THE ROLE OF NITRIC OXIDE AND PROSTAGLANDINS IN TRIGGERING AND POTENTIATION OF THE LIVER REGENERATION CASCADE

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Therapeutics
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Abstract

Liver regeneration has been studied for over 100 years, and yet the trigger of the liver regeneration cascade is unknown. It has been hypothesized that nitric oxide (NO), released secondary to an increase in shear stress in the liver, triggers the liver regeneration cascade. To further test this hypothesis, c-fos mRNA expression after partial hepatectomy (PHX) and left branch portal vein ligation (PVL), a model of liver hyperplasia with similar hemodynamic conditions as PHX, was used as an index of initiation of the liver regeneration cascade. c-Fos mRNA expression increased after PHX and PVL, and was inhibited by the NOS antagonist, L-NAME. The NO donor, SIN-1, reversed the inhibition. Also, the increase in c-fos expression after PVL was prevented by ligation of the superior mesenteric artery, thus causing a 2/3 decrease in portal venous blood flow. These results support the hypothesis that a hemodynamic change after PHX or PVL causes shear stress and NO release in the remaining liver, which triggers the liver regeneration cascade.

Prostaglandins (PGs) are released after PHX and in response to shear stress. It was hypothesized that NO and PGs are released in response to increased shear stress after PHX, and work together to trigger the liver regeneration cascade. NOS or COX inhibition prevented the increase in c-fos expression after PHX. This inhibition was reversed by the NO donors, SIN-1 or SNAP, and PGE_2 or PGI_2, suggesting that NO and PGs work together and cause the increase in c-fos mRNA expression after PHX, and that excess amounts of exogenous NO or PGs can compensate for the absence of the other.

SNAP, the phosphodiesterase V antagonist, Zaprinast (ZAP), PGI_2, and the combination of all three, potentiated c-fos mRNA expression after PHX. Also, liver weight restoration, which includes the entire regeneration cascade, was potentiated by ZAP, 6-keto-$PGF_{1\alpha}$, a stable metabolite of PGI_2, and the combination of ZAP and 6-keto-$PGF_{1\alpha}$, 48 hours after PHX. Thus, NO and PGs can potentiate the liver regeneration cascade, and represent potential therapeutic targets for patients undergoing liver resection.

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AP-1: activator protein-1

cAMP: cyclic adenosine monophosphate

cGMP: cyclic guanosine monophosphate

cdk: cyclin dependent kinase

CRE: cAMP-response element

CREB: cAMP-response element binding protein

COX: cyclooxygenase

EGF: epidermal growth factor

GC: guanylate cyclase

HGF: hepatocyte growth factor

IL-1: interleukin-1

IL-6: interleukin-6

6-keto-$PGF_{1\alpha}$: 6-keto-prostaglandin $F_{1\alpha}$

L-NAME: N^{ω}-nitro-L-arginine methyl ester

NO: nitric oxide

NOS: nitric oxide synthase

eNOS: endothelial/constitutive nitric oxide synthase

iNOS: inducible nitric oxide synthase

nNOS: neuronal nitric oxide synthase

O_2^-: superoxide anion

PDE: phosphodiesterase

PFs: proliferative factors

PG(s): prostaglandin(s)

PGE_2: prostaglandin E_2

PGI_2: prostacyclin

PHX: partial hepatectomy

PVL: selective left branch portal vein ligation

PVP: portal venous pressure

SIN-1: 3-morpholinosydnonimine

SNAP: s-nitroso-n-acetylpenicillamine

TGF-α: transforming growth factor-α

TGF-β: transforming growth factor-β

TNF-α: tumor necrosis factor-α

ZAP: Zaprinast, a phosphodiesterase type V antagonist

Hypothesis

An increase in the blood flow-to-liver mass ratio following 2/3 partial hepatectomy (PHX) causes shear stress and the release of nitric oxide (NO), which triggers the liver regeneration cascade. In addition, prostaglandins (PGs) are also released in response to shear stress, and these PGs work together with NO to trigger the liver regeneration cascade.

NO and PGs represent possible therapeutic targets that can potentiate the liver regeneration cascade.

Specific Hypotheses

1. An increase in the blood flow-to-liver mass ratio causes shear stress-induced NO release, which triggers the liver regeneration cascade.
2. c-Fos mRNA expression is a valid index for the initiation of the liver regeneration cascade.
3. The increase in portal venous pressure (PVP), and therefore shear stress, after PHX and ligation of the left branch of the portal vein (PVL) will be similar.
4. Manipulation of blood flow to the liver by ligation of the superior mesenteric artery, which reduces portal venous blood flow by 2/3, prior to PVL will prevent the increase in c-fos mRNA expression that occurs after PVL.
5. eNOS is responsible for NO release, which triggers the liver regeneration cascade.

6. PGs are released in response to shear stress in the remnant liver, and, along with NO, trigger the liver regeneration cascade.

7. PGs and NO potentiate c-fos mRNA expression after PHX.

8. PGs and NO potentiate liver weight restoration after PHX.

9. PGs and NO are possible therapeutic targets for potentiation of liver regeneration.

Introduction

The liver accounts for 2 to 5% of body weight in the adult human, and is the largest solid organ in the body (Desmet, 1994). Approximately 30% of liver volume is made up of blood (Lautt and Greenway, 1987). Classically, the human liver is divided into 4 lobes: the left and right lateral, caudate and quadrate lobes (Desmet, 1994). Blood flows into the liver via the hepatic artery and the portal vein, and is drained by the hepatic vein into the inferior vena cava. The liver does not control inflowing blood from the portal vein. The portal vein and hepatic artery enter the liver at the hilum, and branch repeatedly until they reach terminal arterioles and portal venules. These terminal branches open into hepatic sinusoids.

In 1833, Kiernan gave one interpretation of the anatomical arrangement of the liver (reviewed by Sasse et al., 1992). According to this concept, the cells are arranged into lobules, a polygonal areas of parenchyma delineated by a boundary of connective tissue, with corners occupied by portal spaces containing a branch of the portal vein, a branch of the hepatic artery and a bile ductule (portal triad). The sinusoids transport the blood from the portal triad toward the "central" vein, situated in the center of the lobule, which drains the entire lobule. Alternately, Rappaport and co-workers (1954) described an arrangement of parenchyma and sinusoidal cells, termed the "hepatic acinus". This model is based on the 3-dimensional arrangement of hepatocytes and sinusoids, and the direction of blood flow in the microcirculation. The acinus is defined as "a small parenchymal mass irregular in size and shape arranged around an axis consisting of a terminal portal venule, terminal hepatic arteriole, bile ductule(s), lymph vessels, and

nerves which grow out together from similar preterminal structures in a small triangular portal field" (Rappaport et al., 1954). Rappaport also described 3 zones within the sinusoid: zone 1, or periportal, zone 2, mid-lobar (between zones 1 and 3), and zone 3, or perivenous (Rappaport, 1973).

The liver consists of several different cell types. The hepatic parenchyma, composed of hepatocytes, accounts for 80% of the cells in the liver. The hepatocytes are arranged in plates, usually 1 cell thick, and have on one side the sinusoid, and on the other the bile caniculus (Ekataksin and Kaneda, 1999) (Figure 1). The hepatic sinusoid consists of fenestrated endothelial cells, which line the sinusoid, Kupffer cells, which are the resident macrophages of the liver and are attached to the luminal surface of the endothelial cells, and fat storing (stellate) cells interspersed between the endothelium and hepatic parenchymal cells in the space of Disse (McCuskey et al., 1986). The diameter of the sinusoid is more narrow in the periportal region and wider in the perivenous region (Wisse et al., 1983). The endothelial cells in the hepatic sinusoid are unique, in that they possess fenestrae in their cell wall. These fenestrae are organized in "sieve plates" and have no diaphragms controlling their size (McCuskey et al., 1986). The fenestrae in the periportal region are slightly wider than those in the perivenous region, and play a role in transport of fluid, particles and lipid droplets from the passing blood (Wisse et al., 1983). Stellate cells are located in the space of Disse (the space between the endothelial cells and hepatocytes), surrounding the sinusoid (Tanikawa, 1995). The stellate cells have long cytoplasmic processes, which contain large amounts of actin and myosin, suggesting that these cells can contract and play a role in regulation of sinusoidal circulation (Tanikawa, 1995).

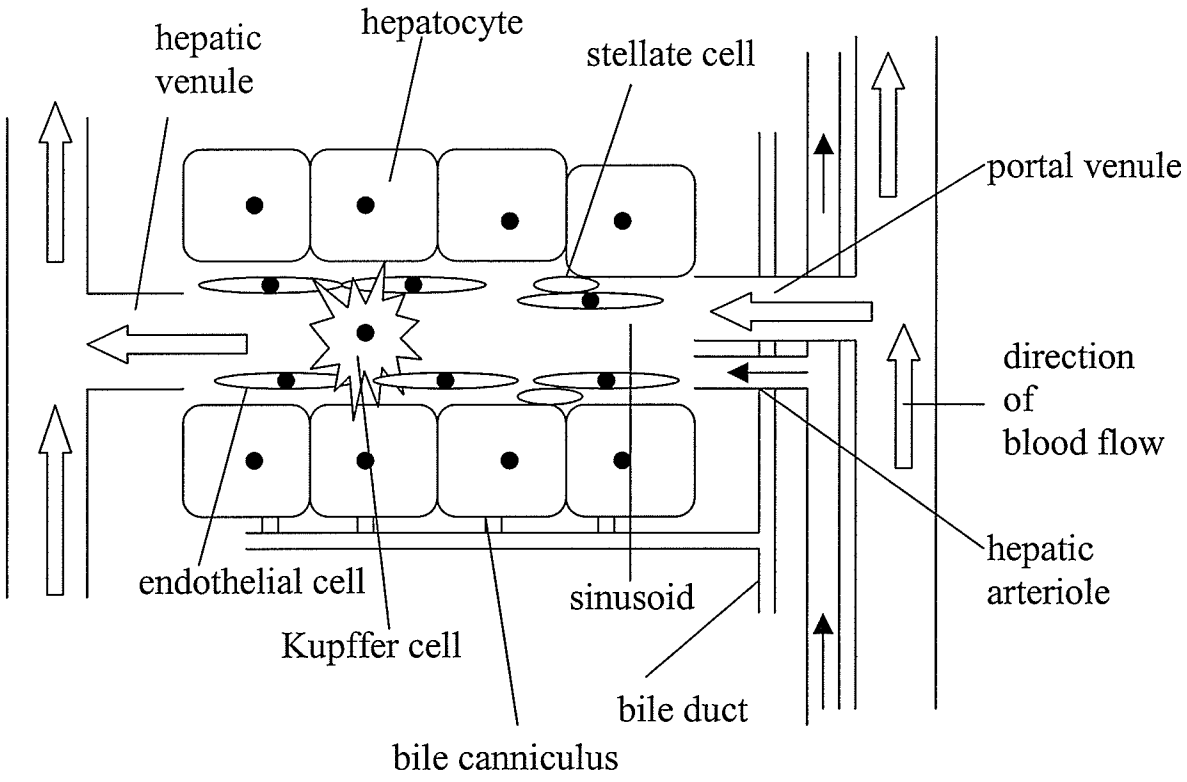

Figure 1: The hepatic sinusoid. Cells in the hepatic sinusoid include hepatocytes, endothelial cells, stellate cells and Kupffer cells. Blood flows in from the portal vein, through portal venules, and from the hepatic artery, via hepatic arterioles. Arterial and venous blood enter the sinusoid, where they mix. Nutrients and toxins and other xenobiotics are taken up by the cells stored, modified, or excreted.

I. Liver Regeneration

In addition to its many physiological functions, the liver has a remarkable ability to regenerate following surgical resection or chemical damage. The "regeneration" is more correctly described as "compensatory hyperplasia", as the remnant liver grows and enlarges, rather than restoration of liver tissue in the area from where it was removed (Fishback, 1929). The enlargement is due to cellular proliferation, with regeneration of the liver cells occurring directly from previously existing cells (Milne, 1909), with no dependence on progenitor (or stem) cells (Michalopoulos and DeFrances, 1997). Liver regeneration is a rapid process, and hepatic hypertrophy occurs most rapidly when large amounts of the tissue are removed.

A common model for studying the liver regeneration cascade is 2/3 partial hepatectomy (PHX), developed by Higgins and Anderson in 1931. This model involves ligation and surgical resection of the left lateral and median lobes of the liver, which account for 2/3 of the liver weight. Following PHX, the remaining liver tissue rapidly grows, with 50% of weight restoration complete within the first 48 hours in the rat. Liver regeneration is usually complete within 14 days (Higgins and Anderson, 1931). In addition, the immense chronic regenerative capacity of the liver has been demonstrated (Fishback, 1929). Three sequential PHXs were performed, and on each occasion, liver regeneration occurred, restoring the liver to its original size.

Compensatory hyperplasia also occurs in response to an alteration in blood flow to the liver lobes. Ligation of the left branch of the portal vein (PVL), which supplies portal venous blood to the left lateral and median lobes, the same lobes removed during PHX (Rocheleau et al., 1999), causes hypertrophy of the nonligated lobes and atrophy of

the ligated lobes in a reciprocal manner. The concomitant hypertrophy and atrophy results in a constant liver weight, which is not different from that of the original liver (Rozga et al., 1986). Liver weight restoration (or hypertrophy of the nonligated lobes) after PVL occurs in a similar manner as that of PHX. Within 48 hours after PVL, the nonligated lobes hypertrophy, accounting for 50% of the total liver weight, while the ligated lobes begin to atrophy. Within 14 days, the nonligated lobes account for 100% of original liver weight, while the ligated lobes have completely atrophied (Um et al., 1994). DNA synthesis also occurs in a similar manner in both models, peaking at 24 hours after PHX or PVL (Lambotte et al., 2000).

In order for DNA synthesis and cell replication to take place, a complex cascade of events is initiated. Cell replication in the normal liver is very low, with approximately 1 in 1000 hepatocytes undergoing DNA synthesis (Diehl and Rai, 1996). However, following PHX or PVL, DNA synthesis increases about 10-fold (McNeil et al., 1985; Bilodeau et al., 1999). In order that cells synthesize new DNA and replicate, they must enter into, and proceed through, the cell cycle. Briefly, the cell cycle consists of 4 phases: G_1, or gap phase 1, an interval between cell division and DNA synthesis; S, or synthesis phase, where DNA synthesis occurs; G_2, or gap phase 2, an interval between DNA synthesis and mitosis during which DNA repair also occurs; and M, or mitosis phase, where mitosis and cell division take place. The majority of adult cells are in a quiescent state in G_1, also known as G_0. Cells must be stimulated, and adequate growth conditions must be met for cells to exit G_0 and enter G_1 and the cell cycle to divide (Alberts et al., 1989). Following PHX or PVL, cells enter the cell cycle resulting in increased DNA synthesis, which peaks at 24 hours (Lindroos et al., 1991; Zarnegar et al.,

1991; Tomiya et al., 1998; Lambotte et al., 2000) and remains elevated for 96 hours (Tomiya et al., 1998). The largest proportion of hepatocytes in S phase at 24 hours after PHX are found in the periportal and midzonal areas (zone 1 and 2), and relatively less in the perivenous area (zone 3) (Post and Hoffman, 1964). Within the first 24 to 48 hours after PHX, most hepatocytes in the remaining liver will enter into the cell cycle and replicate at least once (Higgins and Anderson, 1931).

I.i. The Liver Regeneration Cascade

The liver regeneration cascade, which will be illustrated using the PHX model, can be divided into two phases: 1) a priming phase, where quiescent hepatocytes transition into the cell cycle; and 2) a progression phase, which includes DNA synthesis and cell proliferation (Figure 2) (Fausto and Webber, 1994). Initial events in the priming phase include activation of immediate early and delayed early genes, transcription factors and growth factors, while the progression phase consists of liver cells synthesizing DNA and replicating via completion of the cell cycle. The priming phase takes place during the G_1 phase, which lasts for 18 hours after PHX. Progression includes the S phase, during which DNA synthesis occurs, that lasts from about 18 to 28 hours after PHX. G_2 phase occurs approximately 28 to 30 hours after PHX, followed by cell division during the M phase at about 32 hours (Loyer et al., 1994).

I.i.i. Gene Expression

Immediate early genes are genes whose mRNA expression is rapidly upregulated without the need for transcription. That is, these genes can be upregulated in the

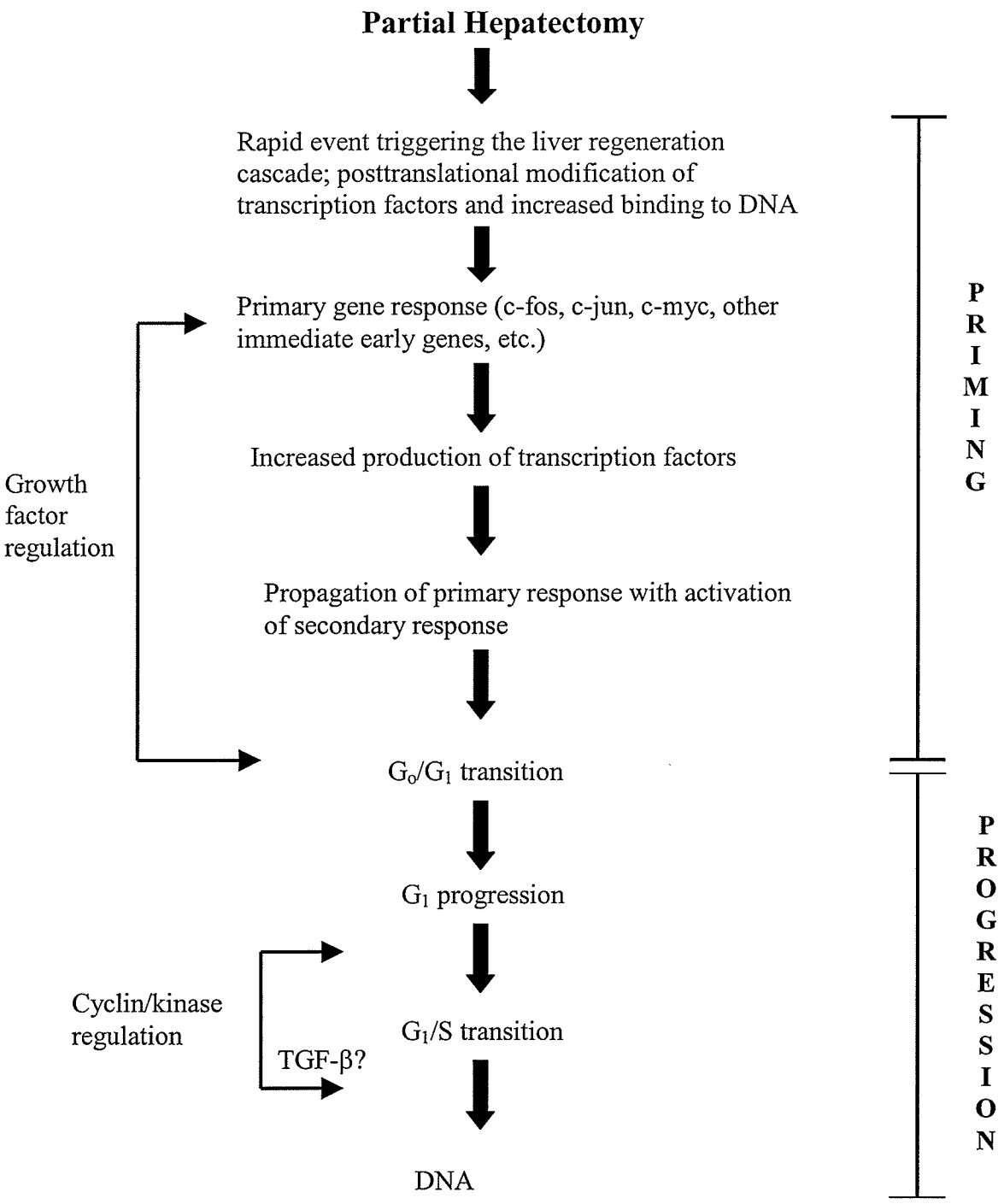

Figure 2: scheme of the liver regeneration cascade.

