GENE EXPRESSION AND CELL SIGNALING DURING HEMORRHAGIC SHOCK: BENEFICIAL EFFECTS OF PICROLIV AND CURCUMIN

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CERTIFICATE

This is to certify that the thesis entitled " Gene Expression and Cell Signaling During Hemorrhagic Shock: Beneficial Effects of Picroliv and Curcumin" submitted by Shirin Sundar ID.No.94PF29228 for award of Ph.D degree of the Institute, embodies original work by her under my supervision.

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AP-1 – activator protein 1

ATF-2 - activating transcription factor-2

ALT – alanine transaminase

AST – aspartate transaminase

ATP – adenosine triphosphate

COX-2 – cycloxygenase-2

Egr-1 - early growth response-1

GADD45 - growth arrest and DNA-damage inducible 45

GAPDH - glyceraldehydes-3-phosphate dehydrogenase

GPx – glutathione peroxidase

GR – glutathione reductase

Grp78 - glucose regulated protein 78

GSH – glutathione

H/R – hemorrhage/resuscitation

HSF-1 – heat shock factor-1

HSP – heat shock protein

IEG - immediate early gene

IL – interleukin

iNOS – inducible nitric oxide synthase

LR – lactated Ringer's solution

MAP – mean arterial pressure

MAPK - mitogen activated protein kinase

MDA - malondialdehyde

MOF – multiple organ failure

NF-κB – nuclear factor kappa B

NO – nitric oxide

ROS – reactive oxygen species

RNS - reactive nitrogen species

SEM – standard error of mean

SOD – superoxide dismutase

TNF-α - tumor necrosis factor alpha

TRPM-2 - testosterone repressed prostate message-2

ABSTRACT

Current strategies for fluid resuscitation have deleterious side effects caused by the generation of reactive oxygen and nitrogen intermediates. These harmful metabolites significantly affect cell signaling and consequent gene expression. Since the liver is a vital organ of metabolism and homeostasis, it is highly sensitive to hemorrhagic shock-induced changes in homeostasis. Hence comprehensive knowledge of the gene expression changes in liver induced by hemorrhagic shock injury will greatly improve prognosis and therapy. Therefore the present study elucidates the gene expression pattern following hemorrhage and demonstrates the beneficial effects of phytochemicals picroliv and curcumin in countering hemorrhage-induced liver injury. We also propose a hypothesis for their mode of action.

We used a mouse model of non-resuscitated hemorrhagic shock to study the pattern of stress-induced genes in liver at various time points. Anesthetized mice were bled approximately 37% of the estimated total blood volume through the femoral artery. Animals were sacrificed 1, 4 and 24 h following surgery. Genes induced by hemorrhagic shock were examined by cDNA macroarray analysis. We found that heat shock protein genes, stress-induced immediate early genes, as well as signaling molecule genes in the apoptotic pathway were differentially regulated following shock compared with mice that underwent sham surgery or normal mice. We have identified nine genes previously unreported as being regulated during hemorrhagic shock: activating transcription factor-2 (ATF-2), αB-crystallin, growth arrest and DNA-damage inducible 45 (GADD45),

GADD45β, Hsp86, Mdm2, p21Waf1, p53 and testosterone repressed prostate message-2 (TRPM-2). The specificity of these results was confirmed using Northern blot and RT-PCR. Normal and sham mice also expressed certain stress-induced genes. The pattern of expression of the transcription factors activator protein-1 (AP-1), early growth response-1 (Egr-1), heat shock factor 1 (HSF1) and nuclear factor kappa B (NF-κB) at the protein level was different from that at the mRNA level as revealed by gel shift assay. Differences in mRNA expression were transient but the effect of hemorrhage on protein expression was noticeable at later time points. Hemorrhage also caused membrane damage, apoptosis and the increased release of liver injury markers into the serum. Our findings show that traumatic stress causes immediate upregulation of genes involved in a variety of cellular defense pathways. Complex interactions among them might determine the ultimate fate of the cell.

Resuscitation from hemorrhagic shock initiates profound changes in liver that are likely to contribute to end organ damage and resultant dysfunction after shock. Inflammatory cytokines interleukin-1 (IL-1), IL-2, IL-6 and tumor necrosis factor-alpha (TNF-α) have been recognized as important mediators of pathophysiological and immunological events associated with shock. These inflammatory events after hemorrhage and resuscitation are characterized by the activation of transcription regulators such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). Extensive research in this area has indicated the potential of anti-inflammatory and antioxidant strategy for better management of the pathophysiology following hemorrhage-resuscitation (H/R) injury. We studied the effect of novel pharmacological agents picroliv (derived from the roots of

Picrorhiza kurrooa) and curcumin (isolated from the rhizomes of *Curcuma longa*) on hepatocellular injury and redox status as well as its possible mechanism of action in a H/R model in adult rats. Anesthetized rats were subjected to hemorrhagic shock by bleeding 30ml/kg body weight. After 60 min of shock, rats were resuscitated with twice the shed blood volume of lactated Ringer's solution and sacrificed 2 h or 24 h after resuscitation.

We observed that picroliv (12 mg/kg) pretreatment, given orally for seven days, resulted in a significant decrease in serum aspartate transaminase and gamma-glutamyl transpeptidase levels. Picroliv also inhibited the lipid peroxidation and nitric oxide release that occurred following H/R and altered the activity of glutathione reductase (GR) in a favorable manner, thereby suggesting better antioxidant status. It did not have any effect on GR and glutathione peroxidase (GPx) levels. Picroliv significantly downregulated the stress-sensitive transcription factor AP-1 and decreased the level of c-fos mRNA as well as c-jun and c-fos proteins in liver tissue. Inflammatory mediators iNOS, IL-1β and IL-6 (all modulated by AP-1) were also downregulated by picroliv, indicating that its actions could be mediated through AP-1 and associated signal transduction pathways. These findings suggest that picroliv has the potential to be developed as a protective agent against H/R injury.

