ANIMAL MODELS FOR THE STUDY OF LIVER REGENERATION: ROLE OF NITRIC OXIDE AND PROSTAGLANDINS.

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1. ABSTRACT

The mechanisms that permit adult tissues to regenerate are the object of intense study. Liver regeneration is a research area of considerable interest both from pathological and from physiological perspectives. One of the best models of the regenerative process is the two-thirds partial hepatectomy (PH). After PH, the remnant liver starts a series of timed responses that first favor cell growth and then halts hepatocyte proliferation once liver function is fully restored. The mechanisms regulating this process are complex and involve many cellular events. Initiation of liver regeneration requires the injury-related cytokines tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6), and involves the activation of cytokine-regulated transcription factors such as NF-κB and STAT3. An important event that takes place in the hours immediately after PH is the induction of nitric oxide synthase 2 (NOS-2) and cyclooxygenase 2 (COX-2), and the consequent release of nitric oxide (NO) and prostaglandins (PGs). NO is involved in the vascular readaptation after PH, favoring a general permeability to growth factors throughout the organ . This review examines the mechanisms that regulate NO release during liver regeneration and the animal models used to identify these pathways.

2. INTRODUCTION

Liver regeneration is a complex, evolutionarily conserved process directed at restoring liver mass after liver injury by toxic and xenobiotic compounds or after surgical resection. In humans, liver regeneration occurs most frequently after liver damage by ischaemia or hepatitis. This regenerative capacity of the liver has provided new treatment options for patients with liver damage, and in recent years the use of partial livers from living donors has greatly increased. In this context, understanding the molecular bases of liver regeneration might enable us to explain aspects of clinical liver disease that require liver regeneration, and could lead to the development of new pharmacological therapies and surgical approaches.

3. PARTIAL HEPATECTOMY AS A MODEL OF LIVER REGENERATION

The rodent PH model has been used extensively for the study of liver regeneration (1). In this model, two thirds of the liver are surgically removed, and the remaining liver initiates a series of timed responses that first favor cell growth and inhibit apoptosis, and later halt hepatocyte proliferation once the original liver mass and liver function are restored (2-5). Under these conditions, liver regeneration is accomplished by proliferation of all existing mature cell populations resident in the remaining organ. These populations include hepatocytes, biliary epithelial cells, fenestrated endothelial cells and Kupffer and Ito cells. Nonetheless, hepatocytes are the first cells to proliferate (6), which suggests that these cells provide the mitogenic stimuli for proliferation of the other cell types (7-9).

Rapid changes in gene expression and the activation of receptors and transcription factors occur immediately after PH (6,10). Indeed, it is estimated that ~70 genes increase their expression in the period immediately after PH (11). The signaling pathways underlying the early priming phase of liver regeneration are thought to be triggered by the synergistic effect of a wide array of stimuli released into the portal circulation, including cytokines (12), prostaglandins (13), hormones (6), reactive oxygen species (14), and lipopolysaccharides (15). This complex network acts in an orderly manner,

involving cytokines (TNF-α and IL-6) and growth factors (hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factors (TGFs), insulin and glucagons, as well as their downstream transcription factors STAT3 and nuclear factor NF–κB (figure 1).

3.1. Use of modified mouse models in liver regeneration studies.

The sequence of events triggering liver regeneration after acute loss of hepatic mass has been extensively studied in modified mouse models: transgenic mice that overexpress specific genes and knockout animals with functional inactivation or gene deletion. These models have contributed to a better knowledge of the mechanisms that initiate liver regeneration and the genes and proteins that are rapidly activated in the remnant liver after partial hepatectomy. However, these models do not always provide accurate information about the crucial role of genes because signaling pathways involved in liver regeneration are coordinated and redundant; and in general, deletion or inactivation of a gene delays but does not impair regeneration. Indeed, few models have been described in which liver regeneration after PH is impaired to the extent to promote animal death.