Adapted from: Fausto and Webber, 1994

presence of cyclohexamide, which inhibits transcription. The immediate early genes c-fos, c-myc and c-jun are rapidly upregulated after PHX (Morello et al., 1990; Haber et al., 1993). c-Fos mRNA increased in the liver within 1 hour of PHX (Cressman et al., 1996), peaking 15 minutes after PHX (Moser et al., 2001). C-Jun is also upregulated within 30 minutes (Moser et al., 2001) and c-myc increases within 1 hour of PHX (Cressman et al., 1996). Another class of genes upregulated during liver regeneration is the delayed early genes. These genes are activated after induction of the immediate early genes, and their activation requires transcription. An example is Bcl-2, the main antiapoptotic gene expressed by the liver. Bcl-2 is upregulated after PHX, peaking at 8h (Tzung et al., 1997). This gene could potentially suppress apoptosis in the early phase of liver regeneration, but its exact function is not known.

I.i.ii. Transcription Factors

Transcription factors are factors that bind specific recognition sites in genes to initiate or enhance their transactivation (Fausto, 2000). Activator protein 1 (AP-1) is upregulated during the early phase of liver regeneration. AP-1 is a transcription factor composed of a dimer of either the proteins FOS-JUN or JUN-JUN. AP-1 binds to inducible sites in the promoter region of various genes to cause activation and transcription (Angel and Karin, 1991). AP-1 activates specific AP-1 and transcription factor activator (ATF) site-containing promoters (Taub, 1996). Hepatic AP-1 DNA-binding activity is induced after PHX in mice (Heim et al., 1997) and in rats (Westwick et al., 1995). An additional transcription factor, signal transducer and activator of transcription 3 (STAT3), is also involved in the liver regeneration cascade. It is activated

within 30 minutes, peaking at 3 hours, after PHX, and does not require *de novo* protein synthesis (Cressman et al., 1995; 1996).

Nuclear factor for the kappa chain of B cells (NF-kB) is a transcription factor that is found in most cell types including hepatocytes. It is a heterodimer composed of 2 subunits, p65 and p50 (Tewari et al., 1992; FitzGerald et al., 1995). The p65-p50 complex is assembled in the cytoplasm, but remains inactive due to an inhibitor, IkB, which is bound to the p65 subunit. Once the inhibition is removed, the p65-p50 complex migrates to the nucleus where it activates genes involved in inflammation, cell adhesion, stress responses, cell replication and apoptosis (Fausto, 2000). NF-kB is activated rapidly after PHX, within 30 minutes (Tewari et al., 1992; Cressman et al., 1994; FitzGerald et al., 1995). Plumpe et al. (2000) found that inhibition of NF-kB resulted in an 80% decrease in DNA synthesis after PHX. Thus, transcription factors are involved in the priming and proliferation events of the liver regeneration cascade.

I.i.iii. Growth Factors and Cytokines in the liver regeneration cascade

Numerous growth factors are activated after PHX, and play an important role in the liver regeneration cascade. These include hepatocyte growth factor (HGF), epidermal growth factor (EGF) and transforming growth factor α (TGF-α), along with the cytokines interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α), and cell cycle promoters, such as the cyclins. Along with the growth-stimulating factors, growth-suppressing factors are also stimulated, including transforming growth factor β (TGF-β) and interleukin-1 (IL-1), and cell cycle inhibitory factors.

HGF is a potent mitogen for hepatocytes (Nakamura et al., 1984). Normally inactive in the liver, HGF is rapidly activated in the liver, beginning 15 minutes and peaking 1 hour, after PHX (Mars et al., 1995). Plasma HGF increases within 2 hours, peaking 12 to 18 hours after PHX, and remaining elevated for 36 hours (Zarneger et al., 1991; Tomiya et al., 1998). HGF is a potent stimulator of DNA synthesis in hepatocytes (Lindroos et al., 1995; Michalopoulos and DeFrances, 1997) and is required for cells to progress through the cell cycle and replicate (Fausto et al., 1995). In addition, infusion of HGF increases the rate of liver regeneration by 72 to 96 hours after PHX (Kobayashi et al., 1996; Kaibori et al., 2002). Thus, HGF is an important component of the liver regeneration cascade.

TGF-α and EGF are also important growth factors in the liver regeneration cascade. TGF-α is produced by hepatocytes, causing hepatocyte replication in a paracrine manner, by binding to the EGF receptor (Fausto et al., 1995). TGF-α mRNA increases within 4 hours, peaking 16 to 24 hours after PHX, and remaining elevated for 48 (Evarts et al., 1992; Webber et al., 1993) to 96 hours (Tomiya et al., 1998). Also, EGF mRNA production increases 10-fold, peaking within 15 minutes after PHX (Mullhaupt et al., 1994), and insulin and glucagon also act synergistically with EGF to stimulate DNA synthesis after PHX (Olsen et al., 1988). Infusion of TGF-α or EGF also stimulated liver regeneration after 1/3 PHX (Webber et al., 1994), indicating the essential role of growth factors in the liver regeneration cascade.

In addition, cytokines are required for the liver regeneration cascade to occur normally. Serum levels of IL-6 increase within 12 hours, and peak at 24 hours, after PHX (Akerman et al., 1992; Iwai et al., 2001). IL-6 has been shown to be an important

component of the liver regeneration cascade, as hepatocyte mitosis and DNA synthesis were inhibited in IL-6 deficient mice (Cressman et al., 1996; Wallenius et al., 2000). The inhibition was reversed by administration of IL-6 to these knockout mice (Cressman et al., 1996). Also, TNF-α stimulates hepatocyte growth *in vitro* (Beyer and Theologides, 1993). This cytokine is produced by Kupffer cells in the liver (Iwai et al., 2001), and has also been shown to increase in the liver and plasma within 1 hour after PHX (Rai et al., 1997; Iwai et al., 2001). TNF-α plays an important role in DNA synthesis in the liver regeneration cascade. Antibodies to TNF-α caused a delay in the peak of DNA synthesis from 24 to 48 hours after PHX (Kahn et al., 1994). This is manifest as a reduction in DNA synthesis 24 hours after PHX, and also subsequently decreased hepatocyte and nonparenchymal cell proliferation (Akerman et al., 1992). Thus, production of various growth factors and cytokines are integral events in the liver regeneration cascade.

I.i.iv. Cell Cycle Factors

I.i.iv.i. Cell cycle promoters

The progression through the cell cycle is promoted and regulated by proteins called cyclins. Cyclins D and E, when bound to cyclin-dependent kinases (cdks) play essential roles in the G_1 to S transition in the cell cycle (Grana and Reddy, 1995; Pines, 1995), while cyclin A is necessary for the G_2 to M transition (Fang and Newport, 1991). Cyclin D1 is upregulated in G_1 phase of the cell cycle, and is thought to be a key intracellular mediator of extracellular signals, such as mitogens, that regulate proliferation (Sherr, 1994). For example, administration of HGF, EGF or both stimulated expression of cyclin D1 in hepatocytes (Moriuchi et al., 2001). Cyclin D1 induces DNA

synthesis in hepatocytes *in vitro* (Albrecht and Hansen, 1999), and also peaks at 16 and 36 hours after PHX, which corresponds with the two waves of DNA synthesis in rats (Fausto, 2000). Cyclin E is also activated after PHX, beginning during G_1, between 12 and 18 hours after PHX (Albrecht et al., 1999). In addition, cyclin A is upregulated after PHX, beginning after 12 hours (Rininger et al., 1997), continuing through S phase and into G_2 and M phases, and then decreasing by 96 hours after PHX (Loyer et al., 1994). Activation of cyclins, therefore, contributes to progression through the cell cycle, and thereby to progression of the liver regeneration cascade.

I.i.iv.ii. Cell cycle inhibitors

The growth factor, TGF-β, has inhibitory effects on cell cycle progression and on liver regeneration. TGF-β inhibits DNA synthesis in hepatocytes in primary culture (Nakamura et al., 1985), and also inhibits the increase in HGF secretion and HGF mRNA expression in cultured fibroblasts (Matsunaga et al., 1994). TGF-β transiently decreased, beginning at 1 hour and continuing to its nadir at 24 hours, the peak of DNA synthesis in the liver after PHX. Thereafter, TGF-β levels increased beyond control levels by 48 hours after PHX (Rai et al., 1997), presumably to balance the growth-promoting factors activated in the liver regeneration cascade.

In addition, the cytokine, IL-1, is upregulated at 24 to 48 hours after PHX, when DNA synthesis is decreasing, and is thought to play an inhibitory role in hepatic cell replication. Indeed, exogenous IL-1 administered at 0 and 12 hours after PHX, significantly reduced DNA synthesis 18 hours after PHX (Boulton et al., 1997).

Boulton et al. (1997) suggest that IL-1 plays a role in termination of DNA synthesis after PHX.

A group of cell cycle inhibitory proteins is also activated after PHX, including the tumor suppressor, p53, and the cyclin dependent kinase (cdk) inhibitors p16, p18, p21 and p27. The p53 protein increases biphasically, at 1 hour and 12 hours after PHX (Rininger et al., 1997). The cdk inhibitors bind to cdk, which binds to and activates cyclins, thereby inactivating the cdk enzyme. Expression of p16 initially decreased after PHX, but then increased by day 4 and was still upregulated at day 7 (Jeong et al., 1999). However, p18 showed a biphasic increase 6 hours and 12 hours after PHX (Jeong et al., 1999). The increase in p21 begins by 24 hours, peaking at 48 hours and decreasing to control levels by 120 hours after PHX (Albrecht et al., 1998). The p27 protein increased by day 4 after PHX (Jeong et al., 1999). Thus, regulation of the cell cycle and cell growth in the liver regeneration cascade is maintained by a complex balance between growth promoters (growth factors, cytokines and cyclins), and growth inhibitors (TGF-β, IL-1, p53, p16, p18, p21 and p27).

I.i.v. The trigger of the liver regeneration cascade

Despite over 100 years of research (Milne, 1909), the trigger of the liver regeneration cascade remains unknown. Michalopolous and DeFrances (1997) stated that elucidation of the trigger for the liver regeneration cascade would be akin to the big bang theory of evolution of the universe. Several theories have been proposed as to the trigger of the liver regeneration cascade, including portal venous blood flow, and various growth factors and cytokines.

I.i.v.i. Portal venous blood flow and liver regeneration

Portal venous blood flow has long been known to be an important factor for normal liver regeneration to occur. Partial occlusion of the portal vein prior to PHX resulted in a significantly lower liver weight restoration compared to rats without partial occlusion of the portal vein (Stevenson, 1932). Also, experiments performed using dogs showed that constriction of the portal vein prior to PHX resulted in almost no regeneration of the liver, while complete restoration of liver mass was observed following PHX alone (Grindlay and Bollman, 1952). Mann (1940) shunted portal venous blood to the inferior vena cava (portacaval shunt), thus bypassing the liver, prior to PHX, and found that regeneration in the shunted animals was also inhibited. Further, deprivation of portal venous blood flow to the liver causes a decrease in liver weight in dogs (Weinbren, 1955) and rats (Guest et al., 1977) in a normal liver. Thus, it was initially hypothesized that portal venous blood flow was the most important and determining factor for activation of the trigger for the liver regeneration cascade (Grindlay and Bolman, 1952).

I.i.v.ii Humoral growth factors and liver regeneration

However, the blood flow theory of liver regeneration was soon called into question. When PHX was performed on one of a set of parabiotic twins, attached via their circulatory systems, the liver of the other twin was found to have enlarged (Wenneker and Sussman, 1951). Christensen and Jacobsen (1949) and Bucher et al. (1951) showed that following PHX in one parabiotic twin, the amount of cells

undergoing mitosis was increased in the other twin. Bucher et al. (1951) additionally performed PHX on 2 of 3 parabiotic triplets and found that in the liver of the third, non-PHX, triplet, the number of mitoses occurring was greater than previously seen with the parabiotic twins. Also, after PHX in one twin, there was increased DNA synthesis in the liver of the non-PHX twin (Moolten and Bucher, 1967). Thomson and Clarke (1965) and Clarke et al. (1968) demonstrated no increase in mitotic indices in the normal, non-PHX, liver in response to an increase in blood flow. These researchers therefore stated that the blood flow theory of liver regeneration was "untenable". Thus, it was concluded that a blood-borne factor was released after PHX, which triggered the liver regeneration cascade, and blood flow, while still important, was no longer considered the trigger.

Further support for the blood-borne factor theory was provided using lobes of the liver removed and transplanted to foreign regions of the body. For example, the left lateral lobe that was removed and transplanted to the neck region of a dog underwent DNA synthesis (Sigel et al., 1968). Further, these researchers found that hepatocytes in the periportal region underwent more DNA synthesis than those in the perivenous region. However, when the graft was transplanted in the opposite orientation (i.e. arterial inflow through the hepatic vein and outflow through the portal vein), DNA synthesis occurred predominantly in the perivenous region. These results provide support for the hypothesis that a trophic factor triggers liver regeneration, and that hepatocytes located near the inflowing blood have preferential access to trophic factors from intestinal blood under physiologic conditions.

However, the trophic factor hypothesis remained controversial. Blood flow to the liver was manipulated after PHX, such that the remnant had blood flow from the hepatic

artery and portal vein (normal conditions), portal vein only, hepatic artery only, and portacaval shunt with renal vein transposition (systemic blood) and hepatic artery. The groups receiving normal flow, portal venous flow and systemic flow all showed similar, normal amounts of liver weight restoration, while the hepatic arterial group had minimal restoration (Slapak et al., 1970). It was concluded that "adequate total blood-flow is the important factor for regeneration and not a specific portal substance", and they suggested that any blood-borne trophic factor was either completely or partially removed from the blood by non-hepatic organs, and/or that "a minimum blood-flow to the liver which is greater than that supplied by the hepatic artery alone [is required]" for normal liver regeneration to occur. The same group performed a similar study (Wexler et al., 1970), in which their data confirmed their previous results. In addition, in studies using the PVL model, in all cases, the lobes deprived of portal venous blood atrophy even though they have access to growth factors via recirculation through the hepatic artery (Rozga et al., 1985; Rocheleau et al., 1999). Thus, the trigger of the liver regeneration cascade remains controversial.

I.i.vi. Hypothesis

My hypothesis encompasses the blood flow theory and the humoral theory of liver regeneration, and proposes a mechanism for triggering the liver regeneration cascade. The liver does not control portal venous blood flow, but must accept the entire outflow from other splanchnic organs, such as the intestine, stomach, spleen and pancreas. Removal of 2/3 of the liver (PHX) causes a 3-fold increase in the blood flow-to-liver mass in the remaining 1/3 (Rice et al., 1977; Kahn et al., 1984). It is my hypothesis that

this increase in the blood flow-to-liver mass ratio causes shear stress and release of nitric oxide (NO) and prostaglandins (PGs), which trigger the liver regeneration cascade. This hypothesis suggests that blood flow is the trigger for the liver regeneration cascade, and the hemodynamic change results in the release and/or activation of growth factors, cytokines and other factors that comprise the events in the cascade. The end result is restoration of liver mass and function: i.e. liver regeneration. Further, I will test the hypothesis that NO and PGs are potential therapeutic targets to potentiate the liver regeneration cascade.

II. Nitric oxide

NO has a half-life of less than 5 seconds (Palmer et al., 1987). NO is a free radical formed from the guanidino nitrogen of arginine by the enzyme NO synthase (NOS) (Umans and Levi, 1995). There are three isoforms of NOS: neuronal NOS (nNOS or NOS I), inducible NOS (iNOS, or NOS II) and endothelial NOS (eNOS, or NOS III). nNOS and eNOS are constitutive and their activation is calcium-calmodulin dependent, while iNOS must be activated to produce NO, in a calmodulin-independent manner (Alexander, 1998).

Transduction of NO signaling occurs through a pathway involving guanylate cyclase (GC) and cyclic guanosine monophosphate (cGMP). NO activates GC, which causes an increase in cGMP levels (Kimura et al, 1975). The increase in cGMP activates a cGMP-dependent protein kinase, which causes dephosphorylation of the myosin light chain, leading to relaxation of the smooth muscle cell (Murad, 1986), thus allowing vasodilation in response to stimuli, such as shear stress. In addition, cGMP is involved in

the liver regeneration cascade, peaking within 20 minutes following PHX (Miura et al., 1976).

II.i. Functions of NO

II.i.i Proliferative and antiproliferative effects of NO

NO is known to cause cytotoxicity. For example, the NO donor, 3-morpholinosydnonimine (SIN-1), inhibited DNA synthesis in bovine aortic endothelial cells, while the NOS antagonist, N^{ω}-nitro-L-arginine methyl ester (L-NAME), reversed the inhibition (Lopez-Farre et al., 1997). NO also caused DNA damage in isolated mitochondria, and induced apoptosis through opening of mitochondrial permeability transition pores, thereby releasing apoptogenic factors (Hortelano et al., 1997). Kupffer cells, when stimulated by lipopolysaccharide (LPS), release NO which can inhibit oxidative phosphorylation in adjacent hepatocytes, in a Kupffer cell-hepatocyte coculture, leading to hepatocyte cell death (Kurose et al., 1996).

However, this production of NO by Kupffer cells *in vivo* also has a protective function. Metastasis of cancer cells to the liver via the portal vein is a concern in cancer patients. Jessup et al. (1999) showed that tumor cells enter the liver via the portal vein and bind in the periportal region or wedge into hepatic sinusoids, occluding blood flow. The obstruction in flow causes hypoxia, and the formation of NO and superoxide (O_2^-), thereby causing tumor cell death. Also, when cultured with hepatoma cells, iNOS, and production of NO, is upregulated in Kupffer cells, resulting in mitochondrial and membrane barrier dysfunction in the tumor cells (Fukumura et al., 1996). Thus, NO production may have evolved as a protective measure against metastasis of cancer cells.

NO also has other protective roles in apoptosis, ischemia-reperfusion injury and liver disease. There is evidence that NO protects cells against apoptosis induced by TNF-α (Polte and Schroder, 1998; Li and Billiar, 1999). The presence of NO prevents further injury in the liver in ischemia-reperfusion injury. For example, liver injury occurs after ischemic insult followed by reperfusion, as evidenced by increased plasma alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) levels, in the liver. However, administration of the NOS antagonist, L-NAME, prior to ischemia-reperfusion resulted in greater liver injury than when NO was present (Koken and Inal, 1999; Liu et al., 2000). Peralta et al. (1997) found that administration of the NO donor, NONOate, reversed the increased damage induced by NOS antagonism. In addition, inhibition of NOS in rats with carbon tetrachloride (CCl_4)-induced liver cirrhosis caused more lipid peroxidation, decrease in glycogen stores, and increased bilirubin levels in the plasma compared to CCl_4 treatment alone (Muriel, 1998). L-arginine, the substrate for NOS, was able to reverse the increase in liver damage to CCl_4 control levels.