Administration of curcumin before hemorrhagic shock had salutary effects on cytokines and the redox-sensitive transcription factors NF-κB and AP-1. Significant increases in the levels of liver cytokines IL-1α, IL-1β, IL-2, IL-6 and IL-10 were observed in 2 h post H/R group compared with sham animals. In contrast, oral administration of curcumin (40 mg/kg) for seven days followed by

H/R regimen resulted in inhibition of these cytokine mRNAs; in fact IL-1β levels were lower than sham levels. Curcumin did not affect IL-10 and TNF-α at 2 h. A different pattern was observed 24 h post-resuscitation, with some exceptions. Hemorrhage did not affect the mRNA levels of the cytokines IL-1α, IL-1β and IL-2 but significantly inhibited IL-10 and TNF-α. Curcumin strongly inhibited IL-6 and upregulated IL-10 and TNF-α. NF-κB was differentially activated 2 and 24 h post hemorrhage and was inhibited by curcumin pretreatment. Curcumin caused an increase of IκB protein in the cytoplasm, thus substantiating previous evidence that it inhibits NF-κB by preventing the degradation of IκB. p65 protein levels measured in the cytoplasm and nucleus by Western blotting also confirmed the mechanism of action of curcumin. Serum aspartate transaminase (AST) estimates indicated decreased liver injury in curcumin pretreated hemorrhage animals. These results suggest that protection against H/R injury by curcumin pretreatment may result from the inactivation of transcription factors involved and modulation of cytokines to beneficial levels.

INTRODUCTION

Hemorrhage/resuscitation injury

Hemorrhage or blood loss is a serious complication associated with trauma/shock or complex surgical procedures. Uncontrolled blood loss is a major cause of death in civilian trauma victims. A large number of combat casualties are also complicated by hemorrhage. Uncontrolled bleeding leads to global ischemia and if untreated, ultimately to cellular damage, multiple organ failure (MOF) and death in such cases (Henao, Daes et al., 1991; Rose, Marzi, 1996; Yao, Redl et al., 1998). Traumatic insult is further complicated by sepsis due to severe bacterial and fungal infection.

Hemorrhagic shock is an exogenous hypovolemic shock, one of the many kinds of circulatory shocks (Schwartz, 1999) often associated with traumatic injury and inflammation. Hypovolemic shock is caused by decreased circulating blood volume mainly due to external or internal hemorrhage, eliciting compensatory response from all organ systems (Bongard, Sue, 1994). Primary responses to shock are increased heart rate and peripheral vasoconstriction to increase blood supply to vital organs and maintain blood pressure. Redirection of blood flow causes early decrease in flow to liver and bowel. Hence these organs are extremely sensitive to injury due to ischemic changes.

Fluid resuscitation is the necessary treatment for restoring homeostasis following hemorrhagic shock. Resuscitation replaces lost fluid content, delivers oxygen and improves microcirculation. Resuscitation fluids are generally

classified as crystalloid and colloid depending on the highest molecular weight of the components (Bongard, Sue, 1994). Crystalloids have low viscosity and hence can be rapidly administered through peripheral veins. They are isotonic with plasma and have sodium as the main osmotically active species. Popular crystalloids include lactated Ringer's solution and hypertonic saline. They are safe and effective but excessive administration leads to edema and resuscitation endpoints are difficult to measure. Colloidal resuscitation fluids include those based on albumin or synthetics such as hetastarch. They maintain or restore serum protein levels and also provide more rapid restoration of circulating volume. Their use is restricted due to risk of infection, difficulty of storage and higher expense involved. A systematic review of randomized trials in critically ill patients found that colloid resuscitation was associated with a 4% increased risk of absolute mortality (Schierhout, Roberts, 1998).

One of the most widely used resuscitative fluids today, lactated Ringer's solution (LR), had its origin in 1883 when Sidney Ringer discovered that solutions made with tap water served as better perfusion fluids than those made with distilled water (Ringer, 1883). It was modified in subsequent years to mimic the electrolyte composition of plasma. The presence of sodium lactate in LR allows sodium to bind to excess chloride after the lactate is metabolized to CO_2 and H_2O. Another significant advantage of LR is that it provides a source of bicarbonate when lactate gets metabolized; and unlike bicarbonate, it does not precipitate calcium when it is added to intravenous fluids. Original studies on LR have shown its effectiveness in improving survival time and abating extracellular fluid volume deficits (Fogelman, Wilson, 1960; Shires, Cohn et al., 1964; Middleton, Mathews et al., 1969). Due to these reasons, LR has been an

accepted and effective resuscitation fluid for the past 50 years (Pope, French et al., 1999) and is now routinely used as one of the first-line fluids for resuscitation. Hypertonic saline is also increasingly used since it combines several advantages of crystalloids with those of colloids (Tremblay, Rizoli et al., 2001). Hypertonic saline decreases wound and peripheral edema by displacing water from intracellular compartment to the extracallular space (Bongard, Sue, 1994). Although these and other resuscitative fluids have been accepted as safe and effective in clinical practice, there are distinct disadvantages to them. They need to be modified or used synergistically with other pharmacological agents so as to improve the antioxidant status and homeostatic control of organ systems post-resuscitation.