3.1.1. Cytokine-dependent pathways

Cytokines play a prominent role in the initiation of liver regeneration. A rapid release of TNF-α (16) and IL-6 (17) has been observed after PH, leading to the activation of cytokine-regulated transcription factors such as NF-κB (18) and STAT-3 (19) in the liver remnant. Studies by Yamada et al. with knockout mice lacking either TNF receptor 1 (TNFR-1) or receptor 2 (TNFR-2) demonstrated that TNFR-1, but not TNFR-2, is necessary for liver regeneration (20-22). Indeed, mice lacking TNFR-1 show defects in DNA synthesis after PH, and decreases in IL-6 synthesis and DNA binding by NF-κB and STAT-3 transcription factors. Furthermore, experiments with IL-6-deficient animals demonstrate that IL-6 is a critical component of the regenerative response: after PH, IL-$6^{-/-}$ mice had impaired liver regeneration characterized by liver necrosis and failure (23), and a significantly impaired activation of STAT3 and NF-κB.

The production of IL-6 and TNF-α is mediated, at least in part, by the innate immune system. Release of IL-6 from Kupffer cells can be triggered by C5a (a complement component) in concert with LPS, thereby mediating the expression of acute-phase genes in cultured hepatocytes (24). Moreover, C3a is can modulate prostaglandin synthesis (25) and alter production of IL-6, TNF-α, and IL-1β (26-28). Results from C3 or C5 knockout mice demonstrate the essential role of complement in liver regeneration. After PH, these mice show a diminished liver regeneration accompanied by transient or fatal liver failure; and in mice with dual C3 and C5 deficiency the phenotype is more exacerbated (29,30). Additionally, inhibition of C5a receptor signaling suppresses IL-6/TNF-α induction; and lack of C3 and C5a receptor stimulation attenuates NF-κB/STAT3 activation after hepatectomy.

3.1.2. Growth-factor-pathways activated during liver regeneration.

In addition to cytokines, growth factors regulate liver regeneration by providing both stimulatory and inhibitory signals for cell proliferation. Among the extracellular growth factors shown to induce hepatocyte proliferation, hepatocyte growth factor (HGF/SF), transforming growth factor α (TGF-α), and epidermal growth factor (EGF), as well as their receptors, have been particularly well studied. These growth factors stimulate DNA synthesis in hepatocytes, and are strong candidates for important roles in liver regeneration (31,32).

HGF is a potent mitogen for a variety of cells, including hepatocytes. HGF is synthesized by non-parenchymal cells, particularly stellate cells, and activates the receptor tyrosine kinase Met and various downstream pathways, including those that involve PI3K, ERK, S6 kinase and AKT (33,34). Gene knockout of HGF (35,36) or its receptor, Met, is embryonic lethal (31):(32) these animals die *in utero* between embryonic days 13.5 and 16.5 with multiple abnormalities, including signs of underdeveloped liver. Therefore, in order to determine the contribution of these factors to liver regeneration, studies have been conducted ina liver-specific conditional knockout of Met. The results of these studies demonstrate that the HGF–Met pathway is important for DNA synthesis after liver injury (31).

The initiation of liver regeneration by HGF first requires the activation of HGF from its precursor, pro-HGF, by proteases such as uPA (urokinase-type plasminogen activator) (37,38). In mice deficient in uPA the appearance of HGF is delayed, which in turn delays liver regeneration (39). However, liver regeneration is unaffected in mice deficient in the uPA receptor (uPAR), indicating that whatever role uPA may play in liver regeneration it does not require binding to uPAR.

In the regenerating liver, TGF-α is expressed in hepatocytes a few hours after the HGF surge in the circulation, but before the start of DNA synthesis. In spite of the strong correlative evidence linking TGF-α expression and hepatocyte proliferation, TGF-α knockout mice grow normally and have no deficit in liver regeneration (36). TGF-α is a member of the epidermal growth factor family, and shares homology with EGF. These growth factors shares a common receptor, the EGF receptor (EGFR) (40); and it is possible that liver regeneration requires activation of the EGFR *per se*, but that the specific identity of the ligand is unimportant. However, mice with a null mutation in the EGFR(41) die either at the blastocyst stage or *in utero* from placental failure, and so cannot provide information on the requirement for EGFR signaling in liver regeneration. Recent results have demonstrated that HB-EGF (heparin-binding epidermal growth factor-like growth factor) may be an important factor in PH. HB-EGF is produced earlier after PH than HGF, and acts by a paracrine

mechanism (42). HB-EGF knockout mice manifest a delay in DNA replication after PH (43).