NO appears to have both cytotoxic and protective effects. The effect of NO, positive or negative, seems to be a function of the amount of NO produced, as a high concentration induces apoptosis, while low amounts contribute to cell survival (Shen et al., 1998). Additionally, it has been suggested that the positive effects of NO may be due to the ability of NO to combine with other free radicals, such as O_2^-, to form nitrate or nitrite (NO_3 or NO_2), although this combination may also result in the extremely reactive radical, peroxynitrite (Muriel, 1998; Koken and Inal, 1999). The protective effects of NO may also be due to inhibition of the apoptosis pathway (Li and Billiar, 1999). I hypothesize that NO plays a beneficial role in triggering the liver regeneration cascade.

II.i.ii. Physiological Roles of Nitric Oxide

NO plays a role in many important biological processes. These include regulation of basal vascular resistance, distribution of blood flow and oxygen delivery (Umans and Levi, 1995), as well as control of arterial pressure (Forzard and Part, 1991; Bauer et al., 1997), sodium balance and urine flow (Mattson et al., 1998), and it also has a role in maintenance of normal fibrinogen levels (Kawabata, 1996) and prevention of leukocyte adhesion (Kubes, 1999). Production of NO also aids in the body's defense against microbes, parasites and tumor cells (Suzuki et al., 1995). In addition, NO is released in response to shear stress (Shah et al., 1997). It is the constitutive isoform, eNOS, which is involved with regulation of blood flow (Stauss et al., 2000), and is also activated to produce NO by fluid shear stress (Gallis et al., 1999; Fisslthaler et al., 2000). Shear stress causes upregulation of eNOS in endothelial cells (Redmond et al., 1998), and the release of NO from the endothelial cells has been shown to be proportionate to the amount of shear stress applied (Kelm et al., 1991).

III. Shear stress

Shear stress is defined as a viscous drag at the surface of endothelial cells created by adjacent blood flow (Kamiya and Togawa, 1980; Busse and Fleming, 1998). An increase in shear stress causes modifications to endothelial cells adjacent to the flow. Endothelial cells reorient with the direction of flow, form actin stress fibres that also orient in the direction of flow, and increase adhesion to their substrate (Wechezak et al., 1989; Girard and Nerem, 1995). It has been suggested that these changes serve to protect

the endothelial cells from shear stress-induced damage (Franke et al., 1984; Wechezak et al., 1989). Endothelial cells also release vasoactive mediators, such as NO and PGs in response to shear stress (Ballerman et al., 1998). These vasodilators may also serve to protect the vessel against damage caused by increased shear stress.

III.i. Shear stress and NO in the liver

All three isoforms of NOS are present in the liver (Muriel, 2000; Esteban et al., 1997). NO is produced by hepatocytes (Zhang et al., 1997; Nicholls-Grzemski et al., 1999), Kupffer cells (Shiratori et al., 1998) and endothelial cells (Shah et al., 1997; Zhang et al., 1997). Indeed, eNOS and iNOS are located in hepatocytes, Kupffer cells and endothelial cells in the hepatic sinusoid (Shah et al., 1997), while nNOS has been localized to the terminal hepatic nerves located in the periportal region (Esteban et al., 1997; Estaban et al., 1998). NO is produced in the liver in response to increased flow. Shah et al. (1997) showed that, in the isolated perfused liver, shear stress causes an increase in production of nitrate/nitrite (NO_x), which was blocked by a NOS antagonist. *In vivo*, shear stress in the hepatic circulation is reflected as an increase in portal venous pressure (PVP). Macedo and Lautt (1998) showed that prevention of elevation of PVP by reducing portal blood flow during vasoconstriction prevented NO release. However, if PVP was allowed to rise by holding flow steady, NO caused compensatory vasodilation. If vascular perfusion pressure rises, either as a result of increased flow or increased resistance, the elevated perfusion pressure reflects increased shear stress in venous as well as arterial vascular beds (Macedo and Lautt, 1996; 1998). It is known that PVP increases immediately after PHX (Heikkinen and Larmi, 1968) and remains elevated

until liver mass is restored (Grindlay and Bollman, 1952; Um et al., 1994). Rabinovici and Weiner (1963) also observed the increase in PVP after PHX and during the entire regeneration process, correlating the highest PVP values with the periods of most active growth in the liver.

The amount of shear stress is proportional to the blood flow through a vessel and the inverse of the cube of the vessel radius (Busse and Fleming, 1998). The PVP is related to hepatic blood flow by the equation F = P/R, where F is total blood flow, P is pressure and R is resistance in the liver. The PVP is thus directly proportional to flow and inversely proportional to resistance. An increase in pressure is directly related to an increase in flow when resistance does not change.

Flow is also related to wall shear stress (τ) by the equations:

$$\tau = \eta\gamma$$

and

$$\gamma = (m+2)F/\pi r^3$$

where γ is shear rate, m is 2 for laminar flow (or >2 for turbulent flow), r is the radius of the vessel, and η is the blood viscosity (Kamiya and Togawa, 1980). Thus, wall shear stress is directly proportional to flow, and it can be concluded that an increase in blood flow results in increased shear stress in the liver, and that the rise in PVP can serve as an indirect index of the rise in shear stress under constant flow conditions. The index is not, however, based on a linear relationship because of the passive distensibility of the portal resistance vessels. This means that as flow increases, the hepatic resistance passively decreases (Lautt and Legare, 1992). Similarly, after PHX, increased perfusion of the remaining liver results in a decrease in resistance (Gertsch et al., 1997).

III.ii. Shear stress, NO and liver regeneration

NO is required for normal liver regeneration to occur. Liver regeneration is inhibited in iNOS knockout mice (Rai et al., 1998), and NOS antagonism was also shown to inhibit DNA synthesis *in vivo* after PHX (Carnovale et al., 2000). In addition, it has been postulated that shear stress triggers, and is required for, liver regeneration to occur (Sato et al., 1997; Sato et al., 1999), although no explanation or mechanism for the involvement of shear stress was provided. Previous experiments have implicated shear stress-induced NO as the trigger for the liver regeneration cascade. Inhibition of NOS using L-NAME, a nonselective NOS antagonist, inhibits liver mass restoration 48 hours after PHX (Figure 3) (Wang and Lautt, 1998). In addition, production of proliferative factors (PFs), detected in the blood four hours after PHX are inhibited by administration of L-NAME prior to PHX. This inhibition is reversed by administration of L-arginine, the substrate for NOS (Figure 4) (Wang and Lautt, 1998). Shear stress also increases after PVL, a model similar to PHX, which uses hemodynamic manipulation only to stimulate compensatory hyperplasia, as reflected by an increase in PVP (Um et al., 1994). In situations where blood flow to the liver is decreased, for example by portacaval shunt or constriction of the portal vein, liver weight decreases in normal livers, and neither an increase in PVP nor regeneration is seen under these hemodynamic conditions after PHX (Mann, 1940; Grindlay and Bolman, 1952). Thus, as has been hypothesized (Wang and Lautt, 1997a; Sato et al., 1997; Wang and Lautt, 1998; Sato et al., 1999), an increase in PVP and development of shear stress in the liver are required for liver regeneration to occur.

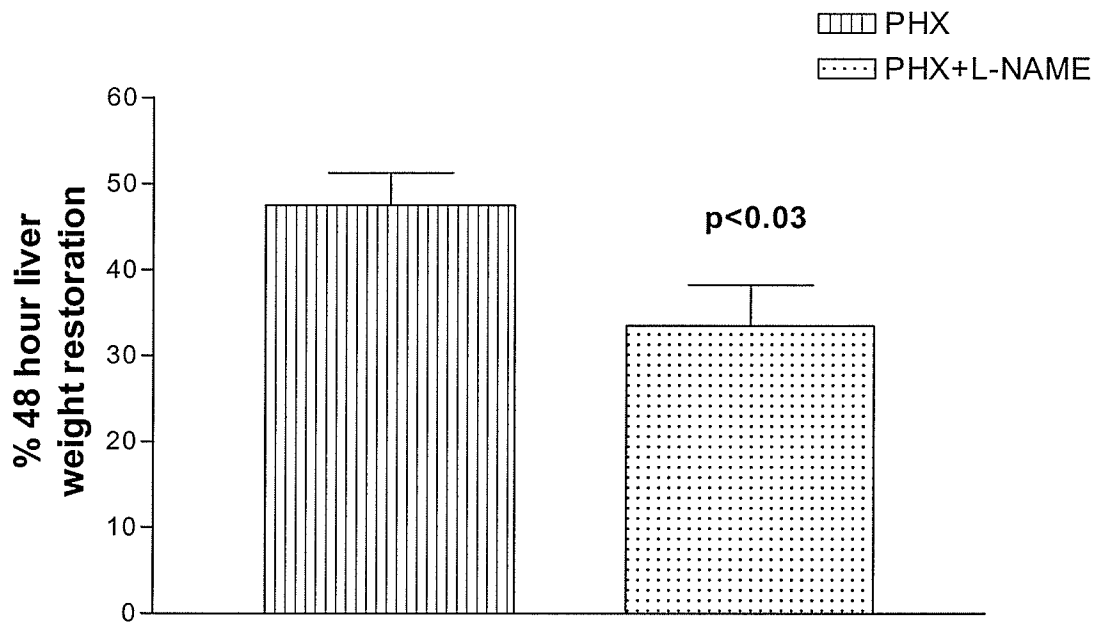

Figure 3: Liver weight restoration after PHX. The NOS antagonist, L-NAME, inhibits liver weight restoration after PHX, providing support for the hypothesis that NO triggers the liver regeneration cascade (Wang and Lautt, 1998).

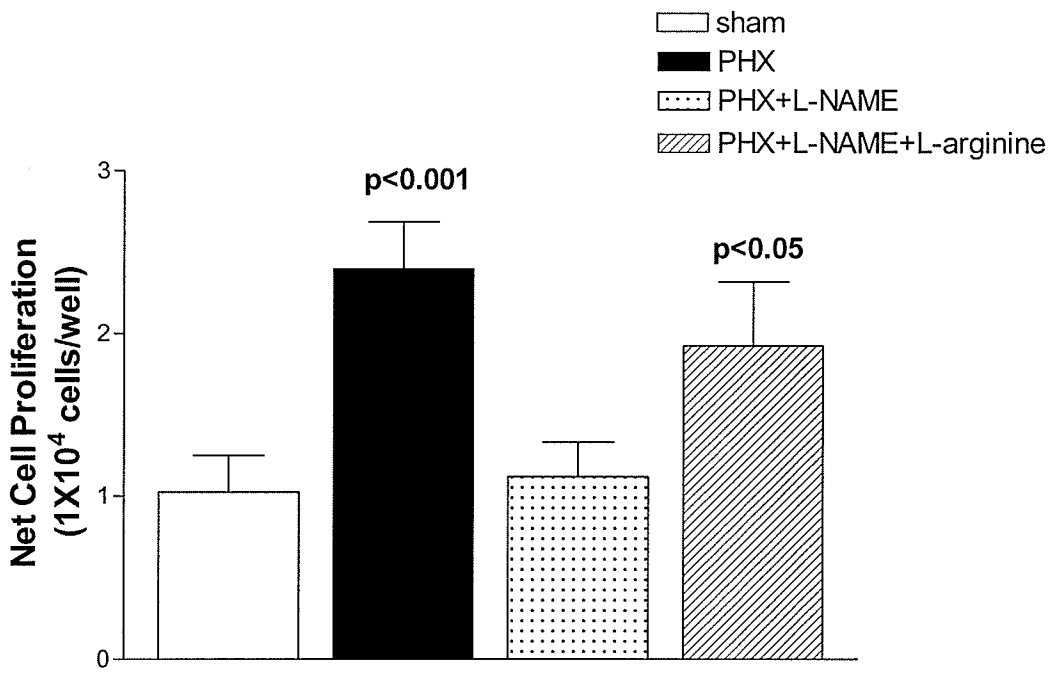

Figure 4: Proliferative factor (PF) production after PHX, and with NOS antagonist. The NOS antagonist, L-NAME inhibits the increase in PFs after PHX, and the inhibition is partially reversed by the substrate for NOS, L-arginine. These results provide support for the hypothesis that NO triggers the liver regeneration cascade (Wang and Lautt, 1998).

IV. Prostaglandins

Prostaglandins (PGs) are potent vasoactive substances that are synthesized from arachidonic acid, via activation of cyclooxygenase (COX), which gives rise to PGE_2, $PGF_{2\alpha}$, PGD_2 and prostacyclin (PGI_2) (Figure 5) (Moncada and Vane, 1979). There are two isoforms of COX: COX-1, which is present and constitutively active in most tissues, and COX-2, which is the inducible isoform, which is undetectable in most tissues until stimulated by inflammatory mediators (Langenbach et al., 1995; Langenbach et al., 1997). PGE_2 is a vasodilator while $PGF_{2\alpha}$ is a vasoconstrictor (Moncada and Vane, 1979). PGI_2 inhibits platelet aggregation and relaxes smooth muscle (Gryglewski et al., 1976; Hohn et al., 1981), and this PGI_2-induced relaxation of smooth muscle causes a decrease in blood pressure and total peripheral resistance (Rubin et al., 1982).

Actions of PGs are mediated through cyclic adenosine monophosphase (cAMP) (MacManus and Whitfield, 1974). PGs activate adelylate cyclase (AC), which increases cAMP levels (Schlondorff et al., 1978; Muallem et al., 1989), and this cAMP can then stimulate gene expression. Many genes, such as c-fos (Ohki et al., 1995), contain cAMP-response element (CRE) sequences in their promoter region, which allows stimulation of transcription in response to increased cAMP levels (Della Fazia et al., 1997). cAMP-responsive genes are stimulated by transcription activators, such as CREM, which binds to CRE sequence in the promoter region in response to increased levels of cAMP (Della Fazia et al., 1997). Stimulation of CREM is known to occur after PHX (Servillo et al., 1997).

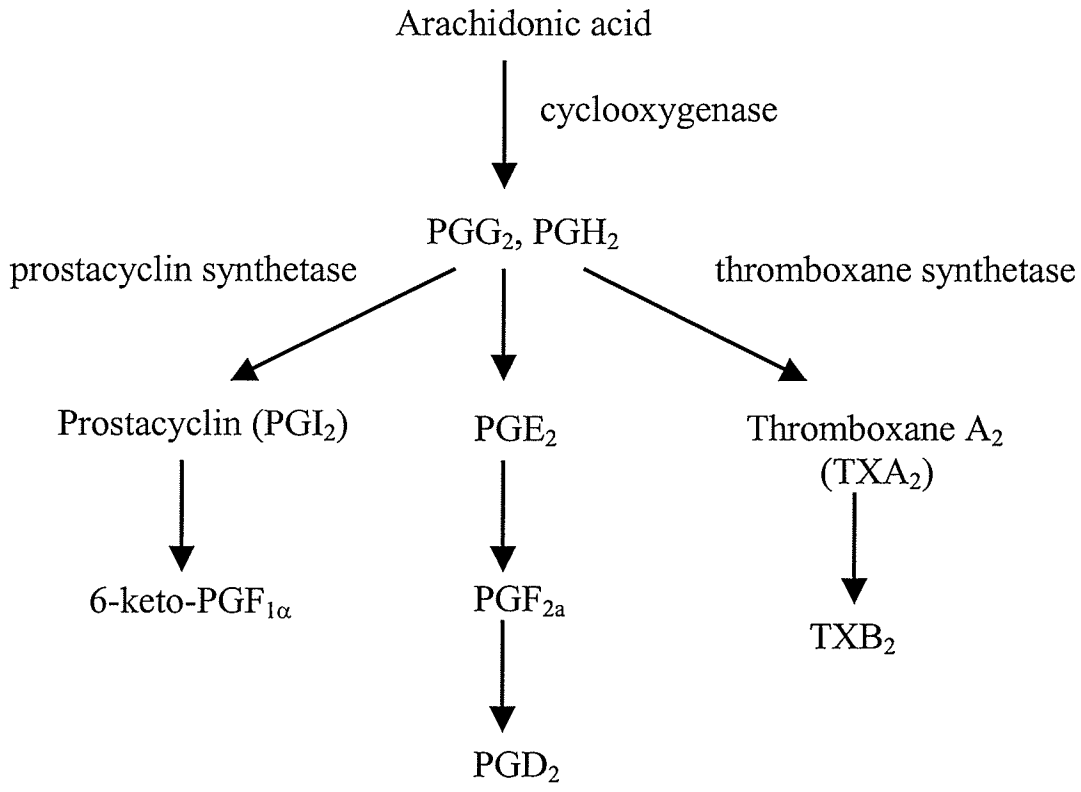

Figure 5: Synthetic pathway of prostaglandins and thromboxane

Adapted from Moncada and Vane, 1979

IV.i. Prostaglandins and shear stress

Shear stress causes the release of vasoactive PGs from endothelial cells. PGI_2 is released in response to an increase in flow, and inhibited by the COX antagonist, ibuprophen (Frangos et al., 1985). PGI_2 production, which is mediated by G proteins (Berthiaume and Frangos, 1992), peaks within 1 to 2 minutes of application of shear stress, and increases in proportion to the amount of shear stress applied (Grabowski et al., 1985). PGE_2 is also released in response to shear stress (Wang and Tarbell, 2000), but shear levels must be of a longer duration and higher intensity compared to levels required to stimulate PGI_2 (Alshihabi et al., 1996).

IV.ii. Prostaglandins and cell proliferation

DNA synthesis is stimulated by PGI_2 (Besse et al., 1991) and PGE_2 (MacManus and Braceland, 1976; McNeil et al., 1985), and inhibited by the COX antagonist, indomethacin (MacManus and Braceland, 1976; McNeil et al., 1985). Following PHX, PGE_2 and PGI_2 levels increase (Callery et al., 1991; Tsujii et al., 1993; Lai et al., 2000), and the increase in PGE_2 was shown to correspond to elevated levels of cAMP, and thereby DNA synthesis (MacManus and Braceland, 1976; Miura et al., 1976; Tsujii et al., 1993). It has also been suggested that PGE_2 and PGI_2 are important stimulators of liver regeneration (McNeil et al., 1985; Besse et al., 1991), and are potentially involved in triggering the liver regeneration cascade (Miura and Fukui, 1979). Indeed, arachidonic acid and PGE_2 were shown to stimulate hepatocyte growth *in vitro* (Skouteris et al., 1988), and PGE_2 and PGI_2 were also shown to stimulate HGF expression (Matsumoto et al., 1995). Blockade of COX-1 and COX-2 suppresses the increase of PGE_2, PGI_2 and

phosphorylated CREB after PHX, and also inhibits DNA synthesis (Rudnick et al., 2001). Previous studies demonstrated that PFs are also inhibited by indomethacin, a COX antagonist, suggesting that PGs are also involved in triggering the liver regeneration cascade (Figure 6) (Wang, 1997). The blockade of PF production implies blockade of all of the factors that are known to be generated after PHX and that can cause hepatocyte proliferation. For this reason, PF production is taken as a powerful tool to indicate whether the liver regeneration cascade has been activated. Thus, PGs play in important role in DNA synthesis and in the liver regeneration cascade.