Involvement of liver in shock

The liver is responsible for maintaining homeostasis in the body and is the main source of energy to peripheral organs (Arias, 2001). It plays an important role in metabolism, immunomodulation and the detoxification of foreign chemicals. Hence, it is readily susceptible to ischemic injury associated with hemorrhagic shock, alcoholic liver disease and transplantion (Thurman, Gao et al., 1995; Jarrar, Chaudry et al., 1999). Growing evidence suggests that disturbances in liver function profoundly affect other body systems leading to MOF or multiple organ dysfunction syndrome and death. Kupffer cells form integral elements in the response of liver to hemorrhagic and other forms of shock. These tissue-macrophages are constantly exposed to various immunomodulators and antigens released during hypotension. Enhanced release

of inflammatory cytokines observed following hemorrhage is largely due to the action of Kupffer cells (Ayala, Perrin et al., 1992). At the molecular level, studies using the calcium-channel blocker diltiazem have shown hepatocellular Ca^{2+} overload and impaired Ca^{2+} signaling during hemorrhage/resuscitation (Rose, Pizanis et al., 1997). This could considerably impact downstream signaling and gene expression. It has also been shown that hepatocellular function following hemorrhage remains depressed despite resuscitation with LR (Wang, Hauptman et al., 1990). This may form the basis of MOF after traumatic shock and additional pharmacological intervention might be needed in addition to fluid resuscitation to restore cellular homeostasis. Study of ischemic changes in the liver is also important for liver transplantation since ischemia/reperfusion injury is a significant risk factor for both rejection and primary graft dysfunction. Therefore the elucidation of hepatic responses to shock is of great importance in the pathogenesis and treatment of traumatic injuries.,

Cellular responses to shock

The pathophysiology of hemorrhage is the result of ischemia/hypoxia, reperfusion and subsequent inflammation. When a tissue is subjected to ischemia due to blood loss, a series of chemical reactions and biological events are initiated, leading to cell injury. Neuroendocrine mechanisms are activated, which gear organ systems toward compensatory methods including vasoconstriction, translocation of fluid into the vascular space and maintenance of cardiac output (Bongard, Sue, 1994). Blood flow is diverted to critical organs, causing decrease in visceral, splanchnic and cutaneous circuits. The biochemical

changes following decreased oxygen delivery and ischemic injury include the release of lysosomal enzymes, histamine, prostaglandins, alterations in blood clotting, acidemia, and hepatocellular dysfunction (Schwartz, 1999).

Decrease of oxygen supply due to hypoperfusion leads to impaired mitochondrial function and a failure of aerobic metabolism, forcing the cell to switch over to anaerobic glycolysis. This type of metabolism produces much less adenosine triphosphate (ATP) and leads to accumulation of toxic metabolites like lactate, which causes acidosis (Bongard, Sue, 1994). It has been shown that the probability of survival decreases with increase in blood lactate in patients with circulatory shock (Weil, Afifi, 1970). Thus the accumulation of lactic acid provides a quantitative measure of oxygen deficit. Another consequence of the decrease in ATP production is the disruption of ATP-dependent membrane pumps, which keep the cellular pH and osmolarity optimal. This includes the Na^+-K^+ pump and the Ca^{2+} pump. Cell swelling occurs due to defective Na^+-K^+ transport across the cell membrane. Loss of cellular calcium homeostasis plays an important role in the pathogenesis of ischemic cell damage since Ca^{2+} is both a signaling molecule and a cofactor for important cellular enzymes. Breakdown of the Ca^{2+} gradient (which is normally 1:10,000 times in favor of the extracellular space) causes massive Ca^{2+} influx into the cell (Siesjo, 1992; Pope, French et al., 1999). Lowered ATP level and altered Ca^{2+} homeostasis cause changes in the second messenger system, activating signal transduction pathways inside the cell. Signaling leads to the activation of transcription factors and subsequent gene expression, ultimately causing tissue injury by the action of inflammatory cytokines and reactive free radicals (Chaudry, Ayala, 1992).

Resuscitation supplies oxygen to the ischemic region of the tissue. The paradox of resuscitation with blood or other fluids is that reperfusion exacerbates tissue injury by explosive generation of reactive oxygen and nitrogen metabolites (Cuzzocrea, Riley et al., 2001). These toxic oxygen and nitrogen species are of central importance in cellular damage (Jaeschke, 1995). The free radical species released by the vascular endothelium and activated neutrophils accumulate at the site of ischemic injury. They significantly affect Ca^{2+} signaling inside endothelial cells, effecting extensive downstream gene expression (Lounsbury, Hu et al., 2000).

Reactive oxygen species (ROS) such as hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$), and reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite anion (ONOO-) are extremely toxic because of their effects on lipid bilayers, intracellular enzymes, structural proteins, nucleic acids, and carbohydrates. But they are also normal byproducts of cellular metabolism. In the course of routine biochemical process, antioxidant enzymes detoxify ROS and RNS. During ischemia and reperfusion of tissue, there is an exceedingly high amount of reactive species generation. As ATP production decreases, precursors of ATP get degraded by the enzyme xanthine oxidase to generate ROS as byproducts. The cellular antioxidant defense system is activated in response to this free radical insult. Hydroxyl radical is generated by various pathways, mainly the arachidonate cascade involving the enzyme cycloxygenase-2 (COX-2). Superoxide anion, released by neutrophils and lipid peroxidation, is converted by the cellular antioxidant enzyme superoxide dismutase (SOD) to hydrogen peroxide, which is converted to harmless water and oxygen by catalase (Fig. 1).

ROS formation causes early hepatocellular injury following hemorrhagic shock and triggers the activation of cellular compensatory mechanisms (Rensing, Bauer et al., 1999). An important reactive species produced during hemorrhagic shock is the bioregulatory molecule NO (Szabo, Billiar, 1999). NO is a potent vasodilator. Its physiological functions include neurotransmitter-like action and the regulation of blood flow and kidney function. NO can enhance ROS toxicity by rapid reaction with oxygen to form the peroxynitrite anion (Beckman, Beckman et al., 1990). This extremely reactive radical can modify the structure and activity of enzymes by nitrating the tyrosine residues and oxidating sulfhydryl groups. Nitration of structural proteins such as actin by peroxynitrite can disrupt the cytoskeletal assembly and exacerbate cell injury (Beckman, Koppenol, 1996). It also causes direct DNA damage (Ducrocq, Blanchard et al., 1999). Excess reactive intermediates also oxidize membrane lipids and deplete the antioxidant enzymes in the cell. These changes cause cell signaling and the release of proinflammatory cytokines, which recruit neutrophils and cause additional release of ROS, thus initiating a vicious cycle.