3.1.3. Transcription factors.

The activation of growth factor and cytokine signaling pathways after PH induces the activities of several transcription factor complexes. These include NF-κB and STAT3 (which are both strongly induced by TNF-α), AP-1, c-Myc, CREM and C/EBPβ (CCAAT/enhancer-binding proteins). These transcription factors play major roles in the initiation of liver regeneration (11,18,19).

NF-κB activation is particularly rapid, occurring within 30 minutes after PH. NF-κB activates multiple target genes that have an NF-κB recognition sequence, such as the gene for IL-6, which itself causes activation of STAT3. Blockage of NF-κB activity, by infecting livers of rats with an adenovirus expressing a truncated form of IκB (44), has a major apoptotic effect in the regenerating liver.

STAT3 is ubiquitously expressed and transiently activated by a variety of different ligands, including IL-6, leukemia-inhibitory factor (LIF), EGF, and platelet-derived growth factor (PDGF), as well as by a number of oncogenic receptor and non-receptor tyrosine kinases (45-49). STAT3 plays a crucial role in the embryonic development of various organs and in cell proliferation (49). Knockout of *Stat3* is embryonic lethal, so experiments have been conducted with liver-specific *Stat3* knockouts. These experiments demonstrate that STAT3 promotes cell-cycle progression and cell proliferation under physiological growth conditions.

The expressions and activities of several C/EBP isoforms fluctuate in the regenerating liver. Among them, increased expression of C/EBPβ after PH seems to indicate an important role in liver regeneration. Indeed, Greenbaum *et al.* have presented evidence showing that C/EBPβ knockout mice exhibit impaired hepatocyte proliferation and decreased liver regeneration after PH (50).

The DNA binding activity of AP-1 is rapidly induced in response to PH (51). AP-1 is a heterodimeric sequence-specific transcription factor most commonly composed of the products of the *jun* and *fos* families of genes. AP-1 activity is increased both by growth factor and by cytokine signaling pathways, although through different mechanisms. A TNF-α/IL-6-dependent signal stimulates c-Fos (6), and c-Jun is stimulated by a second mechanism, possibly emanating from growth factor receptors. JNK activity increases after PH; and, in contrast to c-*fos* (knockout animals deficient in this gene have a normal regenerative response), c-*jun* is likely to be important for hepatocyte proliferation. However, study of c-Jun's role is not possible in standard knockout models: fetuses lacking c-*jun* die at mid-gestation with defects in heart morphogenesis and increased apoptosis of both hepatoblasts and hematopoietic cells in the fetal liver (52). To bypass this embryonic lethality, conditional mice have been developed to suppress c-*jun* expression in adult mice (53). These experiments show that c-Jun is a critical regulator of hepatocyte proliferation and survival after liver injury.

4. ROLE OF NOS-2 AND COX-2 IN LIVER REGENERATION

The rapid activation of transcription factors induces the expression of key enzymes that control the regenerative process downstream of this point. Among these enzymes are NOS-2 (6,18,54), which catalyses the formation of NO, and cyclooxygenase-2 (COX-2), which catalyzes the rate-limiting step in the synthesis of prostaglandins (PGs). Both proteins are expressed after PH in the remnant tissue, and serum levels of PGE_2 increase. PGs produced by COX-2 are important for the early steps of liver regeneration, participating in the regulation of the cell cycle after PH.

4.1. Regulation of liver regeneration by NO.

NOS-2 is expressed after PH by hepatocytes and by Kupffer cells, and the NO released plays a regulatory role in liver regeneration (54,55). Indeed, inhibition of NOS-2 delays early cell-cycle progression after PH (54). NO release seems to be a local effect, because hepatic NOS activity and levels of NOS mRNA have been detected exclusively in liver. Although the relative contributions of each liver cell type to NO synthesis differ (Kupffer cells produce more NO than hepatocytes), the origin of NO is not critical, since it can diffuse through the cells. NOS-2 levels are mainly controlled at the transcriptional level; and activation of NF-κB has been reported to be essential for NOS-2 transcription (56).