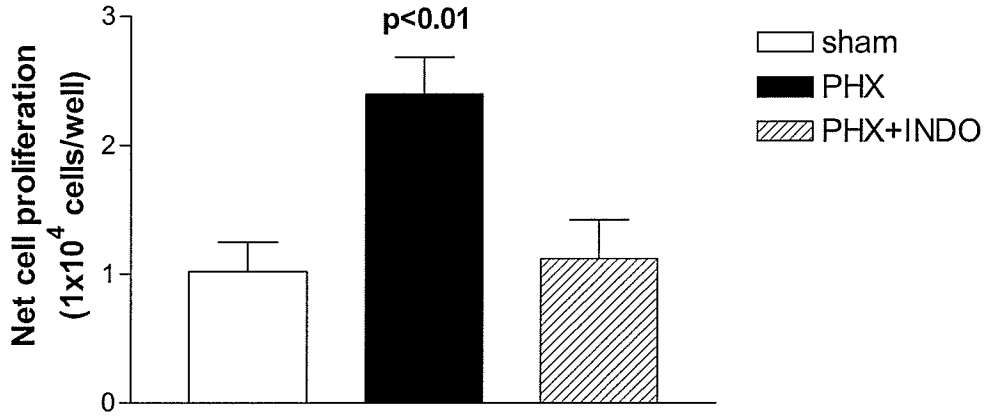

Figure 6: Proliferative factor production after PHX, and with COX antagonist. The COX antagonist, indomethacin, inhibits the increase in PFs after PHX. This result supports the hypothesis that PGs are involved in triggering the liver regeneration cascade (Wang, 1997).

Chapter 1

NO induces c-fos mRNA expression, an index of initiation of the liver regeneration cascade

1.1 Introduction

1.1.1 c-fos mRNA expression

To more fully test the hypothesis that NO and PGs trigger the liver regeneration cascade, an additional index, closer to the actual triggering event, was required. Numerous early events in the liver regeneration cascade take place, including changes in expression of several immediate- and delayed-early genes (Haber et al., 1992). Among the immediate early genes, c-fos mRNA expression is increased, peaking 15 minutes after PHX (Moser et al., 2001). Moser and co-workers (2001) also observed that c-fos mRNA expression increases proportionate to the degree of PHX performed. In addition, c-fos mRNA expression increases in response to shear stress (Hsieh et al., 1993), and proportional to the shear stress applied (Ranjan and Diamond, 1993). NO also stimulates expression of c-fos mRNA (Haby et al., 1994; Morris, 1995; Ohki et al., 1995; Pilz et al., 1995). For these reasons, c-fos mRNA expression was selected as an index of initiation of the liver regeneration cascade.

1.1.2 The c-fos gene

c-Fos is a proto-oncogene homologous to the oncogene v-fos. Finkel, Biskis and Jinkin first identified the v-fos oncogene from mouse osteosarcoma viral particles, later named FBJ particles, in 1966. v-Fos is part of the FBJ viral complex which is

responsible for transformed morphology in cells infected in tissue culture (*in vitro*) and for the induction of tumors *in vivo* (Verma and Graham, 1987).

The c-fos gene is located on chromosome 14 and consists of 3 introns and 4 exons (Verma and Graham, 1987). The promoter region of c-fos contains several inducible sequences that activate c-fos transcription. The serum response element (SRE) induces c-fos transcription in response to a protein present in serum, serum response factor (SRF), which binds to the SRE. A second, but less responsive serum responsive element, SRE-2, is also present in the c-fos promoter. In addition, CRE is a cAMP response element that is present in the promoter region of c-fos. This sequence activates c-fos transcription in response to increased cAMP. Once c-fos has been transcribed, the resulting mRNA is translated into a protein, FOS. This protein, having a molar weight of 55,000 Daltons, is also referred to as p55, and is 380 amino acids long (Verma and Graham, 1987). FOS dimerizes with the protein product of c-jun, JUN, to form the transcription factor AP-1. AP-1 and other transcription factors bind to inducible sites in the promoter region of various genes to cause activation and transcription (Angel and Karin, 1991).

1.1.3 NO and c-fos mRNA expression

There is evidence that NO activates the transcription of c-fos. For example, NO activates c-fos mRNA expression in cultured striatal neurons (Morris, 1995) and PC12 cells (Haby et al., 1994). In addition, systemic administration of the NOS antagonist, L-NAME, significantly attenuated c-fos expression in neurons of the hypothalamic paraventricular nucleus (Amir et al., 1997) and suprachiasmatic nucleus (Amir and Edelstein, 1997) in the brain. NO also caused an increase in c-fos mRNA expression in

rat retinal cells 45 minutes after injection of the nitric oxide donor, sodium nitroprusside (SNP) (Ohki et al., 1995). In addition, c-fos mRNA increased in cultured fibroblasts 30 minutes after exposure to SNP (Pilz et al., 1995) and in lung epithelial cells 1 hour after exposure to another NO donor, sodium NONOate (Janssen et al., 1997).

In this chapter, I will test the hypothesis that an increase in the blood flow-to-liver mass ratio, causes shear stress and the release of NO in the remaining liver, triggering the liver regeneration cascade. In addition, it is hypothesized that eNOS is the isozyme responsible for NO release, which triggers the liver regeneration cascade. c-Fos mRNA expression will be used as an index and both the PHX and PVL models will be employed. Since the PVL model is used as a model of the liver regeneration cascade with similar hemodynamic conditions as after PHX, the similarity of the hemodynamic change after PHX and PVL will first be validated.

1.2 Methods

1.2.1 Animals

Male Sprague-Dawley rats, 250 g, were fed standard laboratory chow *ad libitum* until the day before the experiment, when they were fasted for 8 hours and fed for two hours prior to experimentation. Animals were treated according to the guidelines of the Canadian Council on Animal Care, and all protocols were approved by the Ethics Committee on Animal Care at The University of Manitoba.

1.2.2 Surgical preparation

1.2.2.1 Partial hepatectomy (PHX)

Animals were anesthetized by sodium pentobarbital (6.5 mg/100 g, i.p.; MTC Pharmaceuticals). Anesthesia was maintained using a continuous i.v. infusion (Harvard infusion pump, model 2720) of sodium pentobarbital (1.08 mg/ml saline, 1.0 ml/100 g/hr) via a cannula (PE50, Clay Adams) inserted into the femoral vein. All surgical procedures were performed between 10:00 am and 2:00 pm. Arterial blood pressure was monitored via a cannula (PE50, Clay Adams) inserted into the femoral artery and recorded by an R-611 SensorMedics Dynograph Recorder. To facilitate breathing, a tracheotomy was performed, using a polyethylene tube (PE240, Clay Adams) inserted into the trachea. Body temperature was maintained at 37.5 $\pm$ 0.5°C by the use of a rectal thermometer (Hanna Instruments) and a heated surgical table (Harvard Apparatus). Laparotomy was

performed and a 24G catheter (Johnson and Johnson) was inserted into the portal vein. The rat was then allowed to stabilize for 30 minutes.

The appropriate drug, or saline, was infused and, 10 minutes later, PHX (resection of the left lateral and median lobes as described by Higgins and Anderson (1931)) or gentle manipulation of the liver lobes, was performed. Resected lobes were ligated using black braided surgical silk (size 0, Ethicon) and excised. Fifteen minutes after PHX, or sham manipulation, the remaining liver was removed, slashed and blotted to remove blood, and immediately frozen on dry ice for RNA analysis. Portal venous pressure (PVP) was measured via the portal venous catheter, connected to a R-611 SensorMedics Dynograph Recorder, immediately before and after PHX.

1.2.2.2 Selective Portal Vein Branch Ligation (PVL)

Surgical procedures were the same as described for PHX, except that liver lobes were not ligated and resected. Rather, the left branch of the portal vein was carefully isolated from the surrounding hepatic artery, hepatic nerves, bile duct and lymphatics. A thread was slipped around the left branch of the portal vein and the animal was allowed to stabilize for 1 hour. Following the stabilization period, the appropriate drug or saline was administered as described for the PHX group. The left branch of the portal vein was then ligated and liver tissue was removed 15 minutes after PVL, slashed and blotted, and immediately frozen on dry ice for RNA isolation. For rats in the sham PVL group, the left branch of the portal vein was isolated, but not ligated, and the liver lobes were gently manipulated in a similar manner to the experimental groups. The extent of PVL was verified by injecting India ink into the portal vein of PVL and sham operated animals

immediately before removal of the liver. Only livers with complete ligation of portal venous blood supply to the left lateral and median lobes were included in the experimental groups. PVP was measured immediately before and after PVL, as described for PHX surgery.

1.2.3 Drugs

L-NAME (Sigma) (5 mg/kg, i.v., 0.5 ml bolus infused over 2 minutes), a non-selective NOS antagonist, was dissolved in saline, and the rat was allowed to stabilize for 10 minutes following administration. Aminoguanidine (Sigma) (75 mg/kg, i.v.), a selective iNOS antagonist, was also dissolved in saline, and the rat was allowed to stabilize for 30 minutes following administration. The NO donor, SIN-1 (5 mg/kg) (Alexis Corp) was also dissolved in saline, and a 0.2 ml bolus was infused intraportally over 2 minutes. PHX, PVL or sham procedures were performed immediately following NO donor administration.

1.2.4 RNA isolation and northern blot analysis

Total RNA was isolated by a method modified from Chirgwin et al. (1979). Liver tissue was homogenized (Brinkman Polytron) in lithium chloride/urea (3M/6M; Sigma). The homogenate was centrifuged at 25000 rpm (Beckman L8-70M Ultracentrifuge) for 20 minutes at 4°C. Total RNA was extracted using phenol/chloroform (Fisher Biotech/Sigma) and allowed to precipitate overnight in sodium acetate/ethanol (Sigma) at –70°C. The samples were then centrifuged at 15000 rpm (Beckman Microfuge E) for 15 minutes at 4°C. The supernatant was decanted and the pellet was resuspended in sterile

distilled water. The concentration of the RNA was then determined using a spectrophotometer (Ultrospec2000, Pharmacia Biotech). Twenty micrograms of total RNA was loaded onto a 1% agarose (Gibco BRL), 2.2 mol/L formaldehyde (Sigma) and 1X MOPS denaturing gel. RNA was separated by electrophoresis at 100 mV for 90 minutes and then transferred overnight to a GT-Zeta nylon membrane (Bio-Rad) by capillary action. The RNA was crosslinked to the membrane using a UV crosslinker (UV GS Genelinker, Bio-Rad). The membranes were prehybridized at 42°C for 3 hours in prehybridization buffer consisting of 5x SSC, 7% SDS (Sigma), sheared denatured salmon sperm DNA (100 μg/ml, Gibco BRL), and 5x Denhardt's solution (Sigma). c-Fos mRNA was detected using a 1.8 kb cDNA probe (ATCC). The cDNA probe was labeled, by the random prime method using a commercial kit (Gibco BRL), with α-dCTP ^{32}P (NCN Isotopes) and added to the prehybridization buffer. The membranes were hybridized overnight at 42°C, and washed using solutions of 2x SSC and 0.1% SDS, 0.5x SSC and 0.1% SDS, and 0.1x SSC and 0.1% SDS at 65°C. Autoradiography was performed using Kodak XAR film at –70°C using an intensifying screen. The density of the c-fos mRNA band was quantitated using an HP scanner and the density of the c-fos mRNA band analyzed with the NIH Image 1.6 Densitometric Analysis Program (National Institute of Health, Bethesda, MD). c-Fos mRNA expression is reported relative to 18S rRNA. Results were analyzed using ANOVA, with $p<0.05$ deemed significant.

1.3 Results

1.3.1 Portal venous pressure after PHX and PVL

The similarity of hepatic hemodynamics in the PHX and PVL models was evaluated by comparing changes in PVP before and immediately following both PHX and PVL. The PVP increased immediately following PHX (10.25 ± 0.43 mmHg, vs. 5.63 ± 0.43 mmHg, n = 4, $p<0.001$) (Figure 7). A similar increase in PVP was observed after PVL (10.50 ± 0.54 mmHg vs. 5.75 ± 0.32 mmHg, n = 4, $p<0.001$). The PVP was not different before PHX or PVL, and there was also no difference between the elevated PVP after PHX and PVL (Figure 7), indicating that both PHX and PVL cause similar increases in PVP. Thus, the shear stress load in the portal circulation is judged to be similar between the 2/3 PHX and selective 2/3 PVL models.

1.3.2 c-Fos mRNA expression after PHX

The hypothesis was further tested using c-fos mRNA expression as an index of initiation of the liver regeneration cascade. c-Fos mRNA expression increased 15 minutes after PHX (1.30 ± 0.20; n = 5) compared to sham operated rats (0.27 ± 0.07, n = 7, $p<0.001$) (Figure 8). This increase was blocked by administration of L-NAME (n = 6; 5.0 mg/kg i.v.) to sham levels (0.60 ± 0.08, N.S. from sham), and reversed by the NO donor, SIN-1 (n = 5; 5.0 mg/kg i.p.v.) (1.32 ± 0.16, $p<0.001$ vs. sham; Figure 8). These results suggest that NO is involved in the increase in c-fos mRNA expression after PHX.

As shown in Figure 8, c-fos mRNA expression is reduced to sham levels by

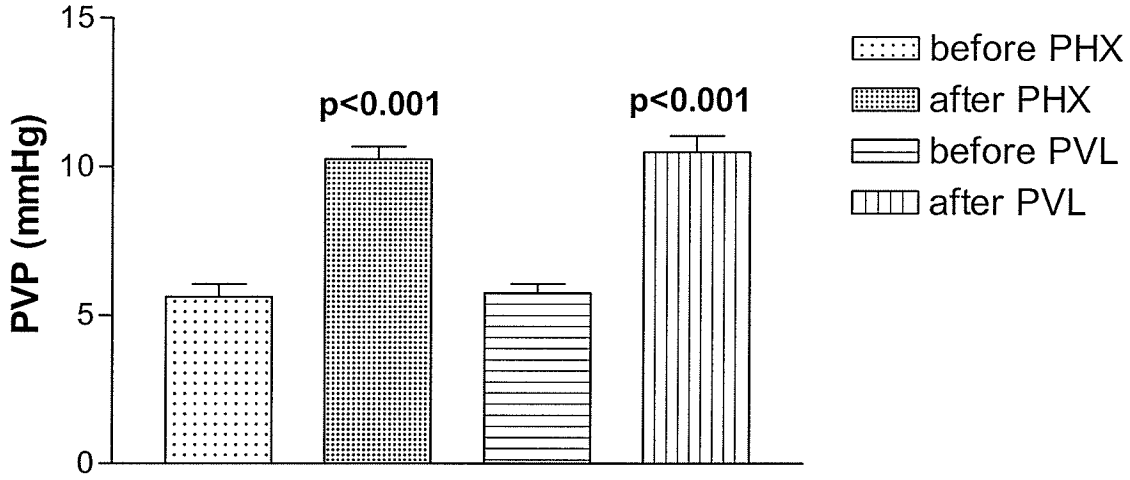

Figure 7: Portal venous pressure (PVP) after PHX and PVL. The PVP increases to a similar extent after PHX and PVL, suggesting similar hemodynamic conditions in the liver in both models, and also a similar amount of shear stress. (data shown as mean $\pm$ SEM)

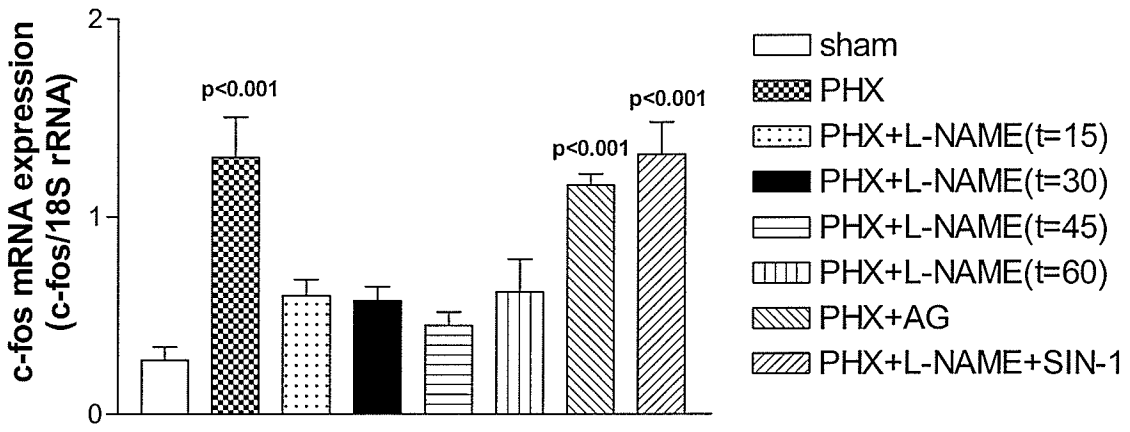

Figure 8: c-Fos mRNA expression after PHX with selective and nonselective NOS antagonists, and the NO donor, SIN-1. c-Fos mRNA expression increases 15 minutes after PHX, and the increase is blocked by the nonselective NOS antagonist, L-NAME. The iNOS selective antagonist, aminoguanidine (AG), has no effect on c-fos mRNA expression. The blockade of c-fos mRNA expression is reversed by the NO donor, SIN-1. These results provide support for the hypothesis that NO triggers the liver regeneration cascade, and are consistent with the hypothesis that eNOS is responsible for the production of NO, which triggers the liver regeneration cascade. (t = time; data shown as mean $\pm$ SEM)

inhibition of NOS by the nonspecific NOS inhibitor, L-NAME. To test the hypothesis that L-NAME inhibits, and does not simply delay, the peak of c-fos mRNA expression, L-NAME (5.0 mg/kg i.v.) was administered to rats prior to PHX and the liver was removed at 30, 45 and 60 minutes after PHX (n = 5 each group). c-Fos mRNA expression was then evaluated and compared to that at 15 minutes after PHX. There was no difference in c-fos mRNA expression at any of these four time points after PHX (Figure 8), suggesting that NOS blockade inhibits c-fos mRNA expression after PHX. In addition, the iNOS antagonist, aminoguanidine (AG, 75 mg/kg i.v.) did not affect the increase in c-fos mRNA expression after PHX (1.16 ± 0.06, $p<0.001$ vs. sham) (Figure 8). This suggests that iNOS is not the isoform of NOS responsible for production of NO after PHX, which triggers the liver regeneration cascade.

1.3.3 c-Fos mRNA expression after PVL

To further evaluate the hemodynamic involvement in triggering the liver regeneration cascade, c-fos mRNA expression was evaluated 15 minutes after PVL. The PVL model was used as it is similar to PHX, but no liver tissue is resected. The only manipulation is a hemodynamic one, ligation of the left branch of the portal vein, which results in similar hemodynamic conditions as after PHX (Figure 7). c-Fos mRNA expression increased significantly after PVL (1.04 ± 0.15, n = 8) compared to sham (0.29 ± 0.05, n = 8, $p<0.01$) (Figure 9). This increase in c-fos mRNA expression was inhibited by L-NAME (n = 4; 5.0 mg/kg i.v.) (0.38 ± 0.12, N.S. from sham), and the inhibition was reversed by the NO donor SIN-1 (n = 8; 5.0 mg/kg i.p.v.) (1.56 ± 0.13, $p<0.001$ compared to sham). These results suggest that c-fos mRNA expression increases in

response to hemodynamic changes in hepatic blood flow. Also, the results provide further support for the hypothesis that a hemodynamic event causes an increase in shear stress and the release of NO, which then triggers the liver regeneration cascade.

c-Fos mRNA expression was evaluated in the ligated (left lateral or median) lobes 15 minutes after PVL. There was no change in c-fos mRNA expression from sham levels in the ligated lobes of the liver following PVL (0.40 ± 0.11, n = 5, N.S.) (Figure 9). These results suggest that c-fos mRNA was selectively expressed in the nonligated lobes, but not in the ligated lobes, following PVL.