NO is generated in the cell catalytically by three enzymes collectively termed as NO synthases (NOS). NOS exists as three isoforms nNOS, eNOS and iNOS, of which iNOS gets activated by inflammatory injury. It has previously been shown that the inducible NOS (iNOS) is upregulated in liver during shock (Menezes, Hierholzer et al., 1999; Szabo, Billiar, 1999; Hua, Moochhala, 2000). iNOS over-expression during hemorrhagic shock significantly contributes to vascular decompensation. Even at low concentrations, iNOS enhances the inflammatory response that follows tissue reperfusion and may be capable of catalyzing the sustained production of NO after fluid resuscitation.

iNOS inhibitors have been shown to be protective against hemorrhagic shock injury (Menezes, Hierholzer et al., 1999; Mota-Filipe, McDonald et al., 1999; Hua, Moochhala, 2000).

The glutathione redox system is part of another potent antioxidant defense mechanism of the cell. In this cycle, H_2O_2 is detoxified by the oxidation of glutathione (GSH) by the enzyme glutathione peroxidase (GPx) and oxidized glutathione is reduced back to GSH by glutathione reductase (Sies, 1999). The tripeptide GSH plays a major role in the physiological strategy against oxidative and nitrosative stress (Sies, 1999). Attack by oxidative free radical species directly or indirectly leads to the formation of glutathione disulfide (GSSG), which is reduced intracellularly to GSH by glutathione reductase (GR) via a NADPH-dependent reaction (Fig. 1). GSH/GSSG ratio determines the redox status in the cell. The ratio is critical for various biological events that include transcriptional activation of specific genes and modulation of redox-regulated signal transduction. GSH imbalance has also been implicated in inflammatory response and following hemorrhagic shock (Bauer, Walcher et al., 1999; Sies, 1999; Silomon, Pizanis et al., 1999). Hepatocyte oxidant injury as evidenced by increased glutathione oxidation is prevented by diltiazem (Rose, Pizanis et al., 1997) and allopurinol (Silomon, Pizanis et al., 1999). The shock-associated generation of oxygen radicals is prevented by resuscitation with deferoxamine-conjugated hydroxyethyl-starch solution as indicated by restored glutathione and reduced thiobarbituric acid-reactive substances (Bauer, Walcher et al., 1999).

In addition to direct action on lipids, proteins and carbohydrates, reactive oxygen species also recruit inflammatory cells and scavenge other bioactive molecules, thus indirectly mediating tissue injury (Jaeschke, 1995). Neutrophils

also release lysosomal enzymes and leukotrienes resulting in vascular leaks into interstitial space, the activation of signaling cascades and gene expression (Bongard, Sue, 1994). The role of ROS and RNS in oxidative stress-related injury, the intracellular sources of reactive species and the antioxidant defense mechanism of the cell has been reviewed in detail (Li, Jackson, 2002). A schematic showing the major pathways involved in this system is shown in Fig. 1.

Alterations in transcription and translation

Reactive species-mediated damage and subsequent cellular events cause fundamental changes in transcription and translation. Early events after hemorrhage/resuscitation (H/R) are characterized by the increased release of inflammatory mediators and activation of transcription regulators. Activating transcription factors regulate cellular response to injury by binding to their consensus sequences in the promoter region of specific genes and initiating transcription. Hence alterations in transcription factor levels have significant impact on diseases and their treatment. Ischemic or hypoxic injury causes immediate upregulation of the transcription factors nuclear factor kappa B (NF-κB), activator protein-1 (AP-1) and heat shock factor-1 (HSF-1). Fig. 1 describes the action of these three factors during oxidative stress. Hemorrhage rapidly activates NF-κB and AP-1 (Altavilla, Saitta et al., 2001a; Hoetzel, Vagts et al., 2001; Rensing, Jaeschke et al., 2001). They promote the expression of proinflammatory cytokines that mediate the immune response and inflammation that cause cell injury.

AP-1, a homodimeric (Jun/Jun)· or heterodimeric (Jun/Fos) complex formed of the members of the Jun and Fos proto-oncogene family, plays an important role in inflammatory processes and in the up-regulation of antioxidant genes (Wisdom, 1999) and is known to be induced in liver during oxidative stress conditions including hemorrhagic shock (Tacchini, Radice et al., 1999; Hoetzel, Vagts et al., 2001; Rensing, Jaeschke et al., 2001; Kato, Singh et al., 2002; Tacchini, Fusar-Poli et al., 2002). Activation of AP-1 is mainly initiated by calcium-induced activation of G protein coupled receptors activating mainly c-jun and c-fos, which translocate into the nucleus to form AP-1. AP-1 binds to the promoter region of a number of pro-inflammatory cytokines, initiating their transcription. Once the cytokines are synthesized, they act on their receptors to transactivate AP-1 and NF-κB, causing the production of even more inflammatory mediators. c-jun and c-fos are immediate early genes (IEGs) and components of AP-1 that are induced rapidly and transiently in response to oxidative and other forms of stress as part of the acute phase response (Wisdom, 1999; Domenicotti, Paola et al., 2000). Transcription of c-fos and c-jun proto-oncogene families has been shown to increase in liver and kidney following ischemia and H/R (Saito, Matsumoto et al., 1998; Wieland, Oellerich et al., 2000). An inverse correlation between GSH content and AP-1 transactivation has been proposed recently (Domenicotti, Paola et al., 2000). Lipid peroxidation is also known to cause signal transduction and activation of this transcription factor (Camandola, Poli et al., 2000).

NF-κB, which was originally identified as the regulator of κ light chain genes in B-lymphocytes (Sen, Baltimore, 1986), is one of the most pleiotropic

transcription factors. It regulates the synthesis of cytokines and haematopoietic growth factors such as IL-1 (interleukin-1), IL-2, IL-6, IL-8, IL-10 and tumor necrosis factor-α (TNF-α) (Baldwin, 1996). It gets strongly transactivated in the liver following H/R (Hierholzer, Harbrecht et al., 1998; Altavilla, Saitta et al., 2002). Hence changes in cytokine levels following trauma/hemorrhage could largely be due to NFκB activation. NF-κB also mediates deleterious effects by transcribing the inflammatory enzymes COX-2 and iNOS. Mitochondrial superoxide dismutase gene transfer significantly reduces acute liver damage and associated redox activation of both NF-κB and AP-1 (Zwacka, Zhou et al., 1998), proving the efficacy of antioxidant therapy against the transactivation of these factors.