Several animal models have been used to study the role of NO in liver regeneration. Experiments in NOS-2 knockout mice demonstrated that hepatocytes undergo apoptosis and necrosis instead of proliferation when the expression of NOS-2 is prevented in the regenerating liver (57). The PH-induced NOS-2 expression occurs preferentially in hepatocytes (54,55); and the NO released protects regenerating liver cells from the pro-apoptotic effects of increased TNF-α concentration and endotoxemia (57-59) by S-nitrosylating procaspases and active caspase enzymes (60).

Two innovative genetic strategies have been used recently to study the protective role of NO during liver regeneration. Both strategies allow controlled delivery of NO to liver. In the first approach animals express a NOS-2 transgene under the control of the liver-specific phospho(enol) pyruvate carboxykinase promoter (PEPCKNOS2 animals). The expression of this gene, and therefore NO release, is triggered under fasting conditions. The second approach involves the hydrodynamics-based in vivo transfection of animals with a plasmid encoding NOS-2 (61).

4.1.1. PEPCKNOS2 animals

Endogenous production of NO in hepatocytes by expression of an NOS-2 transgene

under the control of the PEPCK promoter (58) is one of the most interesting models for the study of liver regeneration. This model has several advantages. First, NO is specifically and endogenously generated in liver, thus avoiding the many undesirable side effects due to the as yet unresolved difficulty of selective delivery of exogenous NO to the organ. Second, NO synthesis is triggered by a simple process that is independent of proinflammatory stimulation. Finally, because the PEPCK promoter is activated in the postnatal period, interference in the course of animal development is avoided.

With this approach, the expression of NOS-2 in transgenic (Tg) animals, which is undetectable in fed animals, is observed after starvation for 24 h (figure 2). This expression in fasted animals promotes a moderate change in parameters related to oxidative stress, but does not significantly affect life-span, an nor does it involve other systemic alterations of the normal behavior of the Tg animals with respect to the corresponding wild-types. Apart from minor expression in kidney, NOS-2 is not expressed in other organs of PEPCKNOS-2 Tg mice. These data suggest that moderate generation of NO in the liver has no noticeably deleterious effects on the organ.

Study of fasted PEPCKNOS-2 Tg animals shows that preexistent synthesis of NO in the hepatocyte protects against lipopolysaccharide (LPS)-induced liver injury (58). The mechanisms that mediate this protection seem to include the inhibition of NF-κB activation and the impairment of the release of TNF-α, IL-1β,. and presumably of other proinflammatory cytokines whose expression is dependent on NF-κB activity (62). However, preexistent synthesis of NO, derived from expression of the PEPCKNOS-2 transgene impairs the later expression of NOS-2 from the endogenous gene after PH. This delays subsequent early signaling and liver mass recovery, although there is no effect on mouse survival (63). The mechanism underlying this delay in liver mass recovery in Tg mice involves impairment of the degradation of IκB-α and a consequently lowered NF-κB activation. STAT3 phosphorylation and the amounts of cytokines (TNF-α and IL-6) are also significantly reduced in these animals .The local presence of NO before PH also delays hepatocyte proliferation through an inhibition of the cell cycle proteins cyclin E, cyclin D1, and PCNA.

In spite of its antiproliferative and cytostatic effects, NO efficiently protected hepatocytes from apoptosis in Tg animals. These hepatoprotective and antiapoptotic effects of NO have been previously described in several pathological situations such as carbon tetrachloride-induced hepatic injury (64) or Fas-dependent apoptosis, which plays a major role in the pathogenesis of immuno-mediated liver diseases such as viral hepatitis and acute liver failure (65-67).