1.3.4 c-Fos mRNA expression after manipulation of portal venous blood flow

As a critical test of my hypothesis that a hemodynamic change triggers the liver regeneration cascade, blood flow to the liver was manipulated by ligation of the superior mesenteric artery (SMA). Ligation of the SMA produces a 2/3 decrease in portal blood flow to the liver (unpublished observation). Thus, the decrease in portal venous blood flow, due to SMA ligation, should offset the 3-fold increase in the remaining 1/3 of the liver following PVL. C-Fos mRNA expression increased after PVL (1.83 ± 0.25, n=4, $p<0.05$ vs. sham (0.78 ± 0.06, n=4)) (Figure 10). However, ligation of the SMA immediately before PVL inhibited the increase in c-fos mRNA expression (0.89 ± 0.19, n=8, N.S. from sham). Ligation of the SMA only had no effect on c-fos mRNA expression (0.84 ± 0.08, n=6, N.S. from sham). This result supports the hypothesis that a blood flow stimulus is responsible for the increase in c-fos mRNA expression, an index of initiation of the liver regeneration cascade.

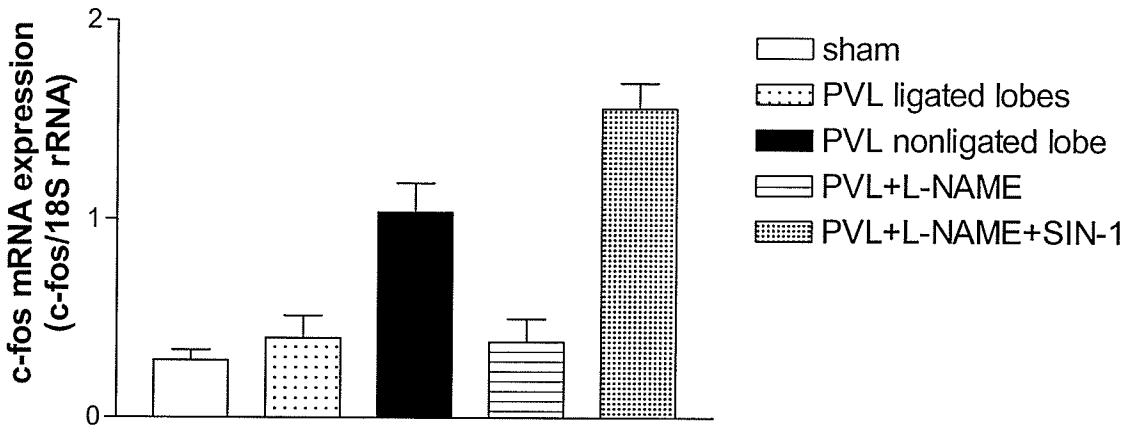

Figure 9: c-Fos mRNA expression after PVL with the NOS antagonist, L-NAME, and the NO donor, SIN-1. c-Fos mRNA expression increases after PVL, and the increase is blocked by L-NAME. The blockade is reversed by the NO donor, SIN-1. Also, there is no c-fos mRNA expression in the ligated lobes, suggesting that c-fos mRNA expression is a valid and selective index for evaluation of the liver regeneration cascade, when sampled appropriately. (data shown as mean $\pm$ SEM)

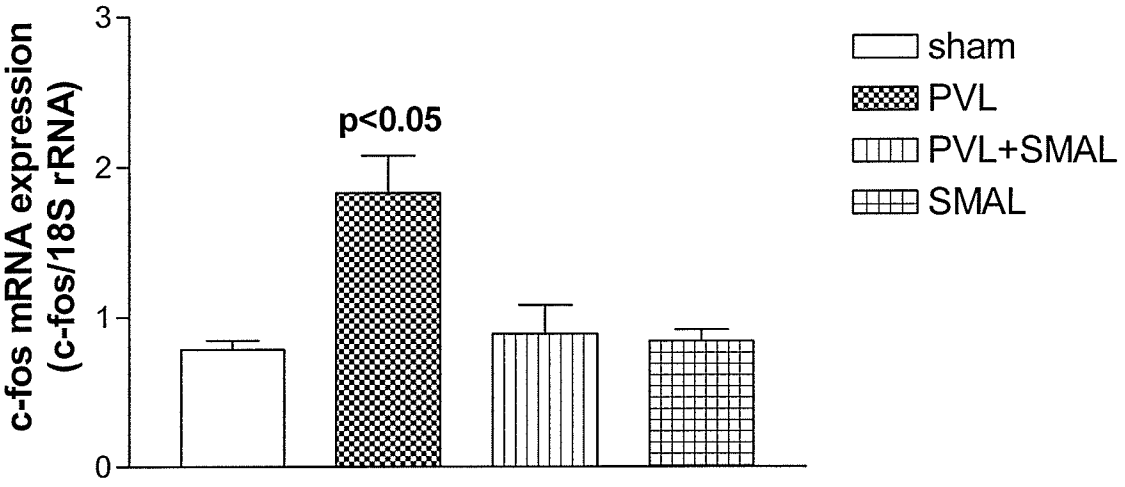

Figure 10: c-fos mRNA expression after manipulation of blood flow to the liver. C-Fos mRNA expression increases after PVL, but the increase is prevented by superior mesenteric artery ligation (SMAL). These results provide support for the hypothesis that a hemodynamic event triggers the liver regeneration cascade. (data shown as mean $\pm$ SEM)

1.4 Discussion

It has been proposed (Wang and Lautt, 1997a, 1998; Sato et al., 1997; Sato et al., 1999) that an increased blood flow-to-liver mass ratio following PHX causes shear stress, which is reflected as an increase in portal venous pressure (PVP) (Macedo and Lautt, 1998). It was further proposed that the mechanism for initiation of the liver regeneration cascade is the shear stress-induced release of NO in the liver (Wang and Lautt, 1997a, 1998). This hypothesis was further tested using a model of liver regeneration, PHX, and a hemodynamic model of compensatory hyperplasia, selective PVL. Resection of 2/3 of the liver or ligation of the left branch of the portal vein, which supplies portal venous blood to 2/3 of the liver, causes all the blood in the portal vein to be shunted through the remaining 1/3 of the liver. Portal blood flow to the liver remains constant before and after PHX, but a 3-fold increase in the blood flow per gram of remaining liver tissue occurs following PHX (Rice et al., 1977).

1.4.1 Portal venous pressure, hepatic blood flow and shear stress

Results of my experiments and others (Heikkinen and Larmi, 1968; Um et al., 1994) have shown that the change in the blood flow-to-liver mass ratio following PHX or PVL causes an increase in PVP. As described above, PVP can be used as an index of the amount of shear stress in the liver (Macedo and Lautt, 1998). The experiment, comparing the increase in PVP in both the PHX and PVL models, showed that the PVP increased to a similar extent in both models (Figure 7), suggesting similar hemodynamic conditions, a similar increase in shear stress in the liver following PHX and PVL, and

therefore similar levels of initiation of the liver regeneration cascade. Shear stress has been recognized by others as essential for liver regeneration to proceed normally (Sato et al., 1997; Sato et al., 1999). However, the mechanism of the triggering event was never determined.

1.4.2 Nitric oxide in the liver

NO is involved in regulation and maintenance of hepatic microcirculation (reviewed by Lautt and Macedo, 2000). Hepatic sinusoidal diameter was found to decrease with NOS inhibition (Bauer et al., 1997), which was reversed by infusion of L-arginine, the substrate for NOS. Conversely, L-arginine caused dilation of sinusoidal diameter and an increase in flow to the liver, which was reversed by administration of N^{ω}-nitro-L-arginine, a NOS inhibitor (Zhang et al., 1997). Within the liver, NO is produced by hepatic sinusoidal cells, including hepatocytes (Zhang et al., 1997; Nicholls-Grzemski et al., 1999), Kupffer cells (Shiratori et al., 1998) and endothelial cells (Zhang et al., 1997; Shah et al., 1997). NO is also known to be released after PHX (Obolenskaya et al., 1994; Hortelano et al., 1995), and is required for liver regeneration to occur normally (Rai et al., 1998). Previous experiments in our laboratory have implicated NO as the trigger of the liver regeneration cascade, as evaluated by PF production four hours after PHX (Wang and Lautt, 1997b; 1998).

1.4.3 NO stimulates c-fos mRNA expression in the liver after PHX and PVL

As mentioned above, c-fos mRNA expression was selected as an index because it increases after PHX, peaking at 15 minutes after resection of 2/3 of the liver, and this

elevation in expression increases in proportion to the degree of PHX (Moser et al., 2001). c-Fos mRNA expression increases in response to shear stress (Hseih et al., 1993), and also proportionately in response to increasing shear stress (Ranjan and Diamond, 1993). In addition, NO has been shown to cause an elevation in c-fos mRNA expression (Haby et al., 1994; Morris, 1995). The increase in c-fos mRNA expression was inhibited by L-NAME, a NOS antagonist, and the inhibition was reversed by administration of the NO donor, SIN-1. Also, using the PVL model, the hemodynamic involvement in c-fos mRNA expression was evaluated. As after PHX, c-fos mRNA expression increased 15 minutes after PVL. This increase was also blocked by L-NAME and the blockade was reversed by SIN-1.

Further support for the hypothesis that a hemodynamic trigger is responsible for activation of the liver regeneration cascade is provided by direct manipulation of blood flow to the liver. Ligation of the superior mesenteric artery (SMA) causes a 2/3 decrease in blood flow through the portal vein. Thus, ligation of the SMA prior to PVL, which, in the normal condition, causes an increase in the blood flow-to-liver mass ratio, should result in normal blood flow to the remaining 1/3 of the liver and should not trigger the liver regeneration cascade. Figure 10 shows that this is, in fact, the case, as c-fos mRNA expression was inhibited after SMA ligation followed by PVL. Thus, the increased blood flow-to-liver mass ratio in the absence of SMA ligation causes the increase in c-fos mRNA expression, an index of initiation of the liver regeneration cascade, in the remaining 1/3 of the liver. These results provide support, through direct manipulation of blood flow to the liver, for the hypothesis that a hemodynamic change results in shear stress and the release of NO, which triggers the liver regeneration cascade.

1.4.4 iNOS is not responsible for shear stress-induced NO release after PHX

As described above, NO is released following PHX (Hortelano et al., 1995), and is required for liver regeneration to occur normally (Rai et al., 1998). However, these studies examined NO production by iNOS, and the role of eNOS and nNOS in the liver regeneration cascade has yet to be determined. All three isoforms of NOS are present in the liver (Kurose et al., 1996; Esteban et al., 1997), and could contribute to NO released to trigger the liver regeneration cascade.

The specific iNOS antagonist, AG, did not inhibit c-fos mRNA expression after PHX (Figure 8), suggesting that iNOS is not the isoform of NOS responsible for production of NO, which triggers the liver regeneration cascade. As described above, 2/3 PHX forces all the portal venous blood to flow through the remaining 1/3 of the liver, resulting in an increase in PVP, and an increase in PVP is an index of shear stress in the liver (Macedo and Lautt, 1998). Shear stress causes the upregulation and activation of eNOS, and results in the production of NO from endothelial cells (Gallis et al., 1999; Fisslthaler et al., 2000), which is an immediate event. An increase in phosphorylation of eNOS, which is correlated with an increase in NO production by the enzyme, was noted 30 seconds after application of fluid shear stress to endothelial cells (Gallis et al., 1999). Hepatic sinusoidal endothelial cells also contain eNOS and can release NO to regulate sinusoidal resistance (Shah et al., 1997). The NO that triggers the liver regeneration cascade may be of sinusoidal endothelial cell origin. This NO may diffuse to immediately adjacent hepatocytes as a signal to initiate the liver regeneration cascade. These results are consistent with the hypothesis that an increase in the blood flow-to-liver

mass ratio causes shear stress and the release of NO, secondary to activation of eNOS, which triggers the liver regeneration cascade. However, further work is needed to more fully examine the involvement of individual isoforms of NOS in triggering the liver regeneration cascade.

1.4.5 Evaluation of c-fos mRNA expression as a suitable index of the initiation of the liver regeneration cascade

It has recently been suggested that c-fos mRNA expression would not be a good index of initiation of the liver regeneration cascade. Starkel et al. (1999) found that c-fos mRNA expression increased both in the ligated and nonligated lobes of the liver following PVL. They concluded that c-fos mRNA expression is nonspecifically expressed as a stress response, rather than, as we suggest, a specific index for initiation of the liver regeneration cascade. However, in our model, c-fos mRNA expression in the ligated lobes was not different from that in sham operated animals, while there was a significant increase in c-fos mRNA expression in nonligated lobes (Figure 9). The difference between our, and others', results is most likely due to the 60 minute stabilization period given to all rats, which underwent PVL in our experiments, in order to eliminate surgical stress as a potential stimulator of c-fos mRNA expression. Preliminary experiments revealed an increase in c-fos mRNA expression in the liver of sham rats, which underwent PVL surgery without ligation of the left branch of the portal vein (data not shown), when the animals were allowed only 30 minutes to stabilize after surgery. Increasing the length of stabilization following surgery from 30 to 60 minutes allowed c-fos mRNA expression in response to ligation of the left branch of the portal

vein to be isolated from that in response to surgical stress. This finding suggests that c-fos mRNA expression is a good index for the initiation of the liver regeneration cascade, if evaluated at appropriate times. In addition, the lack of effect on c-fos mRNA expression in the ligated lobes suggests a lack of significant blood borne signal at this early time point, since the ligated lobes still receive hepatic arterial flow. In fact, a decrease in portal venous blood flow is known to activate the hepatic arterial buffer response to greatly elevate the hepatic arterial flow by a mechanism recently reviewed (Lautt, 1996). The hepatic arterial buffer response has also been shown to be activated following PVL (Rocheleau et al., 1999), causing an increase in arterial blood flow to the ligated lobes.

1.4.6 Relevance of c-fos in the liver regeneration cascade

The protein product of the c-fos protooncogene, FOS, participates in stimulating cell growth. FOS forms a dimer with the protein product of c-jun, JUN, to form the transcription factor, activator protein-1 (AP-1) (Angel and Karin, 1991). AP-1 is known to increase following PHX (Heim et al., 1997), suggesting a functional basis for the increase in c-fos mRNA expression. Also, there has been AP-1 recognition sites found in the promoters of genes coding for important components of the liver regeneration cascade. For example, there is an AP-1 recognition site in the promoter region of the IL-6 (Kick et al., 1995) gene, a proinflammatory cytokine known to be involved in the liver regeneration cascade (Michalopolous and DeFrances, 1997). In addition, an AP-1 recognition site exists in the promoter region of the inducible NOS (iNOS) gene (Chu et al., 1998), also required for normal liver regeneration to occur (Rai et al., 1998). Thus,

the increase in c-fos mRNA expression, triggered by NO, may lead to further activation of pathways in the liver regeneration cascade.

1.5 Summary

Results of these experiments have provided support for the hypothesis that NO, released secondary to an increase in the blood flow-to-liver mass ratio and shear stress in the remnant liver after PHX and PVL, triggers the liver regeneration cascade. There is a similar increase in PVP, and therefore in shear stress, in the liver after both PHX and PVL, suggesting similar hemodynamic conditions in both models. Inhibition of NOS prevents the increase in c-fos mRNA expression, an index of initiation of the liver regeneration cascade, after PHX and PVL, and the NO donor, SIN-1 reverses the inhibition in both models. In addition, reduction in blood flow to the liver by two-thirds prior to PVL, prevented the increase in c-fos mRNA expression after PVL, indicating that a hemodynamic factor is responsible for the increase in c-fos mRNA expression in the liver regeneration cascade. Also, results are consistent with the hypothesis that the NO that triggers the liver regeneration cascade is produced by eNOS, the constitutive isoform of NOS, in response to shear stress.

Chapter 2
Prostaglandins and NO in liver regeneration

2.1 Introduction

The liver regeneration cascade is a complex interaction between growth factors, cytokines and other mediators, which results in compensatory hyperplasia after removal of liver tissue. An additional mediator that is an important component of the liver regeneration cascade is NO, as described in Chapter 1. Evidence has been presented that NO, released secondary to a hemodynamic event, which causes shear stress, triggers the liver regeneration cascade. Prostaglandins (PGs) are also an essential component of the liver regeneration cascade. Inhibition of cyclooxygenase (COX), the enzyme responsible for PG synthesis, inhibits DNA synthesis and delays liver weight restoration after PHX (MacManus and Braceland, 1976). It is hypothesized that PGs are also released by shear stress, secondary to an increase in the blood flow-to-liver mass ratio, and that NO and PGs work together to trigger the liver regeneration cascade.

2.1.1 PGE_2 and PGI_2 in the liver regeneration cascade

Although the liver produces various types of PGs, the roles of PGE_2 and PGI_2 in the liver regeneration cascade were investigated. These PGs were chosen for several reasons. PGE_2 is the most abundant PG produced by the liver (Wernze et al., 1986), and both PGE_2 and PGI_2 have been shown to be cytoprotective to hepatocytes. This cytoprotection is abolished by indomethacin (INDO) (Guarner et al., 1985). PGE_2 and PGI_2 levels are increased after PHX (Tsujii et al., 1993; Lai et al., 2000), and in addition, these PGs play an important role in DNA synthesis. It was demonstrated that cyclooxygenase (COX) antagonists inhibit DNA synthesis and delay liver regeneration

(MacManus and Braceland, 1976; Besse et al., 1991). PGE_2 and PGI_2 are also released in response to shear stress (Frangos et al., 1985; Grabowski et al., 1985; Shah et al., 1997; Wang and Tarbell, 2000), and both PGE_2 and PGI_2 can stimulate c-fos mRNA expression. PGE_2 has been shown to stimulate c-fos mRNA expression in fibroblasts (Zhuang et al., 2000), osteoblasts (Suda et al., 2000) and prostate cancer cells (Chen and Hughes-Fulford, 2000), while PGI_2 stimulates c-fos mRNA expression in fibroblasts and osteoblasts (Glantschnig et al., 1996a; 1996b). Finally, previous experiments showed that PGs are required for proliferative factor (PF) production after PHX, as INDO inhibited the subsequent increase in PFs (Wang, 1997).

2.1.2 Interaction between NO and PGs

NO and PGs are often synthesized and released together in physiological situations. For example, NO and PGI_2 inhibit platelet aggregation (Starzyk et al., 1999) and cause vasodilation in the coronary circulation of conscious dogs (Zhao et al., 1996). Also, NO, PGE_2 and PGI_2 have a cytoprotective effect in the stomach, as NO was found to cause an increase in PGE_2 and PGI_2 production in gastric mucus-producing cells (Uno et al., 1997). *In vitro*, NO modulates COX activity in cultured macrophages, by increasing COX activity and PGE_2 production in response to lipopolysaccharide (LPS), a potent inflammatory stimulus (Salvemini et al., 1993). In the liver regeneration cascade, inhibition of both NO and PG synthesis blocked production of PF after PHX (Wang, 1997; Wang and Lautt, 1998). Also, both NO and PGs are released in response to shear stress in endothelial cells (Kelm et al., 1991; Frangos et al., 1985; Grabowski et al., 1985; Shah et al., 1997; Wang and Tarbell, 2000). It has also been shown that shear stress

stimulates the release of NO and PGs, which are thought to be involved in triggering bone formation. McAllister et al. (2000) found that shear stress stimulated NO, PGE_2 and PGI_2 production, within 1 hour of stimulation of osteoclast precursor cells. In addition, shear stress stimulated mouse bone cells to produce NO and PGE_2 (Klein-Nulend et al., 1995; Smalt et al., 1997). The increase in NO (Klein-Nulend et al., 1995; Smalt et al., 1997) and PGE_2 (Klein-Nulend et al., 1995) production was inhibited by NOS antagonists, thus suggesting interaction between NO and PGs in stimulation of bone formation.