Heat shock proteins (HSPs) are a group of stress response proteins that protect against cellular injury by facilitating the repair and replacement of denatured proteins. HSP70, an inducible member of this family is upregulated in the liver after post-ischemic reperfusion (Bernelli-Zazzera, Cairo et al., 1992) as well as resuscitated hemorrhagic shock (Ackermann, Reuter et al., 2001). Under stress conditions, monomers of HSF-1 translocate into the nucleus, trimerize and getting activated by phosphorylation, binds to the promoter site of genes such as hsp70, inducing heat shock proteins to counter the malfolding of cellular proteins caused by stress (Santoro, 2000; Christians, Yan et al., 2002). HSF-1 has been shown to be an integral part of the cellular defense against oxidative stress, especially in countering the proteotoxic effects of ROS.

The activation of transcription factors is followed by the production and release of proinflammatory cytokines tumor necrosis factor-alpha (TNF-α),

interleukin (IL)-1 and IL-6 (Chaudry, Ayala, 1992), prominently affecting vital organs including liver. These cytokines play an important regulatory role in inflammation and manifestations of tissue destruction and death. The inflammatory response initiated by hemorrhagic shock has many deleterious consequences. The first wave of cytokines including TNF-α, IL-1 and IL-6 are released by circulating and resident macrophages into the extracellular space. These cytokines bind to specific cell-surface receptors, leading to signal transduction mediated by upstream kinases such as NF-κB inducing kinase (NIK) or mitogen activated protein kinase kinase kinase (MAPKKK or MEKK), ultimately inducing the binding of transcription factors in a positive feedback mechanism (Bowie, O'Neill, 2000).

Cytokine release is followed by accumulation of activated neutrophils (Chaudry, Ayala, 1992; Bongard, Sue, 1994). High levels of circulating cytokines gain access to vital organs and contribute to organ dysfunction and MOF (Bown, Nicholson et al., 2001). Host defense system is severely compromised due to alteration in the levels of cytokines and arachidonic acid metabolites in the body. This increases susceptibility to microbial infections, leading to septic shock. The main mechanism by which infection occurs after hemorrhagic shock is by bacterial translocation from intestine through the portal venous system into visceral organs (Baker, Deitch et al., 1988). Recent evidence also suggests that neutrophil activation and priming during hemorrhagic shock might be closely related to bacterial translocation (Shimizu, Tani et al., 2001). Thus hemorrhagic shock effectively disrupts the gut barrier to allow indigenous bacteria to cause systemic infection leading to MOF and death in trauma patients.

Necrotic cell death is another major causative factor of MOF. Necrosis is a normal consequence of severe ischemic injury of the liver. Decreased blood flow leads to regional hypoxia and if untreated, to subsequent necrotic death of tissue. Recently apoptotic cell death, as opposed to necrosis, has been implicated in the progress of tissue injury and MOF due to shock (Papathanassoglou, Moynihan et al., 2000; Clavien, Rudiger et al., 2001). In contrast to necrosis, apoptosis is tightly controlled by various regulatory factors (Neuman, 2001). A major trigger for this from of cell death is TNF-α, the effect of which has been well-characterized in relation to liver tissue (Jaeschke, Farhood et al., 2000; Sedger, Glaccum et al., 2002). Accumulation of reactive species within the cell also initiates apoptosis by the activation of p53 and its target genes bax, p21Waf1 and GADD45 (growth arrest and DNA-damage inducible 45) (Gottlieb, Oren, 1998). Pro-apoptotic activity of these genes manifests in the positive regulation of caspases (a family of proteases), the actual mediators of the apoptotic process. Inhibition of caspase enzymes prevents liver injury caused by ischemia (Cursio, Gugenheim et al., 2000). Pyruvate decreases hepatic apoptosis after hemorrhagic shock by exerting beneficial effects on the redox environment (Mongan, Capacchione et al., 2002). Hence detailed study of the mechanisms by which apoptosis occurs in the liver might suggest possible treatments for ischemia-related injury.

In this context, we were interested in studying the effect of unresuscitated hemorrhagic shock on mouse liver at different time points compared with normal and sham-operated animals. Hemorrhage induced hepatic injury as evidenced by elevated alanine transaminase (ALT) and aspartate transaminase (AST) levels,

increased membrane damage and high levels of apoptotic cell death. We used a macroarray-based approach to understand the expression pattern of a focused collection of genes involved in stress-response following hemorrhagic shock. Over the course of 24 hours, we found rapid and differential regulation of various genes involved in promoting cell survival as well as apoptosis. Normal mouse liver also expressed certain stress-sensitive genes. These results were confirmed by Northern blotting and RT-PCR. Changes in protein levels of selected transcription factors activator protein-1 (AP-1), early growth response-1 (Egr-1), heat shock factor 1 (HSF-1) and nuclear factor kappa B (NF-κB) were distinct from their mRNA profiles, indicating that complex interconnected regulatory pathways are involved in the cellular response to shock.