4.1.2. Hydrodynamics-based transfection in mice

The second approach to liver-targeted NO delivery consists of an *in vivo* transfection of NOS-2 directly to the liver by the hydrodynamics-plasmid delivery procedure (61). By using GFP-NOS-2 cDNA as a reporter gene, an efficient gene transfer and expression can be achieved by a rapid injection of a large volume of DNA solution into animals via the tail vein. The procedure results in a marked expression of the transfected gene in various major organs including the liver, kidney, lung, heart and spleen. Among these, the highest expression is in the liver, with approximately 40 percent of hepatocytes expressing the transfected gene. The reason for this is the accumulation of the injected DNA solution in the inferior vena cava, which produces a high hydrostatic pressure that forces the flow of a large portion of DNA solution into the liver in a retrograde direction. The time-response curve shows that the level of transfected gene expression in the liver peaks approximately 8 h after injection and decreases thereafter. Peak gene expression can be regained by repeated injection of plasmid DNA. The hydrodynamics-based procedure causes no serious liver damage: a transient increase in serum transaminases has been detected, but the levels of these enzymes return to normal values within few days (61).

The hydrodynamics-based transfection model has been used to determine the role of sustained presence of NO before and during PH. The NOS-2 transfected animals show a response similar to that observed in the PEPCK-NOS-2 Tg mice, with a pattern of delayed cell cycle protein expression with respect to controls. Moreover, a significant protection against Fas-dependent apoptosis (detected as reduced activation of caspases) is triggered by the transient expression of NOS-2.

The two experimental models outlined above highlight the importance of correct regulation of NOS-2 expression in the regenerating liver. Although the role of NO in liver regeneration is essentially protective – favoring hepatic circulation and contributing to the angiogenic activity in the remnant tissue – the presence of NO before liver injury prevents the normal expression of the endogenous NOS-2 gene after injury, thus inhibiting liver mass recovery. To be effective, NOS-2 expression must be timed to release NO at the appropriate moment (figure 3).

4.2. Involvement of prostaglandins in liver regeneration

Liver regeneration is also characterized by an altered pattern of the expression of prostaglandins (PGs). Indeed, an accumulation of PGE_2 has been described in the sera of animals after PH. PGs are synthesized by the cyclooxygenase isoenzymes, COX-1 and COX-2. COX-2, the inducible isoform, may contribute to liver damage and tumorigenesis in several animal models. Expression of COX-2 has been demonstrated in liver regeneration after PH, with a maximal expression after 16 h (2). The expression of COX-2 in regenerating liver is accompanied by a decrease in the level of C/EBP-α and an increase in the expression of C/EBP-β and C/EBP-δ. Inhibition of COX-2 activity, either pharmacologically with NS398 or by gene knockout,

impairs liver regeneration (68) and alters various parameters of cell-cycle progression (PCNA levels, cyclins E and D1) (69).

4.3. Lethal failure of liver regeneration after simultaneous abrogation of NOS-2 and COX-2.

Given the importance of NO and PGs during liver regeneration, the simultaneous suppression of NOS-2 and COX-2 activities is potentially lethal. COX-2 knockout has severe systemic defects, and animals die early. A double knockout approach is therefore unsuitable for the analysis of the combined contribution of COX-2 and NOS-2 to liver regeneration. In place of this, a pharmacological approach using COX-2 inhibitors has been used in NOS-2 knockout animals. Simultaneous suppression of the activities of NOS-2 (by gene knockout) and COX-2 (by pharmacological inhibition) results in an imbalance between regenerating cells and cells undergoing apoptosis (70). This results in massive hepatocyte death, which can be prevented by the exogenous supply of NO or the administration of the broad caspase inhibitor z-VAD, allowing animals to regenerate the liver (figure 4).

5. SUMMARY AND PERSPECTIVE.

The experimental findings reviewed here clearly show that NO and PGs have a profound effect on the liver's ability to regenerate after it has been damaged, and suggest that regeneration requires the induction of factors that protect proliferating cells from death. Principal among these is NO; therefore the development of strategies for local delivery of NO to the liver is an area of great interest for the therapeutic treatment of several hepatopathies.

However, neither NO nor PGs are essential, at least independently, for liver regeneration after PH: other factors can compensate for NOS-2 or COX-2 deficiency. These findings illustrate that the signaling pathways involved in liver regeneration are coordinated and redundant, so that, in general, more than one signal must be inactivated for the effect to be lethal.