In this chapter, I will use the c-fos mRNA expression index to test the hypothesis that PGs are released secondary to an increase in the blood flow-to-liver mass ratio following PHX, which causes shear stress in the remaining 1/3 of the liver, and triggers the liver regeneration cascade. In addition, it is hypothesized that NO and PGs will work together to stimulate c-fos mRNA expression and trigger the liver regeneration cascade.

2.2 Methods

Male Sprague Dawley rats were anesthetized and surgical preparation was performed as described in Chapter 1. Surgical procedures were the same as described in Chapter 1. Briefly, rats were anesthetized using sodium pentobarbital. Tracheotomy was performed, and cannulae were placed in the femoral artery, femoral vein and portal vein for infusion of drugs and anesthetic. Laparotomy was performed, and the animal was allowed to stabilize for 30 minutes. Drug or saline was then administered, and PHX or sham procedures were performed at the appropriate time. The remnant liver was then removed after 15 minutes and immediately frozen on dry ice for RNA analysis.

In the first set of experiments, the NOS antagonist, L-NAME, was employed. Rats were divided into the following experimental groups: sham, PHX, sham+L-NAME, PHX+L-NAME, sham+L-NAME+PGE_2, PHX+L-NAME+PGE_2, sham+L-NAME+PGI_2, PHX+L-NAME+PGI_2, sham+L-NAME+SIN-1, PHX+L-NAME+SIN-1, sham+L-NAME+ s-nitroso-n-acetylpenicillamine (SNAP), and PHX+L-NAME+SNAP. In addition, a second set of experiments, using the COX antagonist, INDO, included the groups: sham, PHX, sham+INDO, PHX+INDO, sham+ INDO+PGE_2, PHX+INDO+PGE_2, sham+INDO+PGI_2, PHX+INDO+PGI_2, sham+INDO+SIN-1, PHX+INDO+SIN-1, sham+INDO+SNAP, and PHX+INDO+SNAP.

2.2.1 Drugs

L-NAME (Sigma) (5 mg/kg, i.v., 0.5 ml bolus infused over 2 minutes), a non-selective NOS antagonist, was dissolved in saline, and the rat was allowed to stabilize for

10 minutes following administration. The NO donors, SIN-1 (5 mg/kg) (Alexis Corp) and S-nitroso-N-acetyl penicillamine (SNAP; 5mg/kg) (Sigma) were also dissolved in saline, and a 0.2 ml bolus was infused intraportally over 2 minutes. PGE_2 (10ug/kg) (Sigma) and PGI_2 (10ug/kg) (Sigma) were dissolved in 95% ethanol in a stock solution, and diluted with the appropriate amount of saline to achieve the desired concentration. A 0.1 ml bolus of either PG was infused intraportally over 1 minute. PHX or sham procedures were performed immediately following NO donor or PG administration.

2.2.2 RNA isolation and northern blot analysis

RNA analysis was the same as that described in Chapter 1. Briefly, total RNA was extracted using a 3M/6M lithium chloride/urea solution, centrifuged at 25000 rpm for 20 minutes at 4°C, and total RNA was extracted using phenol/chloroform. The concentration of the RNA was then determined and 20 ug of total RNA was separated by gel electrophoresis under denaturing conditions, transferred to nylon membrane, and fixed by UV crosslinking. The membranes were prehybridized at 42°C for 3 hours in prehybridization buffer, and c-fos mRNA was detected using a 1.8 kb cDNA probe labeled by the random prime method, with α-dCTP ^{32}P. The membranes were hybridized overnight at 42°C, washed at 65°C, exposed to film, and the density of the c-fos mRNA band was determined by densitometry. c-Fos mRNA expression is reported relative to 18S rRNA. Results were analyzed using ANOVA, with $p<0.05$ deemed significant.

2.3 Results

The interaction between NO and PGs in the liver regeneration cascade was investigated using the NOS antagonist, L-NAME, the COX antagonist, Indomethacin (INDO), the NO donors, SIN-1 and SNAP, and the PGs, PGE_2 and PGI_2. The increase in c-fos mRNA expression after PHX (1.82 ± 0.16, n=5 $p<0.01$ vs. sham, n=5, 0.53 ± 0.03) (Figure 11) was inhibited by the NOS antagonist, L-NAME (0.62 ± 0.09, n=5, N.S. from sham). The inhibition by L-NAME was reversed by the PGs, PGE_2 (1.95 ± 0.32, n=7, $p<0.001$ vs. sham) and PGI_2 (1.78 ± 0.24, n=8, $p<0.01$ vs. sham), and by the NO donors, SIN-1 (1.70 ± 0.17, $p<0.01$ vs. sham, n=9) and SNAP (1.64 ± 0.26, n=7, $p<0.05$ vs. sham). In addition, L-NAME had no effect on c-fos mRNA expression in normal, non-PHX livers (0.47 ± 0.08, n=6, N.S. from sham). Neither PGE_2 (0.68 ± 0.04, n=4), PGI_2 (0.90 ± 0.06, n=5), SIN-1 (0.98 ± 0.24, n=4) nor SNAP (0.87 ± 0.05, n=7) administered after L-NAME had any effect on c-fos mRNA expression compared to normal livers in sham rats. Therefore, the NOS antagonist, L-NAME, inhibits c-fos mRNA expression after PHX, and PGE_2, PGI_2 or the NO donors, SIN-1 or SNAP, reversed the inhibition.

c-Fos mRNA expression increased after PHX (2.12 ± 0.40, n=5, $p<0.001$ vs sham (0.65 ± 0.09)), which was inhibited by INDO, a COX antagonist (1.03 ± 0.07, n=7, N.S. vs. sham) (Figure 12). The inhibition tended to be reversed by PGE_2 (1.52 ± 0.23, n=6), although the trend was not significantly different from sham, and fully reversed by PGI_2 (1.96 ± 0.18, n=6, $p<0.001$ vs. sham). The NO donors SIN-1 and SNAP also reversed the inhibition of c-fos mRNA expression after INDO (2.20 ± 0.17, n=5, $p<0.001$, and 2.09 ± 0.28, n=6, $p<0.001$, respectively). C-Fos mRNA expression in the normal, non-PHX

liver, was neither affected by INDO (0.58 ± 0.08, n=5, N.S. from sham), nor by INDO followed by PGE_2 (0.81 ± 0.15, n=4), PGI_2 (0.96 ± 0.06, n=6), SIN-1 (0.44 ± 0.04, n=6), or SNAP (0.66 ± 0.09, n= 6). The increase in c-fos mRNA expression was not affected by the ethanol-saline vehicle in which PGE_2 and PGI_2 were dissolved (data not shown). Thus, inhibition of PG production by COX inhibits c-fos mRNA expression, an index of the initiation of the liver regeneration cascade, and this inhibition can be reversed by administration of either PGI_2 or a NO donor.

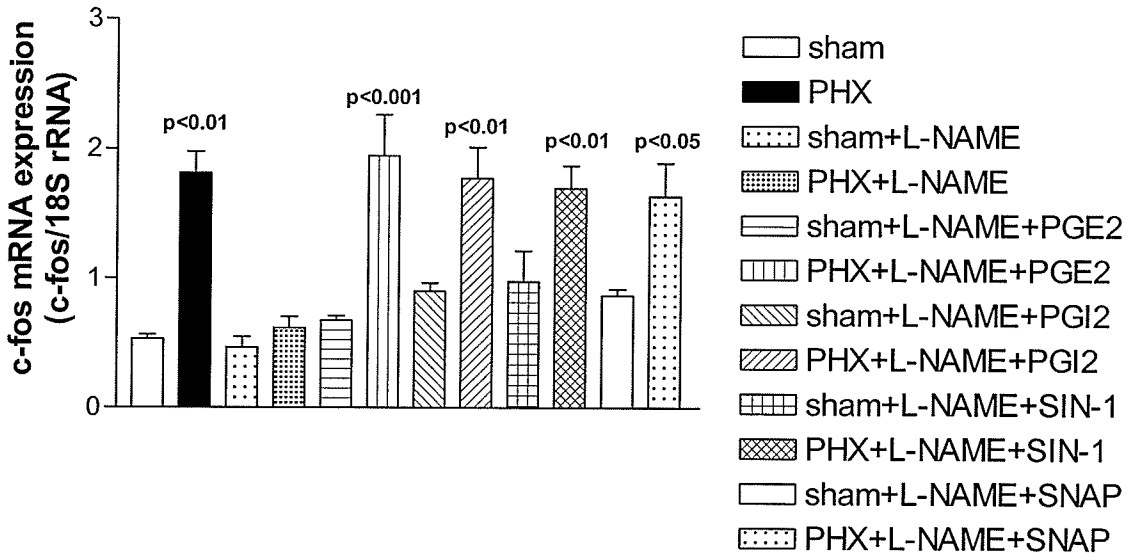

Figure 11: c-fos mRNA expression after PHX and NOS inhibition. The inhibition of c-fos mRNA expression by L-NAME after PHX can be reversed by PGE_2, PGI_2 or the NO donors, SIN-1 or SNAP. These results support the hypothesis that NO and PGS, released secondary to an increase in shear stress in the liver, trigger the liver regeneration cascade. The results are also consistent with the hypothesis that NO and PGs interact to trigger the liver regeneration cascade. (data shown as mean $\pm$ SEM)

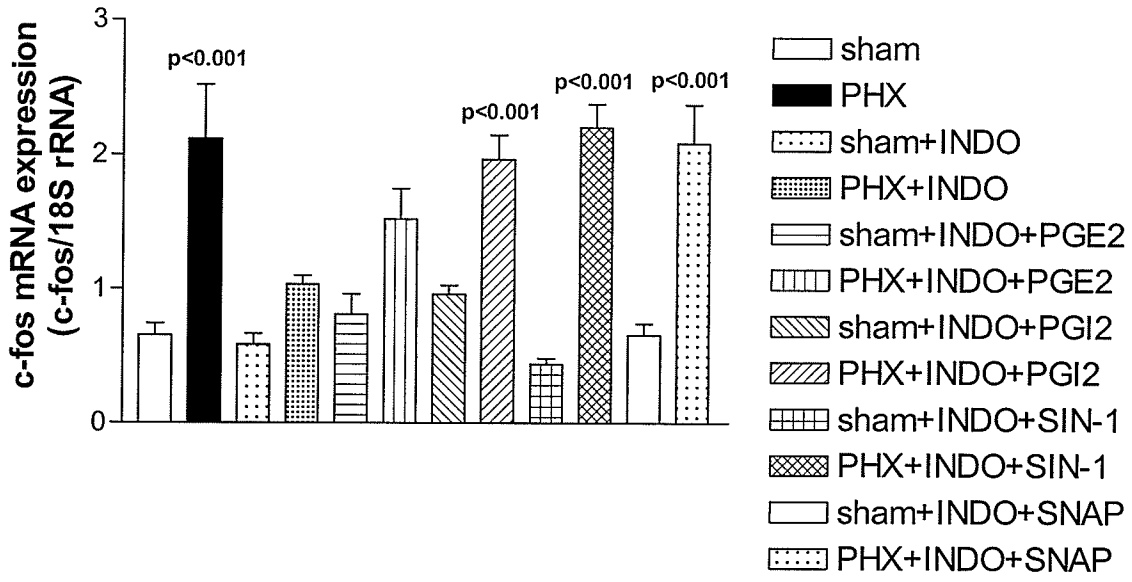

Figure 12: c-fos mRNA expression after PHX and COX inhibition. C-Fos mRNA expression increases after PHX, and this increase is blocked by INDO, a COX antagonist. The inhibition is reversed by PGI_2, or the NO donors, SIN-1 or SNAP. These results also support the hypothesis that an increase in the blood flow-to-liver mass ratio in the remaining 1/3 of the liver after PHX causes shear stress and the release of NO and PGs, which trigger the liver regeneration cascade. In addition, these results also provide evidence that there is interaction between NO and PGs in triggering the liver regeneration cascade. (data shown as mean $\pm$ SEM)

2.4 Discussion

2.4.1 Prostaglandins and the liver regeneration cascade

The inhibition of the increase in c-fos mRNA expression after PHX by L-NAME (a NOS antagonist) is reversed by PGE_2, PGI_2 or a NO donor (SIN-1 or SNAP). Also, the increase in c-fos mRNA expression is inhibited by INDO (a COX antagonist). This inhibition is reversed by PGI_2 or the NO donors, SIN-1 or SNAP. Thus, under conditions where either NO or PG production is blocked, the inhibition of c-fos mRNA expression can be reversed by either PGs or NO donors. This suggests that both NO and PGs are required for c-fos mRNA expression to increase after PHX, and that, in the absence of one, excess exogenous supply of the other can compensate.

Previous experiments suggested an interaction between NO and PGs in triggering the liver regeneration cascade, using proliferative factor (PF) production after PHX as an index of initiation of the liver regeneration cascade. Briefly, PFs include all the growth factors, cytokines, etc. produced after PHX that comprise the liver regeneration cascade. PF production was found to increase, peaking 4 hours after PHX (Wang and Lautt, 1997b). This increase was inhibited by L-NAME and the inhibition was partially reversed by L-arginine, the substrate for NOS (Wang and Lautt, 1998). Administration of INDO also inhibits PF production 4 hours after PHX (Wang, 1997), suggesting that both NO and PGs play a role in triggering the liver regeneration cascade. In the case of both indices, c-fos mRNA expression and PF production, it appears that inhibition of NO or PG synthesis completely blocks the index, which indicates that NO and PG production appear to have an important role in the liver regeneration cascade.

An additional index of initiation of the liver regeneration cascade, liver weight restoration after PHX, was also investigated (Wang and Lautt, 1998). Inhibition of NOS caused a 30% reduction in liver weight restoration 48 hours after PHX, and was also associated with a high mortality rate that was found not to be due to L-NAME itself (Wang, 1997). Blockade of NOS prior to PHX was also shown to inhibit DNA synthesis *in vivo* (Carnovale et al., 2000). Other studies have also implicated PGs in liver weight restoration. Inhibition of COX by INDO causes a decrease in DNA synthesis and delays liver regeneration after PHX (MacManus and Braceland, 1976; Rudnick et al., 2001). Thus, both NO and PGs are an integral part of both the triggering and propagation mechanisms of the liver regeneration cascade.

2.4.2 Mechanism of NO and PG release in the liver regeneration cascade

The mechanism of NO release immediately after PHX is described in Chapter 1. Briefly, the liver does not control inflowing blood from the intestine, and must accept all the portal venous inflow. Removal of 2/3 of the liver tissue results in an 3-fold increase in the blood flow-to-liver mass ratio in the remaining 1/3 of the liver (Rice et al., 1977), and evidence is presented that this increase in blood flow causes shear stress (reflected as an immediate increase in PVP), and the release of NO, which thereby triggers the liver regeneration cascade.

It is also hypothesized that PG production is a result of the same shear stress-dependent mechanism that causes NO release. PGE_2 and PGI_2 are known to increase after PHX (MacManus and Braceland, 1976; Tsujii et al., 1993; Lai et al., 2000), with PGI_2 increasing 3-fold immediately after PHX (Lai et al., 2000). The 3-fold increase in

PGI_2 seems to reflect the 3-fold increase in blood flow in the remaining 1/3 of the liver after PHX. NO and PGI_2 are vasoactive mediators, released in response to an increase in flow, and thereby shear stress, in blood vessels (Grabowski et al., 1985; Kelm et al., 1991). Also, NO, PGI_2 and PGE_2 are released in response to shear stress applied *in vitro* (Gallis et al., 1999; Frangos et al., 1985; Grabowski et al., 1985; Shah et al., 1997; Wang and Tarbell, 2000), and release of these mediators was shown to be proportionate to the amount of shear stress applied (Grabowski et al., 1985; Kelm et al., 1991). Figure 7 shows that PVP, an index of shear stress in the liver, increases after PHX and PVL, indicating that shear stress is increased in the liver, along with the increase in the blood flow-to-liver mass ratio.

2.4.3 Mechanism of interaction between NO and PGs

NO production causes an increase in cGMP, which stimulates c-fos mRNA expression in fibroblasts (Haby et al., 1994) and PC12 cells (Uberall et al., 1994). PG production causes an increase in cAMP, which thereby causes an increase in c-fos mRNA expression in macrophages (Zhuang et al., 2000) and osteocytes (Fitzgerald and Hughes-Fulford et al., 1999). Thus, an increase in either cGMP or cAMP stimulates c-fos mRNA expression, and after PHX, both of these second messengers may be required to stimulate c-fos mRNA expression. However, in the absence of NO, and therefore cGMP, it is possible that administration of excess exogenous PGs caused an increase in the amount of cAMP, thereby stimulating c-fos mRNA expression. Similarly, in the case of COX inhibition, the provision of excess exogenous NO could cause an increase in cGMP, thereby stimulating c-fos mRNA expression after PHX. Thus, in this manner, excess

amounts of NO or PGs could compensate for the actions of the other in situations of either NOS or COX inhibition.

2.4.4 Interaction between NO and PGs

There are several examples of interaction between NO and PGs in other tissues. For example, under normal, non-shear stress conditions, NO stimulates PG production in endothelial cells *in vitro*. Endothelial NO production stimulates PGI_2 production, possibly by directly activating COX, and inhibition of NOS and guanylate cyclase (GC) reduced levels of PGI_2 (Sievi et al., 1997). However, under conditions of shear stress, inhibition of NOS or GC resulted in an increase in PGI_2 production in endothelial and smooth muscle cells undergoing shear stress (Osanai et al., 2000; 2001). Thus, when NOS was inhibited, *in vitro* endothelial cell production of PGI_2 increases and the excess amount of this PG could compensate for the absent effects of NO. In addition, shear conditions caused an increase in eNOS expression and activity in endothelial cells, which was potentiated by the COX antagonist, INDO (Hendrickson et al., 1999). Gastric eNOS is also upregulated in conditions of decreased PGE_2 production and COX inhibition (Fischer et al., 1999). This suggests that in the absense of PGs, endothelial cell NO production increases, and the excess amount of NO could compensate for the lack of effects of PGs. Thus, c-fos mRNA expression could be restimulated under conditions of NOS inhibition after PHX, by excess amounts of PGs, or under conditions of COX inhibition after PHX, by excess amounts of NO.

However, the results from these studies suggest that endogenous production of either NO or PGs cannot compensate for the loss of the other, although provision of

excess exogenous NO or PGs does result in a compensatory effect. Reversal of the inhibition of c-fos mRNA expression in either case occurs only when adequate levels of exogenous NO or PGs are provided. It may be possible, however, that the compensatory mechanism takes some time to upregulate, and therefore c-fos mRNA expression is merely delayed after PHX. This seems unlikely, though, as Figure 8 shows that c-fos mRNA expression after PHX is still inhibited by L-NAME at 60 minutes after PHX. Thus, NO and PGs seem not to compensate for the absence of the other endogenously, at least with regard to c-fos mRNA expression, and/or within the limited time frame examined after PHX. However, this situation would seem to provide an excellent therapeutic opportunity by administration of exogenous NO donors, SIN-1 or SNAP, or the PGs, PGE_2 or PGI_2, to stimulate the liver regeneration cascade.

2.5 Summary

The increase in c-fos mRNA expression after PHX is abolished by either inhibition of NO or PG synthesis, and in either condition, the NO donors, SIN-1 or SNAP, or the PGs, PGE_2 or PGI_2, can reverse the inhibition. This suggests that c-fos mRNA expression after PHX requires both NO and PGs, and in the absence of either NO or PG synthesis, administration of an excess amount of either exogenous NO or PGs seems to be able to compensate for the absent compound. In addition, this interaction between NO and PGs could be exploited as a possible therapeutic target to potentiate the liver regeneration cascade. Either NO or PGs alone, or in combination, could be administered in an attempt to increase the rate of liver weight restoration after tissue

resection. Thus, NO donors and/or PGs seem to be good candidates for therapeutic agents to potentiate the liver regeneration cascade.