Novel approaches to the treatment of shock

Hemorrhage is a major causative factor of battlefield deaths. It is responsible for 30-40% mortality in civilian and military trauma, (Sauaia, Moore et al., 1995; Gofrit, Leibovici et al., 1997). This fact forms the basis of the military's interest in the mechanisms of hemorrhagic shock. Current fluid-replacement protocols like lactated Ringer's solution and various other crystalloid and colloidal solutions have proven to be inadequate on their own. Lactated Ringer's solution causes apoptosis in both small intestine, liver and lung compared with hypertonic saline (Deb, Martin et al., 1999; Deb, Sun et al., 2000). Optimal resuscitation involves the use of a solution that supports oxygen delivery and basic cell function, minimizes reperfusion injury, lack of toxicity and low immunogenicity

(Pope, French et al., 1999). Oxygen therapeutics or blood substitutes, liposomes and perfluorochemicals have been developed in the past few years as part of novel resuscitation fluids. In recent years, antioxidant-scavenging strategies have proven to be very effective in reversing the harmful effects of hemorrhagic shock. (Rensing, Bauer et al., 1999; Mongan, Capacchione et al., 2002). Research has also uncovered new and improved resuscitation fluids such as the oxygen-carrying perfluorocarbons and cell-free hemoglobin (Bongard, Sue, 1994; Pope, French et al., 1999) but they do not address all the requirements of an effective therapeutic agent against hemorrhagic shock. In its recommendations for new and improved resuscitation protocols, the Institute of Medicine's Committee on Fluid Resuscitation for Combat Casualties states that possibly the most interesting, challenging and promising treatment would be to make the soldier less vulnerable to the effects of shock (Pope, French et al., 1999). This could be done effectively by a prophylactic approach, which is attractive in the military situation where blood loss is more severe and adequate care may not be immediately available at the battlefield. Prophylactic drugs that could boost the body's antioxidant defense system with minimum side effects would provide promising treatment. So the search for novel pharmacological agents that could improve prognosis is very important. Ideal drugs should be prophylactic, anti-inflammatory and antioxidant with minimum side effect. Therefore we chose to pretreat the experimental animals with the potent phytochemicals picroliv and curcumin.

Picroliv

Picroliv, an iridoid glycoside mixture of picroside I and kutkoside (1:1.5) (Fig. 2), is derived from the roots and rhizomes of *Picrorhiza kurrooa*, a small perennial herb found in the Himalayan region growing at high elevations. It has been used traditionally in the Ayurvedic system of medicine to treat gastrointestinal and liver disorders (Jain, 1994). Picroliv has potent hepatoprotective, anti-inflammatory and antioxidant properties. It stimulates liver regeneration in rats by inducing protein and DNA synthesis (Singh V., Kapoor et al., 1992). Picroliv has been shown to be hepatoprotective against a variety of toxins including alcohol (Rastogi, Saksena et al., 1996; Saraswat, Visen et al., 1999), galactosamine (Ansari, Aswal et al., 1988; Ansari, Tripathi et al., 1991; Dwivedi, Rastogi et al., 1992b; Dwivedi, Rastogi et al., 1993; Visen, Shukla et al., 1993; Vaidya, Antarkar et al., 1996; Visen, Saraswat et al., 1998), thioacetamide (Dwivedi, Rastogi et al., 1991; Dwivedi, Rastogi et al., 1993; Visen, Saraswat et al., 1998), carbon tetrachloride (Dwivedi, Rastogi et al., 1990; Saraswat, Visen et al., 1993; Santra, Das et al., 1998; Visen, Saraswat et al., 1998), paracetamol (Ansari, Tripathi et al., 1991; Shukla, Visen et al., 1991; Singh V., Visen et al., 1992) and *Amanita phalloides* (mushroom) poison (Dwivedi, Rastogi et al., 1992a). Picroliv offers significant hepatic protection against *Plasmodium berghei* infection in *Mastomys natalensis*, the African soft-furred rat (Chander, Dwivedi et al., 1990; Chander, Kapoor et al., 1992a; Chander, Kapoor et al., 1994), and *Leishmania donovani* infection in golden hamsters(Mittal, Gupta et al., 1998). It protects liver cells from lipid peroxidation (Chander, Kapoor et al., 1992a; Chander, Kapoor et al., 1992b; Chander, Kapoor et al., 1994; Mittal, Gupta et al.,

1998; Santra, Das et al., 1998; Rajeshkumar, Kuttan, 2000; Singh A. K., Mani et al., 2000; Rastogi, Srivastava et al., 2001b; Rastogi, Srivastava et al., 2001a). It possesses immunomodulatory properties (Atal, Sharma et al., 1986; Labadie, van der Nat et al., 1989; Puri, Saxena et al., 1992) and is also a potent antioxidant and free-radical scavenger (Chander, Kapoor et al., 1992b; Rastogi, Saksena et al., 1996; Gaddipati, Madhavan et al., 1999; Rajeshkumar, Kuttan, 2000; Seth, Kumari et al., 2000; Singh A. K., Mani et al., 2000; Rastogi, Srivastava et al., 2001b; Russo, Izzo et al., 2001). It possesses anti-anaphylactic and anti-allergic properties (Baruah, Gupta et al., 1998). Picroliv is active against hepatitis B viral antigen in human serum (Mehrotra, Rawat et al., 1990). Oral administration of picroliv is chemopreventive against hepatocarcinogenesis (Rajeshkumar, Kuttan, 2000; Rajeshkumar, Kuttan, 2001). It is also reported to be protective against myocardial ischemia (Tandon, Rastogi et al., 1995).

Earlier studies from our laboratory have shown picroliv to be protective against hypoxia-reoxygenation injury in various cell lines (Gaddipati, Madhavan et al., 1999). Picroliv differentially modulated the mRNA of the redox-sensitive transcription factor HIF-1 and reduced protein kinase C levels in these cells lines, indicating that its wide-ranging effects could be brought about through diverse cellular pathways. It also modulated insulin-like growth factors in hypoxic rats (Gaddipati, Mani et al., 1999). More recently, our laboratory has shown that picroliv pretreatment lowered lipid peroxidation, improved antioxidant status and reduced apoptosis in renal tissue following ischemia/reperfusion injury. Glutathione (GSH) and glutathione peroxidase (GPx) levels that were reduced following ischemia-reperfusion were improved in the kidneys of picroliv pretreated rats (Seth, Kumari et al., 2000). Picroliv treatment also protects the liver from

ischemia and reperfusion injury as demonstrated by a decrease in the apoptotic process, neutrophil infiltration and lipid peroxidation (Singh A. K., Mani et al., 2000).