The increased use of transgenic and knockout mice has undoubtedly contributed major advances to the understanding of the mechanisms of liver regeneration. Gene manipulation in mice with new technologies that allow conditional and tissue-specific expression of genes will continue to advance knowledge of the mechanisms that regulate and promote regeneration. However, given the redundancy of signaling pathways involved in liver regeneration, strategies directed at simultaneous deletion of two or more genes must be considered.

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Abbreviations: Partial hepatectomy, PH; tumor necrosis factor-α, TNF-α; interleukin 6, IL-6; nuclear factor κ B, NF-κB; hepatocyte growth factor, HGF; epidermal growth factor, EGF; transforming growth factor α, TGF-α; uPA, urokinase-type plasminogen activator; CCAAT/enhancer-binding proteins, C/EBP; phospho(enol)pyruvate carboxykinase, PEPCK; nitric oxide synthase, NOS; nitric oxide, NO; cyclooxygenase, COX; prostaglandins, PGs, Tg, transgenic.

Key Words: partial hepatectomy, nitric oxide, prostaglandins, liver regeneration, apoptosis, animal models.

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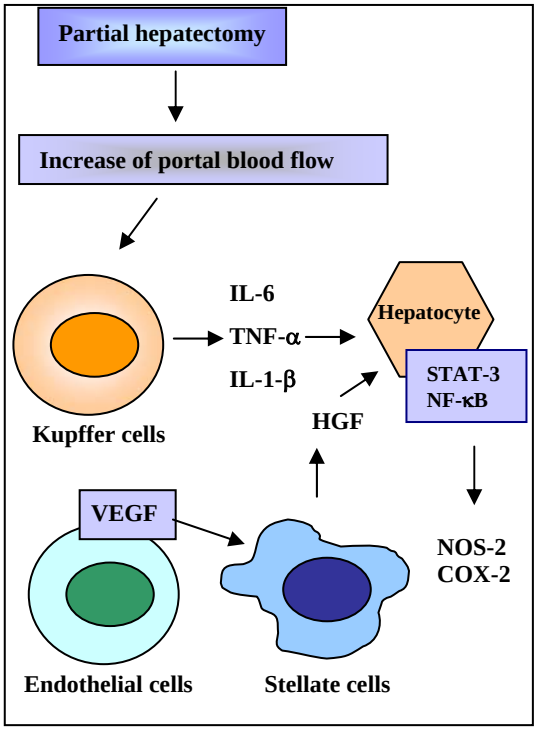

Figure 1. Signaling pathways activated during liver regeneration. After PH or liver injury, several signals are initiated simultaneously in the liver. Cytokine-regulated and growth-factor-pathways are activated, leading to the binding of VEGF to endothelial cells, which triggers the release of HGF from stellate cells, and to the activation of Kupffer cells that release cytokines such as TNF-α, IL-1β and IL-6. Cooperative signals from these stimuli activate transcription factors (NF-κB and STAT3) and, in turn, the expression of genes whose transcription is κB-dependent, such as NOS-2 and COX-2. For a more detailed revision of early signaling involved in liver regeneration see reference 5.