Chapter 3

Potentiation of the liver regeneration cascade by NO and PGs

3.1 Introduction

NO and PGs are important components of the liver regeneration cascade, as described above. Briefly, interaction between NO and PGs has been shown to be an important part of the liver regeneration cascade. Both NO and PGs increase after PHX (Callery et al., 1991; Tsujii et al., 1993; Obolenskaya et al., 1994; Lai et al., 2000), and inhibition of either NO or PG synthesis negatively affects liver regeneration (MacManus and Braceland, 1976; Rai et al., 1998; Carnovale et al., 2000). In addition, in situations where either NOS or COX is inhibited, an excess amount of the other may be able to compensate for the effects of the missing compound. The NO donors, SIN-1 and SNAP, and the PGs, PGE_2 and PGI_2, will be used to test the hypothesis that NO and/or PGs can potentiate the liver regeneration cascade. In addition, to more fully test the hypothesis, the phosphodiesterase V antagonist, Zaprinast (ZAP), will be employed to potentiate the effects of the NO signaling pathway after PHX.

3.1.1 NO donors and cGMP

NO donors cause an increase in cGMP levels in various tissues, including liver (Katsuki and Murad, 1977; Katsuki et al., 1977). This increase in cGMP is caused by stimulation of guanylate cyclase (GC) (Katsuki et al., 1977), an enzyme that converts GTP to cGMP (Murad et al., 1992). The effects of cGMP are regulated by breakdown of

this cyclic nucleotide by phosphodiesterase (PDE) enzymes (Thompson, 1991). There are several isozymes of the PDE enzymes, which hydrolyze not only cGMP but also cAMP, or combinations of the two (Thompson, 1991). PDE I and II hydrolyze cAMP and cGMP, while PDE III is more specific for cAMP but is still involved in cGMP catalysis. PDE IV is specific for cAMP and PDE V is specific for cGMP (Thompson, 1991). Thus, the PDE V antagonist, Zaprinast (ZAP), was chosen to test the hypothesis that potentiation of the NO signaling cascade results in potentiation of c-fos mRNA expression after PHX, and of the liver regeneration cascade.

3.1.2 cGMP and gene expression

In addition, cGMP is known to stimulate gene expression. For example, c-fos and c-jun mRNA expression is stimulated by NO via cGMP (Morris, 1995; Haby et al., 1994; Idriss et al., 1999). Shear stress causes activation of eNOS and thereby an increase in NO and cGMP levels in endothelial cells (Fisslthaler et al., 2000). Also, cGMP has been implicated in the initial stages of hepatocyte proliferation. cGMP levels increase in the liver within 20 minutes of PHX (Miura et al., 1976), which is consistent with the hypothesis that NO is released in response to a hemodynamic change after PHX, and triggers the liver regeneration cascade.

3.1.3 NO donors, Zaprinast and PGs in combination

cGMP levels were increased by either ZAP or SNAP, and incubation of liver slices with both ZAP and SNAP caused a further increase in cGMP production compared to these compounds individually (Wood and Ignarro, 1987). In addition, there is some

evidence that intravenous administration of ZAP and inhaled NO have a synergistic effect on pulmonary arterial relaxation (Thusu et al., 1995). Thus, it is also hypothesized that combination of ZAP and SNAP would further potentiate c-fos mRNA expression and the liver regeneration cascade.

PGs cause an increase in cAMP levels, and cAMP levels are known to increase after PHX (Callery et al., 1991; Tsujii et al., 1993; Lai et al., 2000). Also, cAMP causes an increase in c-fos mRNA expression (Fitzgerald and Hughes-Fulford et al., 1999; Zhuang et al., 2000). In addition, shear stress causes the release of PGs (Frangos et al., 1985; Wang and Tarbell, 2000), and PGs are involved in DNA synthesis after PHX (MacManus and Braceland, 1976). PGE_2 administration has been shown to be hepatoprotective under conditions of hepatitis B infection, by reducing viral load by 47% (Hyman et al., 1999). PGI_2 was shown to be protective to the liver under conditions of ischemia/reperfusion (Totsuka et al., 1998). There is also evidence that PGs and NO administered together have a synergistic effect. For example, administration of PGI_2 and the PDE V antagonist, ZAP, amplifies vasodilation, thereby decreasing pulmonary arterial pressure, in pulmonary hypertension (Schermuly et al., 1999). Therefore, the hypothesis was also tested that PGs and NO, alone or in combination, can potentiate the liver regeneration cascade.

3.2 Methods

3.2.1 c-Fos mRNA expression

Surgical procedures were the same as described in Chapter 1. Briefly, rats were anesthetized using sodium pentobarbital. Tracheotomy was performed, and cannulae were placed in the femoral artery, femoral vein and portal vein for infusion of drugs and anesthetic. Laparotomy was performed, and the animal was allowed to stabilize for 30 minutes. Drug or saline was then administered, and PHX or sham procedures were performed at the appropriate time. The remnant liver was then removed after 15 minutes and immediately frozen on dry ice for RNA analysis. Experimental groups are as follows: sham, PHX, sham+SNAP, PHX+SNAP, sham+SIN-1, PHX+SIN-1, sham+PGE_2, PHX+PGE_2, sham+PGI_2, PHX+PGI_2, sham+Zaprinast (ZAP; 10mg/kg), PHX+ZAP (10mg/kg), sham+ZAP(30mg/kg), and PHX+ZAP (30mg/kg). SNAP (5mg/kg; 0.2 ml bolus i.p.v. over 2 minutes) (Sigma), SIN-1 (5mg/kg; 0.2 ml bolus i.p.v. over 2 minutes) (Alexis Corp.), PGE_2 (10ug/kg; 0.1 ml bolus over 1 minute) (Sigma), PGI_2 (10ug/kg; 0.1 ml bolus i.p.v. over 1 minute) (Sigma) or saline (0.2 ml bolus over 2 minutes) were administered and PHX performed immediately thereafter. Zaprinast (ZAP) (10 or 30 mg/kg; 0.05 ml bolus i.p.v. over 0.5 minutes) was administered and PHX performed 5 minutes thereafter.

Based on the results from the initial potentiation experiments, drug combinations included ZAP and/or SNAP and/or PGI_2. Concentration, volume and route of administration for the combination drugs were the same as for individual drugs. For administration of the double drug combinations, ZAP was administered first and 3 or 4

minutes thereafter, either SNAP or PGI_2, respectively, was administered. In the other combination group, SNAP was administered followed immediately by PGI_2. In the triple combination group, ZAP was administered followed by SNAP and then PGI_2. PHX was then immediately performed, and 15 minutes later, the remaining liver was removed and frozen on dry ice for RNA analysis.

RNA was analyzed as described in Chapters 1 and 2. Results are expressed as c-fos mRNA expression relative to 18S rRNA, and analyzed using ANOVA, with $p<0.05$ deemed significant.

3.2.2 Liver weight restoration

Male Sprague Dawley rats were anesthetized using sodium pentobarbital (0.1 mg/100 g of 65 mg/ml solution, i.p.) and the abdomen was shaved and cleaned with 70% alcohol and betadine. Laparotomy was performed and drug or saline was administered i.p. The left lateral and median lobes were removed (2/3 PHX), or the liver lobes gently manipulated (sham procedures). A micro-renathane (MRE-040, Braintree Scientific Inc.) catheter filled with saline was placed under the skin, with one end inside the abdominal cavity and the other exiting between the shoulder blades on the back of the rat. The abdomen was sutured shut (muscle then skin layers), using Dexon II, 3-0, (Davis & Geck) sutures and ensuring that the catheter was secure. The opening between the shoulder blades was sutured shut, and the catheter secured with sutures. The catheter was capped to allow further i.p. doses of drug or saline. Ketoprophen (an analgesic, 2.5 mg/kg) was administered subcutaneously. The rats were allowed to recover in individual cages placed on heating pads. The animals were monitored until they are awake and able

to move around the cage. They had access to food and water at all times, with some food pellets placed on the bottom of the cage for easier access. Drug or saline administration (0.5 ml bolus i.p.) took place at 5-hour intervals, 3 times per day for a total of 6 doses during the next 48 hours. Forty-eight hours after PHX, the rat was sacrificed by pentobarbital anesthetic overdose and the liver removed, slashed and blotted, and weighed. The percent liver weight restoration after PHX was calculated as follows:

$$\% \text{ liver weight restoration} = \frac{(\text{48 hour liver weight (g)} - \text{remnant liver weight (g)})}{\text{resected liver weight (g)}} \times 100$$

Remnant liver weight = standard total liver weight (g) – resected liver weight (g)

Where the 48 hour liver weight is the weight of the liver 48 hours after PHX; the remnant liver weight is the theoretical remaining liver weight following resection, as calculated above; and resected liver weight is the weight of the left lateral and median lobes removed during PHX. The standard total liver weight was calculated from a regression analysis of the total liver weight versus body weight in control rats (n=40) (data not shown).

3.2.3 Drugs

For the experiments involving c-fos mRNA expression, SNAP (5mg/kg) (Sigma) was dissolved in saline and a 0.2 ml bolus was infused intraportally over 2 minutes. PGI_2 (10ug/kg) (Sigma) was dissolved in 95% ethanol in a stock solution, and diluted with the appropriate amount of saline to achieve the desired concentration. A 0.1 ml bolus of either PG was infused intraportally over 1 minute. ZAP was dissolved in 0.15 M NaOH,

and diluted to the appropriate concentration using saline. PHX or sham procedures were performed immediately following SNAP, ZAP or PGI_2 administration.

For the liver weight restoration experiments, drugs were prepared as described for the c-fos experiments. 6-keto-$PGF_{1\alpha}$, a stable metabolite of PGI_2, was used in the 48 hour liver weight restoration experiments rather than PGI_2, due to the short half-life of PGI_2. The dose of 6-keto-$PGF_{1\alpha}$ was selected based on potentiation of c-fos mRNA expression using PGI_2, and the finding that 6-keto-$PGF_{1\alpha}$ was at least 50% less effective at vasorelaxation of smooth muscle compared to PGI_2 (Levy, 1980). Thus, a dose of 20ug/kg of 6-keto-$PGF_{1\alpha}$, double that of PGI_2 used in the c-fos experiments, was administered. The 6-keto-$PGF_{1\alpha}$ was dissolved in 95% ethanol, and diluted to the appropriate concentration with saline. Doses were based on c-fos mRNA expression experiments, and a volume of 0.5 ml of drug or drug combination was administered each time. The initial dose was delivered i.p. at the time of surgery, and supplemental doses were administered by i.p. injection via the implanted catheter. For each drug or combination, the doses were given at 5-hour intervals, three times per day for two days.

3.3 Results

3.3.1 c-Fos mRNA expression

To test the hypothesis that NO and PGs are possible therapeutic targets to potentiate the liver regeneration cascade, the NO donors, SIN-1 and SNAP, or the PGs, PGE_2 and PGI_2, were administered immediately prior to PHX, and c-fos mRNA expression was evaluated. The increase in c-fos mRNA expression after PHX (1.36 ± 0.17, n=5, $p<0.01$ vs sham (0.34 ± 0.06, n=5)) was potentiated by PGI_2 (2.19 ± 0.08, n=4, $p<0.05$ vs PHX) and by the NO donor, SNAP (2.12 ± 0.21, n=7, $p<0.05$ vs. PHX) (Figure 13). There was no potentiation of c-fos mRNA expression by PGE_2 (1.45 ± 0.21, n=7, N.S. vs. PHX) or by the NO donor, SIN-1 (1.12 ± 0.05, n=6, N.S. vs. PHX). In the normal, non-PHX liver, neither PGE_2 (0.27 ± 0.02, n=4), PGI_2 (0.79 ± 0.06, n=6), SIN-1 (0.85 ± 0.06, n=8) or SNAP (0.41 ± 0.09, n=5) caused a significant increase in c-fos mRNA expression. Thus, PGI_2 and SNAP were able to potentiate c-fos mRNA expression, an index of initiation of the liver regeneration cascade, after PHX.

The phosphodiesterase antagonist, Zaprinast (ZAP), which prevents the breakdown of cGMP, was also used as an alternate means of testing the hypothesis that NO is a possible therapeutic target to potentiate the liver regeneration cascade. Compared to sham, PHX caused an increase in c-fos mRNA expression (1.36 ± 0.18, n=7, $p<0.01$ vs sham (0.49 ± 0.06)), and the phosphodiesterase antagonist, ZAP (10 mg/kg), potentiated the increase in c-fos mRNA expression after PHX (2.07 ± 0.14, n=6, $p<0.001$ vs. PHX) (Figure 14). ZAP at a dose of 30 mg/kg tended to potentiate the increase in c-fos mRNA expression after PHX, but the increase was not significant (2.00

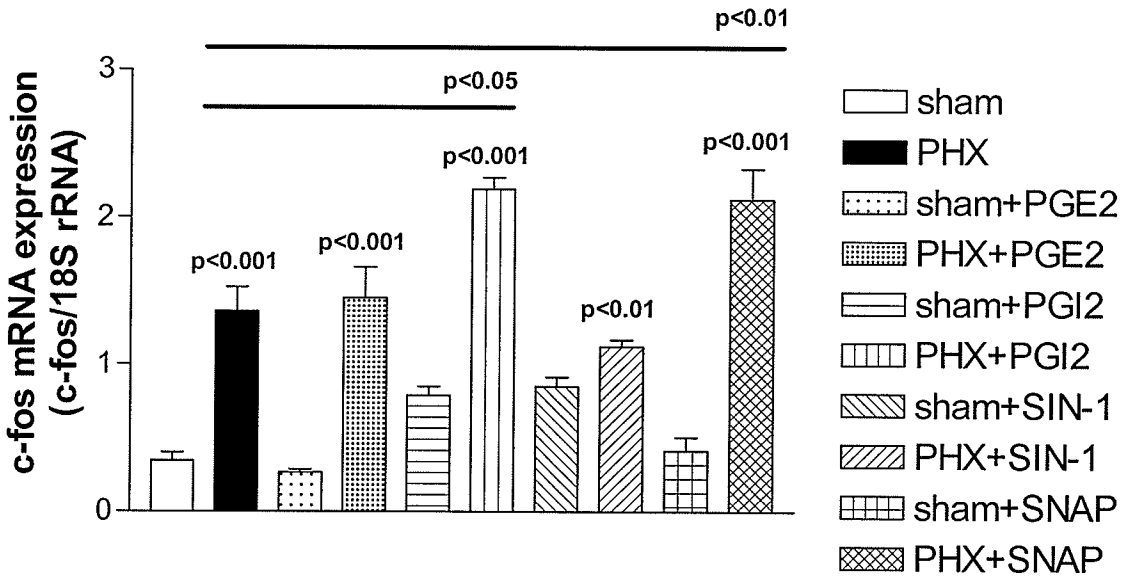

Figure 13: Potentiation of the liver regeneration cascade by NO and PGs. The increase in c-fos mRNA expression, an index of initiation of the liver regeneration cascade, after PHX is further potentiated by PGI_2 and SNAP, and not by PGE_2 and SIN-1. These results provide support for the hypothesis that NO and PGs are possible therapeutic targets for potentiation of the liver regeneration cascade. (PHX, PHX+PGE_2, PHX+PGI_2 & PHX+SNAP $p<0.001$ vs. sham; PHX+SIN-1 $p<0.01$ vs. sham; PHX+PGI_2 $p<0.05$ vs. PHX; PHX+SNAP $p<0.01$ vs. PHX) (data shown as mean $\pm$ SEM)

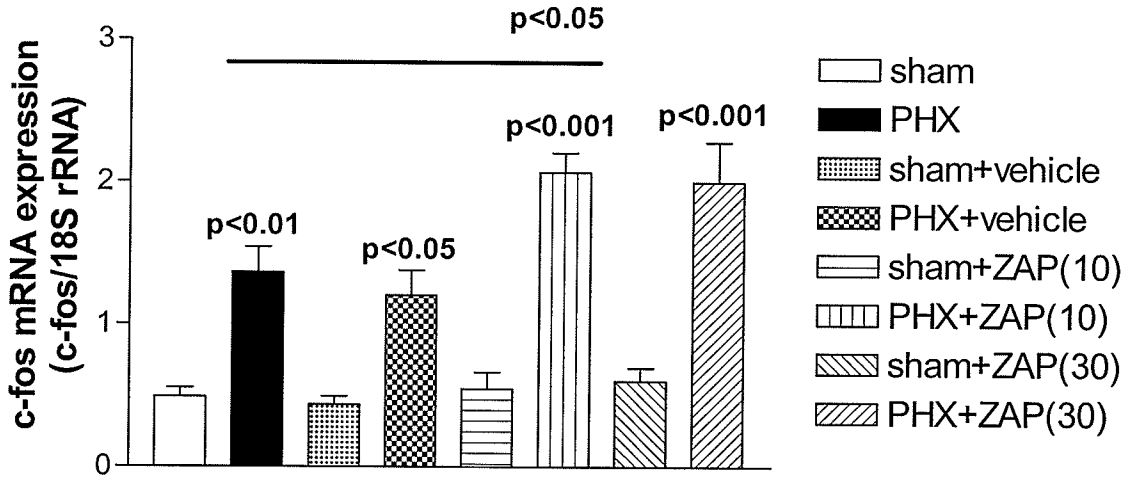

Figure 14: Potentiation of c-fos mRNA expression after PHX by the phosphodiesterase antagonist, Zaprinast (ZAP). ZAP potentiates the increase in c-fos mRNA expression after PHX, providing further support for the hypothesis that NO is a possible therapeutic target to potentiate the liver regeneration cascade. Also, this suggests that ZAP may potentiate liver weight restoration after PHX. (PHX $p<0.01$ vs. sham; PHX+vehicle $p<0.05$ vs. sham; PHX+ZAP(10) & PHX+ZAP(30) $p<0.001$ vs. sham; PHX+ZAP(10) $p<0.05$ vs. PHX). (data shown as mean $\pm$ SEM)

$\pm$ 0.28, n=5, N.S. from PHX). The 0.15 N NaOH and saline vehicle in which ZAP was dissolved had no effect on the increase in c-fos mRNA expression after PHX (1.21 $\pm$ 0.17, n=6; N.S. from PHX). In the normal, non-PHX, liver, neither vehicle (0.44 $\pm$ 0.06, n=6, N.S. from sham), ZAP (10mg/kg) (0.55 $\pm$ 0.12, n=5, N.S. from sham), nor ZAP (30mg/kg) (0.60 $\pm$ 0.09, n=6, N.S. from sham) caused an increase in c-fos mRNA expression. Thus, ZAP is also able to potentiate c-fos mRNA expression after PHX.