Based on our earlier results, the present study was designed to investigate the cytoprotective effects of picroliv and to provide possible molecular mechanism(s) of picroliv-induced protection. We investigated the multifarious effects of picroliv in ameliorating the free radical-induced injury in rat liver due to hemorrhagic shock and resuscitation. Data indicate that picroliv treatment decreased liver injury, lipid peroxidation and nitrosative stress and enhanced cellular GR levels. Further, our studies demonstrate, for the first time, that picroliv pretreatment inhibited the transactivation of AP-1 and the IEGs c-jun and c-fos, suggesting that it protects liver against hemorrhage-resuscitation injury through an antioxidant-mediated anti-inflammatory pathway.

Curcumin

Curcumin is a major component of turmeric. It is isolated from the rhizomes of *Curcuma longa* which have been widely used for centuries in indigenous medicine (Srimal, Dhawan, 1973; Ammon, Wahl, 1991) (Fig. 3). Its anticarcinogenic properties are well documented. Oral administration of curcumin inhibits stomach, skin and colon tumors in mice (Azuine, Bhide, 1992; Huang M. T., Lou et al., 1994). Curcumin could serve as an effective adjunct to other chemotherapeutic agents in the treatment of prostate cancer (Hour, Chen et al., 2002). It is anti-thrombotic (Srivastava, Dikshit et al., 1985) and acts as an angiogenesis inhibitor by modulating protease activity during endothelial

morphogenesis (Thaloor, Singh et al., 1998). It enhances wound healing, suppresses Kupffer cell activation, mixed lymphocyte reaction and mitogen-induced proliferation of mononuclear cells (Huang H. C., Jan et al., 1992; Sidhu, Singh et al., 1998; Lukita-Atmadja, Ito et al., 2002). Curcumin also has anti-inflammatory, antioxidant and bactericidal effects (Satoskar, Shah et al., 1986; Dahl, McGowan et al., 1989; Sreejayan, Rao, 1997). Curcuminoids are effective in suppressing hepatic immune response to bacterial lipopolysaccharide (Lukita-Atmadja, Ito et al., 2002). Curcumin downregulates the production of proinflammatory cytokines TNF-α, IL-1 and IL-8 and inhibits the activation of transcription factors NF-κB and AP-1, which regulate the genes for proinflammatory mediators and protective antioxidant genes (Chan, 1995; Surh, Han et al., 2000; Literat, Su et al., 2001). Curcumin inhibits NF-κB activation by blocking phosphorylation of the inhibitor protein IκBα (Singh S., Aggarwal, 1995) through inactivation of IκB kinase complex (Jobin, Bradham et al., 1999). Suppression of AP-1 by curcumin is due to a direct interaction of curcumin with AP-1 binding to its DNA binding motif (Bierhaus, Zhang et al., 1997; Park, Lee et al., 1998) and also due to inhibition of c-jun or c-fos, components of AP-1 (Huang T. S., Kuo et al., 1995). Curcumin prevents ischemia-induced changes in the cat heart (Dikshit, Rastogi et al., 1995). Pretreatment with curcumin also reduces tubular damage and interstitial inflammation following renal ischemia-reperfusion injury (Shoskes, 1998). In an earlier unpublished study conducted in our laboratory, we monitored the survival benefits of curcumin treatment by observing rats for 72 hours following H/R. Curcumin significantly improved survival. Only 58% of the control hemorrhage animals (n = 24) survived whereas 87% of

curcumin treated animals (n = 23) survived. To examine the possible mechanisms contributing to these protective activities of curcumin, we investigated the extent of liver injury, the effects of curcumin on cytokines and on the activation of transcription factors during hemorrhagic shock using a rat model. The study demonstrates a protective effect of curcumin on liver as evidenced by differential regulation of cytokine expression and inactivation of transcription factors NF-κB and AP-1 following hemorrhagic shock.

