemokine is regulated by pro-inflammatory cytokines in cultured hepatocytes. Iran J. Allergy Asthma Immunol., 6 (3): 115-121.

Hur, G.M., Y.S. Ryu, H.Y. Yun, B.H. Jeon, Y.M. Kim, J.H. Seok and J.H. Lee, 1999. Epatic ischemia/reperfusion in rats induces iNOS gene transcription by activation of NF-κB. Biochem. Biophys. Res. Commun., 261 (3): 917-922.

Jeyabalan, G., A. Tsung and T.R. Billiar, 2006. Linking proximal and downstream signalling events in hepatic ischaemia/reperfusion injury. Biochem. Soc. Trans., 34 (5): 957-959.

Johnson, L.N., E.D. Moliner, N.R. Brown, H. Song, D. Bradford, J.A. Endicott and M.E.M. Noble, 2002. Structural studies with inhibitors of the cell cycle regulatory kinase cyclin-dependent protein kinase 2. Pharmacol. Theor., 93 (2): 113-124.

Joshi-Brave, S., S.S. Brave, W. Butt, J. Klein and C.J. McClain, 2003. Inhibition of proteasome function leads to NF-κB-independent IL-8 expression in human hepatocytes. Hepatology, 38 (5): 1178-1187.

Keller, M., A.L. Gerbes, S. Kulhanek-Heinze, T. Gerwig, U. Grutzner, R.N. Van, A.M. Vollmar and A.K. Kiemer, 2006. Hepatocyte cytoskeleton during ischemia and reperfusion-influence of ANP-mediated p38 MAPK activation. J. Hepatol., 44 (4): 758-767.

Kim, T.H., W. Mars, D.B. Stolz and G.K. Michalopoulos, 2000. Expression and activation of pro-MMP-2 and pro-MMP-9 during rat liver regeneration. Hepatology, 31 (1): 75-82.

Lee, W.M. and W.C Maddrey, 1995. Acute liver failure and transplantation: A symposium. Liver Transplant Surg., 1 (3): 176-177.

Maroni, P., P. Bendinelli, C. Zuccorononno, L. Schiaffonati and R. Piccoletti, 2000. Cellular signalling after *in vivo* heat shock in the liver. Cell Biol. Int., 24 (3): 145-152.

Matthias, P.W., Z. Marketa and L. Muriel, 2003. Phosphoinositide 3-kinase signalling-which way to target? Trends Pharmacol. Sci., 24 (7): 366-376.

Ohmori, Y. and T.A. Hamilton, 1993. Cooperative interaction between interferon (IFN) stimulus response element and kappa B sequence motifs controls IFN gamma and lipopolysaccharide-stimulated transcription from the murine IP-10. J. Biol. Chem., 268 (9): 6677-6688.

Paine, A.J. and E. Andreakos, 2004. Activation of signalling pathways during hepatocyte isolation: Relevance to toxicology *in vitro*. Toxicol. *In vitro*, 18 (2): 187-193.

Rana, B., D. Mischoullon, Y. Xie, N.L. Bucher and S.R. Farmer, 1994. Cell-extracellular matrix interactions can regulate the switch between growth and differentiation in rat hepatocytes: Reciprocal expression of C/EBP alpha and immediate-early growth response transcription factors. Mol. Cell Biol., 14 (9): 5858-5869.

Rovin, B.H., W.A. Wilmer, M. Danne, J.A. Dickerson, C.L. Dickson and L. Lu, 1999. The mitogen-activated protein kinase p38 is necessary for interleukin 1beta-induced monocyte chemoattractant protein 1 expression by human mesangial cells. Cytokine, 11 (2): 118-126.

Varley, C.L., S. Armitage, G. Hassanshahiraviz and A.J. Dickson, 2003. Regulation of the C-X-C chemokine, mob-1, gene expression in primary rat hepatocytes. Cytokine, 23 (3): 64-75.

Wang, H., X. Gao, S. Fukomoto, S. Tademoto, K. Sato and K. Hirai, 1999. Differential expression and regulation of chemokines JE, KC ands IP-10 gene in primary cultured murine hepatocytes. J. Cell. Physiol., 181 (1): 361-370.