Based on the above results, it was hypothesized that combinations of SNAP, PGI_2 and/or ZAP would further potentiate c-fos mRNA expression after PHX. The increase in c-fos mRNA expression after PHX (1.69 $\pm$ 0.10, n=5, $p<0.01$ vs. sham (0.41 $\pm$ 0.06, n=5)) was not further potentiated by the combinations of SNAP+ZAP (5mg/kg, 10 mg/kg) (1.65 $\pm$ 0.20, n=7, N.S. from PHX), SNAP+PGI_2 (5mg/kg, 10ug/kg) (1.93 $\pm$ 0.27, n=8, N.S. from PHX), or ZAP+PGI_2 (10mg/kg, 10ug/kg) (2.09 $\pm$ 0.40, n=6, N.S. from PHX) (Figure 15). However, this index was potentiated by the combination of SNAP, ZAP and PGI_2 (3.35 $\pm$ 0.17, n=6, $p<0.001$ vs. PHX). In the normal liver, neither combinations of SNAP+ZAP (0.85 $\pm$ 0.20, n=7, N.S. from sham), SNAP+PGI_2 (0.84 $\pm$ 0.08, n=8, N.S. from sham), ZAP+PGI_2 (0.85 $\pm$ 0.12, n=7, N.S. from sham), nor SNAP+ZAP+PGI_2 (0.66 $\pm$ 0.10, n=5, N.S. from sham) had any effect on c-fos mRNA expression. Thus, the combination of all three drugs, SNAP, ZAP and PGI_2, potentiated c-fos mRNA expression after PHX.

3.3.2 Liver Weight Restoration

To more fully test the hypothesis that NO and PGI_2 are possible therapeutic targets that can potentiate the liver regeneration cascade, a more physiologically relevant

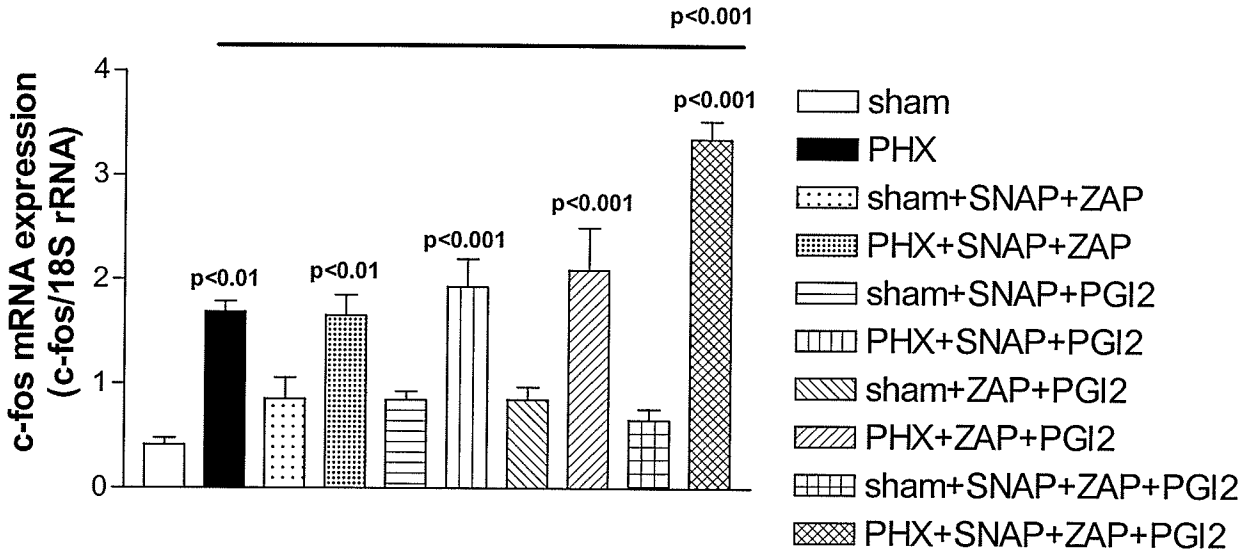

Figure 15: Potentiation of c-fos mRNA expression by the combination of SNAP, ZAP and PGI_2. c-Fos mRNA expression is increased after PHX, and this increase is further potentiated by the combination of the NO donor, SNAP, the phosphodiesterase antagonist, ZAP, and the PG, PGI_2. These results provide evidence that the combination of these 3 drugs potentiate c-fos mRNA expression, an index of initiation of the liver regeneration cascade. In addition, these results also suggest that this combination of drugs may potentiate liver regeneration *in vivo*. (data shown as mean $\pm$ SEM)

index was employed. This index is liver weight restoration after PHX. The drugs SNAP, ZAP, PGI_2, and the triple combination of SNAP, ZAP and PGI_2 were administered to rats immediately prior to PHX, and continuing during recovery after surgery for 48 hours. Forty eight hours after PHX, liver weight was restored by 47.18 ± 1.75% (n=4) (Figure 16). Administration of the phosphodiesterase antagonist, ZAP (10mg/kg, i.p.), as described above, potentiated liver weight restoration to 60.85 ± 2.46% (n=8, $p<0.05$) compared to PHX alone, an increase of 28.97%. Similarly, 6-keto-$PGF_{1\alpha}$ (20ug/kg, i.p.), a stable metabolite of PGI_2, also potentiated liver weight restoration to 61.67 ± 3.19% (n=6, $p<0.05$), an increase of 30.71%, after 48 hours versus PHX only. There was also a trend toward an increase in liver weight restoration 48 hours after PHX following administration of the NO donor, SNAP (5 mg/kg, i.p., n=7) (58.59 ± 2.73%), and following administration of a combination of ZAP (10mg/kg, i.p.), 6-keto-$PGF_{1\alpha}$ (20ug/kg, i.p.) and SNAP (5mg/kg, i.p.) (59.09 ± 3.11%, n=7). Even though these treatments resulted in increases in liver weight restoration of 24.18% and 25.24%, respectively, they were not significantly different from PHX only.

Because both ZAP and 6-keto-$PGF_{1\alpha}$ alone caused an increase in liver weight restoration after PHX, a preliminary experiment was performed in which these two compounds were administered in combination. The combination of ZAP (10mg/kg i.p.) and 6-keto-$PGF_{1\alpha}$ (20ug/kg, i.p.) for 48 hours after PHX potentiated liver weight restoration (73.10 ± 4.50%, n=3, $p<0.001$ vs. PHX only), resulting in a 54.94% increase compared to PHX. However, although the trend is toward further potentiation of liver weight restoration compared to either ZAP or 6-keto-$PGF_{1\alpha}$ only, the increase was not significant. These results suggest that NO and PGI_2, and the administration of ZAP+PGI_2

in combination, can potentiate liver weight restoration after PHX. Thus, these compounds may be of use clinically to potentiate the liver regeneration cascade after surgical resection.

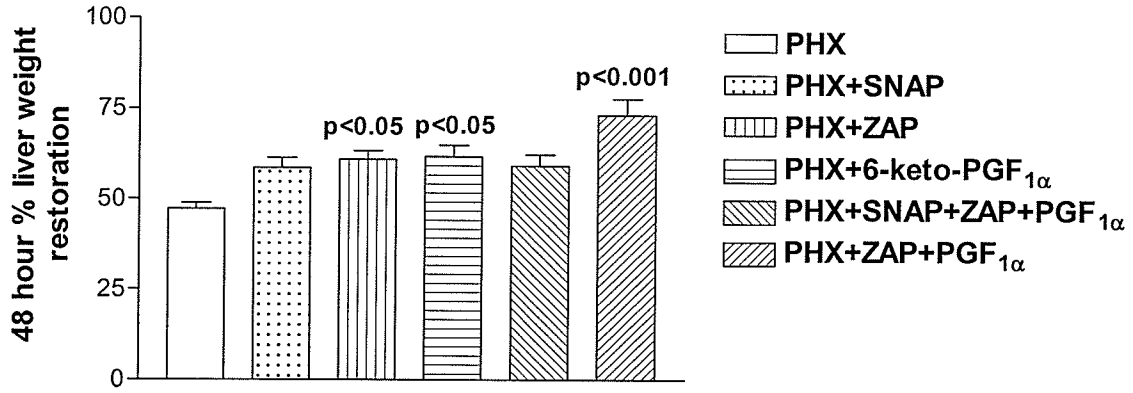

Figure 16: Potentiation of the liver regeneration cascade by ZAP and PGI_2 *in vivo*. Liver weight restoration 48 hours after PHX is enhanced by the phosphodiesterase antagonist, ZAP, by the PG, 6-keto-$PGF_{1\alpha}$, a stable metabolite of PGI_2, and also by the combination of ZAP and 6-keto-$PGF_{1\alpha}$. These results support the hypothesis that NO and PGI_2 are potential therapeutic targets that can potentiate the liver regeneration cascade after PHX. (data shown as mean $\pm$ SEM)

3.4 Discussion

Results indicate that NO and PGI_2 can potentiate the liver regeneration cascade after PHX. The NO donor, SNAP, the PDE V antagonist, ZAP, and PGI_2 potentiated c-fos mRNA expression, an index of initiation of the liver regeneration cascade, after PHX, as did the combination of SNAP, ZAP and PGI_2. These results provide support for the hypothesis that NO and PGs are possible therapeutic targets to potentiate the liver regeneration cascade. In addition, it is interesting that while SNAP, ZAP and PGI_2 administered individually, or as a triple combination, potentiated c-fos mRNA expression after PHX, while combinations of SNAP+ZAP, SNAP+PGI_2 and ZAP+PGI_2 did not. These results require further investigation.

To further test this hypothesis, a more physiologically relevant index was required. C-Fos mRNA expression has proven to be a good index of initiation of the liver regeneration cascade, and has provided excellent information regarding possible therapeutic targets to potentiate liver regeneration. However, liver weight restoration after PHX was chosen as a more physiologically relevant index than c-fos mRNA expression, as liver weight restoration encompasses the entire liver regeneration cascade. The end point evaluated using this index provides information on whether NO or PGs can actually potentiate the liver regeneration cascade *in vivo*, rather than just one pathway involved in liver regrowth.

Liver weight restoration was evaluated 48 hours after PHX, when 50% of liver weight is normally restored in the rat (Higgins and Anderson, 1931). Indeed, 47.18% of the liver weight was restored in rats that underwent PHX only. ZAP and 6-keto-$PGF_{1\alpha}$, a

stable metabolite of PGI_2, caused an increase in liver weight restoration 48 hours after PHX, while SNAP and the combination of SNAP, ZAP and 6-keto-$PGF_{1\alpha}$ also tended to cause an increase in liver weight restoration. In addition, because ZAP and 6-keto-$PGF_{1\alpha}$ caused a significant increase in liver weight restoration 48 hours after PHX, the effect of the combination of ZAP and 6-keto-$PGF_{1\alpha}$ on liver weight restoration was also determined. The combination of ZAP and 6-keto-$PGF_{1\alpha}$ caused a significant increase in liver weight restoration 48 hours after PHX. However, while this increase tended to be greater than either ZAP or 6-keto-$PGF_{1\alpha}$ alone, the difference was not significant. Thus, these results demonstrate that ZAP, PGI_2, or a combination of ZAP+PGI_2 can potentiate liver weight restoration after PHX.

It is also interesting that, while the triple combination of SNAP, ZAP and PGI_2 potentiated c-fos mRNA expression after PHX, the same combination did not cause a significant increase in liver weight restoration. The difference in results could be due to variation between the c-fos mRNA expression and liver weight restoration indices directly. A combination of these 3 compounds may affect an index involving one triggering pathway in a different manner than the effect on the entire liver regeneration cascade. However, these results require further investigation.

3.4.1 Interaction between NO and PGs in disease conditions

Administration of ZAP, PGI_2 or a combination of ZAP+PGI_2 could be of benefit to patients undergoing hepatic resection. ZAP plays a role in dilation of capacitance and resistance vessels in the heart (Ng and Pang, 1998), and has been shown to be effective in the treatment of conditions such as erectile dysfunction (Gibson, 2001). PGI_2 is also

effective in treating chronic pulmonary hypertension, by reducing pulmonary arterial pressure (Abe et al., 2001), and is being tested for use in patients with peripheral vascular conditions (Fink et al., 1999). In addition, it has been shown that the combination of ZAP+PGI_2 can be used to treat some conditions. In patients with pulmonary hypertension, Hill and Pearl (1999) found that inhaled NO and PGI_2, administered together, had an additive effect on decreasing pulmonary arterial pressure. Also, Ziesche et al. (2000) demonstrated that in patients with pulmonary hypertension, who have become refractory to NO treatment, intravenous epoprostenol (PGI_2 analog) administration for 13 to 29 months eliminated the refractoriness to NO treatment. The authors suggest that treatment with NO and epoprostenol might further improve oxygen saturation, cardiac index, mean pulmonary artery pressure and total pulmonary vascular resistance. PGI_2 has been administered to patients undergoing orthotopic liver transplant, and it was found that in patients who received PGI_2, liver oxygenation was increased and liver damage decreased (Neumann et al., 2000). In addition, PGI_2 may play a role in preservation of liver grafts for transplantation, after removal from the donor (Kishida et al., 1997). Thus, administration of either NO, a PDE V antagonist, or PGI_2 may play an important role in treating symptoms of some disease conditions. In addition, administration of NO and/or ZAP and/or PGI_2 in combination may further potentiate the effects of these compounds.

3.4.2 Potentiation of liver regeneration by NO and PGI_2

There has been no suggestion in the literature of administering a combination of ZAP and PGI_2 to patients undergoing liver resection to potentiate liver regeneration. The

experiments described above are the first results suggesting that therapy with ZAP, which increases the effects of the NO signaling cascade, PGI_2, or a combination of both compounds will potentiate liver regeneration after surgical resection, and they provide a potential option for patients whose liver must regenerate. Further research is required to determine the optimal dose and dosing schedule to potentiate liver regeneration, but results obtained in this study provide an excellent starting point and the potential for a new and exciting therapy to improve liver regeneration.

3.5 Summary

The increase in c-fos mRNA expression, an index of initiation of the liver regeneration cascade, after PHX was potentiated by the NO donor, SNAP, the PDE V antagonist, ZAP, and PGI_2, and by the combination of SNAP, ZAP and PGI_2. These results support the hypothesis that NO and PGs, alone or in combination, can potentiate the liver regeneration cascade. An additional index, liver weight restoration, which encompasses all the pathways, growth factors and events in the liver regeneration cascade, was also used. Liver weight restoration 48 hours after PHX was potentiated by ZAP and 6-keto-$PGF_{1\alpha}$, a stable metabolite of PGI_2. In addition, the combination of ZAP and 6-keto-$PGF_{1\alpha}$ also potentiated liver weight restoration after PHX, in a manner that suggested that combination of these two compounds could further increase liver regeneration beyond either ZAP or 6-keto-$PGF_{1\alpha}$ alone. These results provide the first evidence that ZAP, PGI_2 or a combination of the two, are potential therapeutic targets to potentiate the liver regeneration cascade after surgical resection.

Conclusions and Future Directions

4.1 Conclusions

Results presented in this thesis provide support for the hypothesis that an increase in the blood flow-to-liver mass ratio following PHX causes shear stress and the release of NO, which triggers the liver regeneration cascade. There is a similar increase in PVP, and therefore in shear stress, in the liver after both PHX and PVL, suggesting similar hemodynamic conditions in both models. The PVL model was used to further test the hypothesis that a hemodynamic event triggers the liver regeneration cascade. Results obtained using c-fos mRNA expression as a valid index of initiation of the liver regeneration cascade demonstrated that a reduction in blood flow to the liver by two-thirds prior to PVL prevented the increase in c-fos mRNA expression after PVL. This indicates that a hemodynamic factor is responsible for the increase in c-fos mRNA expression in the liver regeneration cascade. In addition, inhibition of NOS also prevented the increase in c-fos mRNA expression after PHX and PVL. This inhibition was reversed by the NO donor, SIN-1, which supports the hypothesis that NO triggers the liver regeneration cascade, secondary to a hemodynamic event. Also, results are consistent with the hypothesis that the NO that triggers the liver regeneration cascade is produced by eNOS, the constitutive isoform of NOS, in response to shear stress.

The increase in c-fos mRNA expression after PHX is inhibited by either NOS or COX antagonists, and in either situation, the NO donors, SIN-1 or SNAP, or the PGs, PGE_2 or PGI_2, can reverse the inhibition. This suggests that c-fos mRNA expression after PHX requires both NO and PGs. However, in the absence of either NO or PG synthesis, administration of an excess amount of either exogenous NO or PGs seems to

be able to compensate for the absent compound. These results support the hypothesis that NO and PGs are released secondary to an increase in the blood flow-to-liver mass ratio in the remaining liver, and that NO and PGs work together to trigger the liver regeneration cascade.

The increase in c-fos mRNA expression after PHX was potentiated by the NO donor, SNAP, the PDE V antagonist, ZAP, and PGI_2, and by the combination of SNAP, ZAP and PGI_2. These results support the hypothesis that NO and PGs, alone or in combination, can potentiate the liver regeneration cascade. Liver weight restoration, which encompasses all the pathways, growth factors and events in the liver regeneration cascade, 48 hours after PHX was potentiated by ZAP and 6-keto-$PGF_{1\alpha}$, a stable metabolite of PGI_2, and by the combination of ZAP and 6-keto-$PGF_{1\alpha}$. These results support the hypothesis that NO or PGs can potentiate the liver regeneration cascade, and provide the first evidence that ZAP, PGI_2 or a combination of the two, are potential therapeutic treatments to potentiate the liver regeneration cascade after surgical resection.

4.2 Future Directions

Future experiments would include determination of the optimal dose, dosing schedule and route of administration of ZAP, 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, or ZAP+6-keto-$PGF_{1\alpha}$. These experiments would be performed in rats using the liver weight restoration index. First, the optimal dose, dosing regimen and route of administration would be determined. The 48-hour time point would be used for the initial experiments to allow comparison to the results obtained above. Alternate routes of administration would include intravenous (i.v.) and intraportal (i.p.v.) infusion. Using

the intraperitoneal (i.p.) route described above, and the intravenous routes, the efficacy of a bolus injection versus an infusion should be evaluated. An infusion would be maintained using an osmotic pump implanted subcutaneously, and a catheter would be implanted i.p. for intraperitoneal infusions and i.v. for intravenous infusions. The i.p.v. route would be evaluated using an osmotic pump with continuous infusion, implanted into the portal vein. It is hypothesized that a constant infusion directly into the portal vein or intraperitoneally, will provide the greatest stimulus for potentiation of the liver regeneration cascade.

The potentiation of the liver regeneration cascade over time should also be evaluated. This could also be done using the liver weight restoration index. Once the optimal dose and route of administration are determined, liver weight restoration would be determined at 24, 72 and 96 hours, and also at 7, 10 and 14 days after PHX using ZAP, 6-keto-$PGF_{1\alpha}$, or ZAP+6-keto-$PGF_{1\alpha}$. It is hypothesized that ZAP, 6-keto-$PGF_{1\alpha}$ and ZAP+6-keto-$PGF_{1\alpha}$ will potentiate liver weight restoration, and cause complete liver regeneration within 7 days, and that the combination of ZAP+6-keto-$PGF_{1\alpha}$ will result in the fastest liver weight restoration after PHX.

In addition, the function of the liver must be evaluated. Serum concentrations of bilirubin, alanine aminotransferase (ALT), and aspartate aminotransferase (AST), indicators of liver injury, should be evaluated at various time points after PHX. The effect of ZAP, 6-keto-$PGF_{1\alpha}$ and ZAP+6-keto-$PGF_{1\alpha}$ treatment on liver function after PHX should be evaluated. It is hypothesized that all 3 treatments will result in improved liver function, along with increased liver weight restoration, after PHX.

The mechanism of initiation of the liver regeneration described above has been determined using a model of surgical removal of liver tissue. This model induces liver regeneration without prior toxic or inflammatory damage to the liver tissue. However, there are other instances where the liver regenerates in the absence of surgical resection. These include liver injury, due to inflammation and/or toxins, and diseases of the liver, such as cirrhosis. A future goal for this research is to apply treatments and mechanisms determined in the non-injured or non-diseased liver (i.e. after PHX) to conditions involving inflammation and liver disease.

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