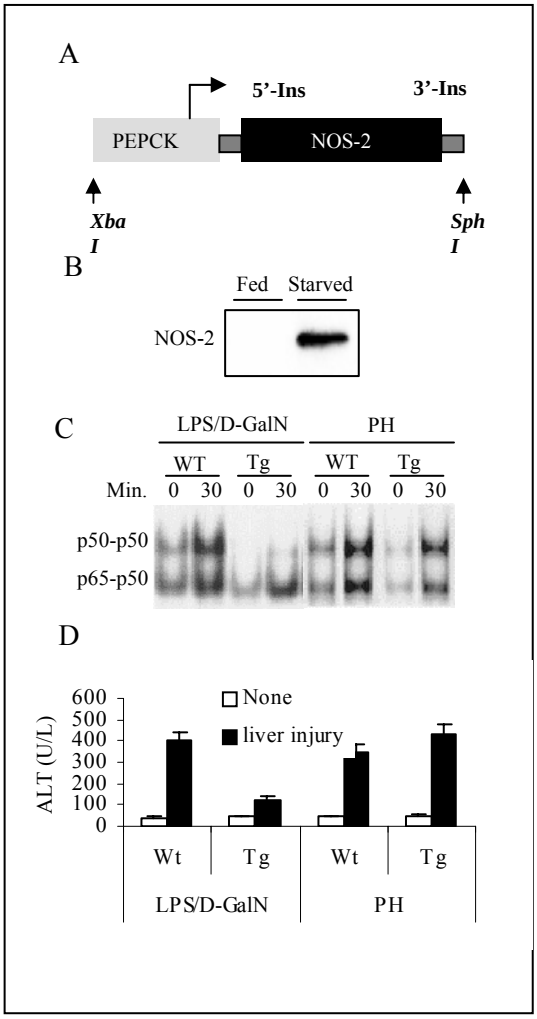

Figure 2. Effects of sustained NO synthesis on liver regeneration. (A) 6.2-kb fragment containing the NOS-2 gene under the transcriptional control of the PEPCK promoter (PEPCKNOS-2). **(B)** Western blot of liver extracts showing the expression of NOS-2 in PEPCKNOS-2 transgenic (Tg) animals fasted for 24 h. **(C)** EMSA showing activation of NF-κB after liver injury (by administration of LPS/D-GalN or resection by PH) in wild-type (WT) and Tg mice**. (D)** Liver injury caused by these treatments, detected as serum levels of alanine aminotransferase (ALT).

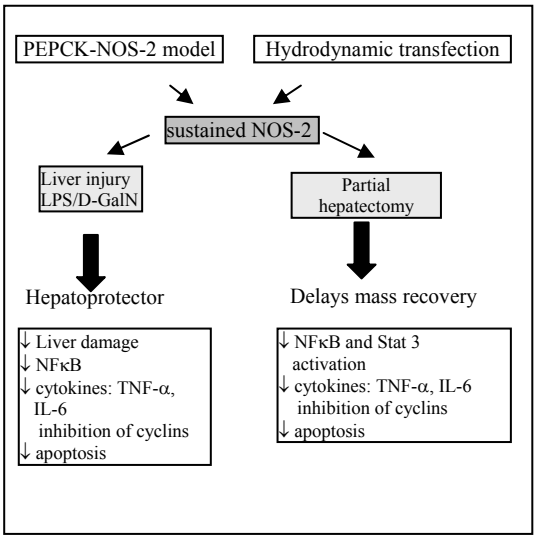

Figure 3. Dual action of NO after liver injury. Sustained NOS-2 expression in liver can be achieved by two experimental delivery methods: the PEPCK-NOS-2 model; and the hydrodynamic transfection procedure. The presence of NO before and after liver injury (LPS/D-GalN or PH) exerts different effects via a common mechanism. NO inhibits activation of NF-κB, cytokine release, and apoptosis, leading to the protection against LPS/D-GalN injury; however, preexisting NO delays liver mass recovery after PH.

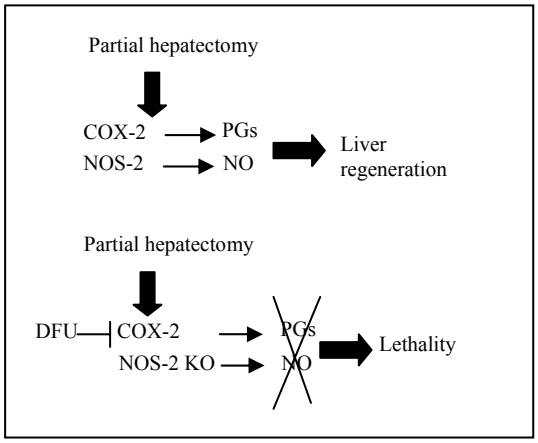

Figure 4. Simultaneous abrogation of NOS-2 and COX-2 activities is lethal after PH. After PH several signaling pathways are activated. Important among these events are increased expression of NOS-2 and COX-2, which release NO and PGs, respectively. When these enzymes are simultaneously inhibited through pharmacological inhibition of COX-2 activity with DFU and gene knockout (KO) of NOS-2, liver mass recovery is prevented, resulting in death.