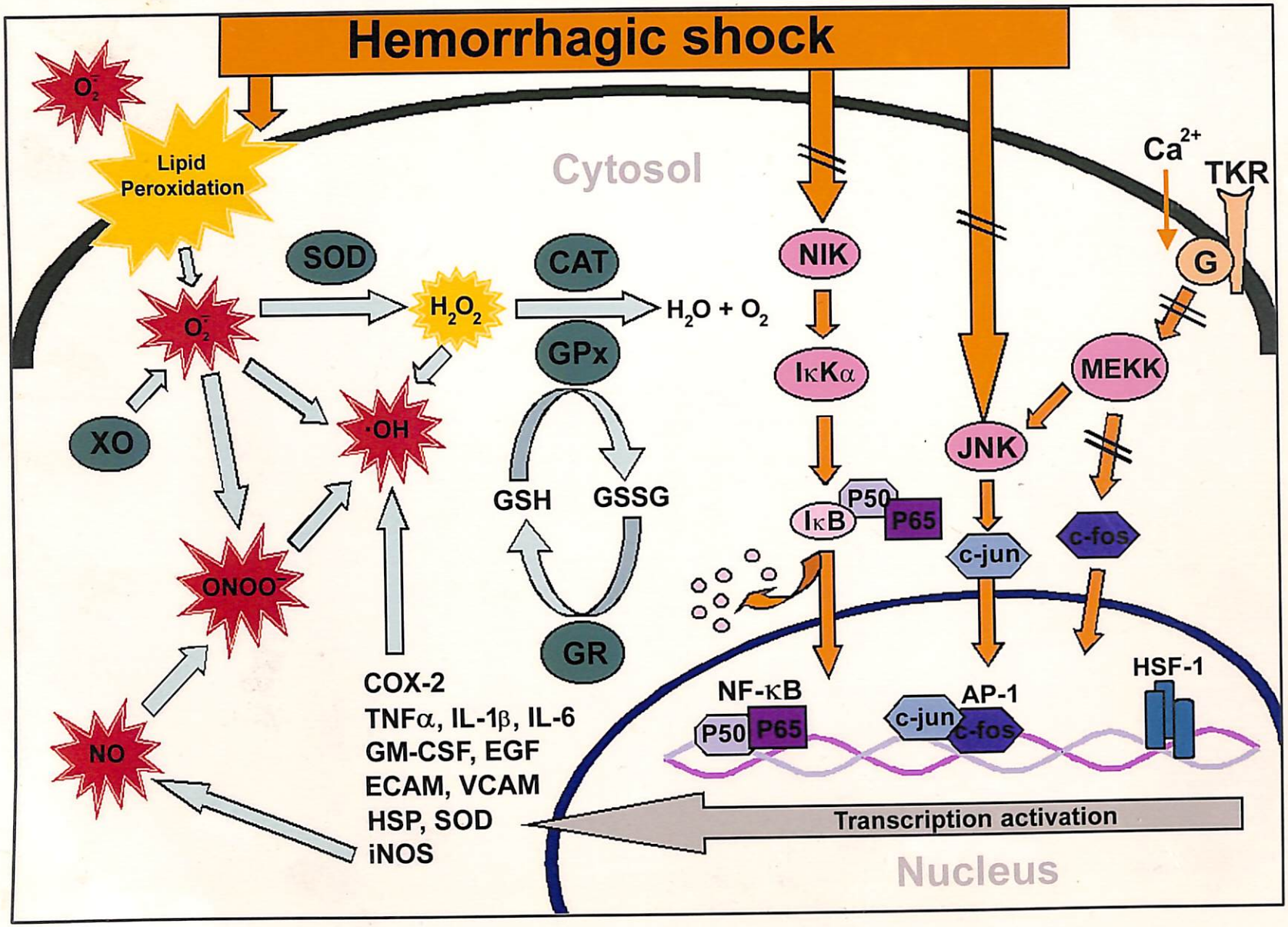
Objectives and rationale

The identification and characterization of transcription factors, cytokines and enzyme systems induced by oxidative stress and elucidation of the role of protective agents such as picroliv and curcumin in their activation would greatly aid the understanding of the mechanism of stress response. Changes at cellular level following hemorrhage and resuscitation are not fully understood. Understanding the cellular mechanisms induced by hemorrhage will help create effective treatment strategies for maintaining fluid volume and homeostasis. Hence we sought to study the oxidative-stress-related genes and signaling pathways activated by hemorrhage and resuscitation and to evaluate rigorously the molecular mechanisms by which picroliv and curcumin would improve the outcome of hemorrhagic shock.

A variety of animal models have been used to study hemorrhagic shock, including rodent, canine and swine models. The degree to which experimental results for animal models can be extrapolated to the human shock condition

should consider several factors including the species, initial experimental condition of the animal, the shock model and the dynamic character of the shock process itself (Pope, French et al., 1999). There are two basic types of hemorrhage models: controlled and uncontrolled with respect to blood loss. Uncontrolled hemorrhagic shock models are relevant where experimental hypotheses involve the effect of treatment on homeostasis. Controlled are either fixed volume or fixed pressure. Controlled models are generally more reproducible compared with uncontrolled ones. Fixed volume models are more realistic and a better measure of shock than fixed pressure models. We chose a mouse model of controlled fixed-volume hemorrhagic shock to evaluate the gene-expression pattern following hemorrhage without resuscitation so as to arrive at a better understanding of the cellular changes leading to organ dysfunction. We used a rat model of fixed-volume hemorrhage resuscitated with lactated Ringer's solution to study the effect of novel pharmacological agents on gene expression and cell signaling.

Fig. 1: Schematic of cellular response to hemorrhagic shock injury: Calcium influx into the cell activates G protein coupled receptors. Pro-inflammatory cytokines bind to cell-surface receptors, activating signaling pathways leading to gene expression. Growth factors such as granulocyte macrophage- colony stimulating factor (GM-CSF) and epidermal growth factor (EGF) bind to tyrosine kinase receptors (TKRs) and induce signaling. These diverse signals converge to induce the expression of transcription factors NF-κB and AP-1. In the NF-κB pathway, NF-κB inducing kinase (NIK) activates IκB kinase (IKK) causing site-specific phosphorylation and degradation of IκB, thus allowing NF-κB to move into the nucleus. c-jun and c-fos, components of AP-1 are activated by mitogen activated protein kinase kinase kinase (MEKK) and jun N-terminal kinase (JNK). Oxidative stress also causes activation of HSF-1 leading to the production of heat shock proteins (HSPs). NF-κB and AP-1 transcribe inflammatory mediators TNF-α, IL-1β, IL-6, COX-2, iNOS, vascular cell adhesion molecule (VCAM) and endothelial cell adhesion molecule (ECAM). iNOS generates NO, which combines to form peroxynitrite anion ($ONOO^-$), a highly harmful radical. Decrease in oxygen supply causes the degradation of ATP by the enzyme xanthine oxidase to release superoxide anion (O_2^-). Hydroxyl radical ($^{\cdot}OH$) is another reactive species formed either from O_2^- or as a byproduct of the arachidonate cascade involving COX-2 enzyme. Peroxidation of membrane lipids also releases O_2^-, which is converted to H_2O_2 by SOD. H_2O_2 is rendered harmless by catalase (CAT) and the glutathione redox system.


Hemorrhagic shock
Cytosol
Lipid Peroxidation
SOD
CAT
H_2O_2
$H_2O + O_2$
GPx
GSH
GSSG
GR
XO
·OH
ONOO⁻
NO
COX-2
TNFα, IL-1β, IL-6
GM-CSF, EGF
ECAM, VCAM
HSP, SOD
iNOS
NIK
IκKα
IκB
P50
P65
JNK
c-jun
MEKK
c-fos
Ca^{2+}
TKR
G
NF-κB
AP-1
HSF-1
Transcription activation
Nucleus

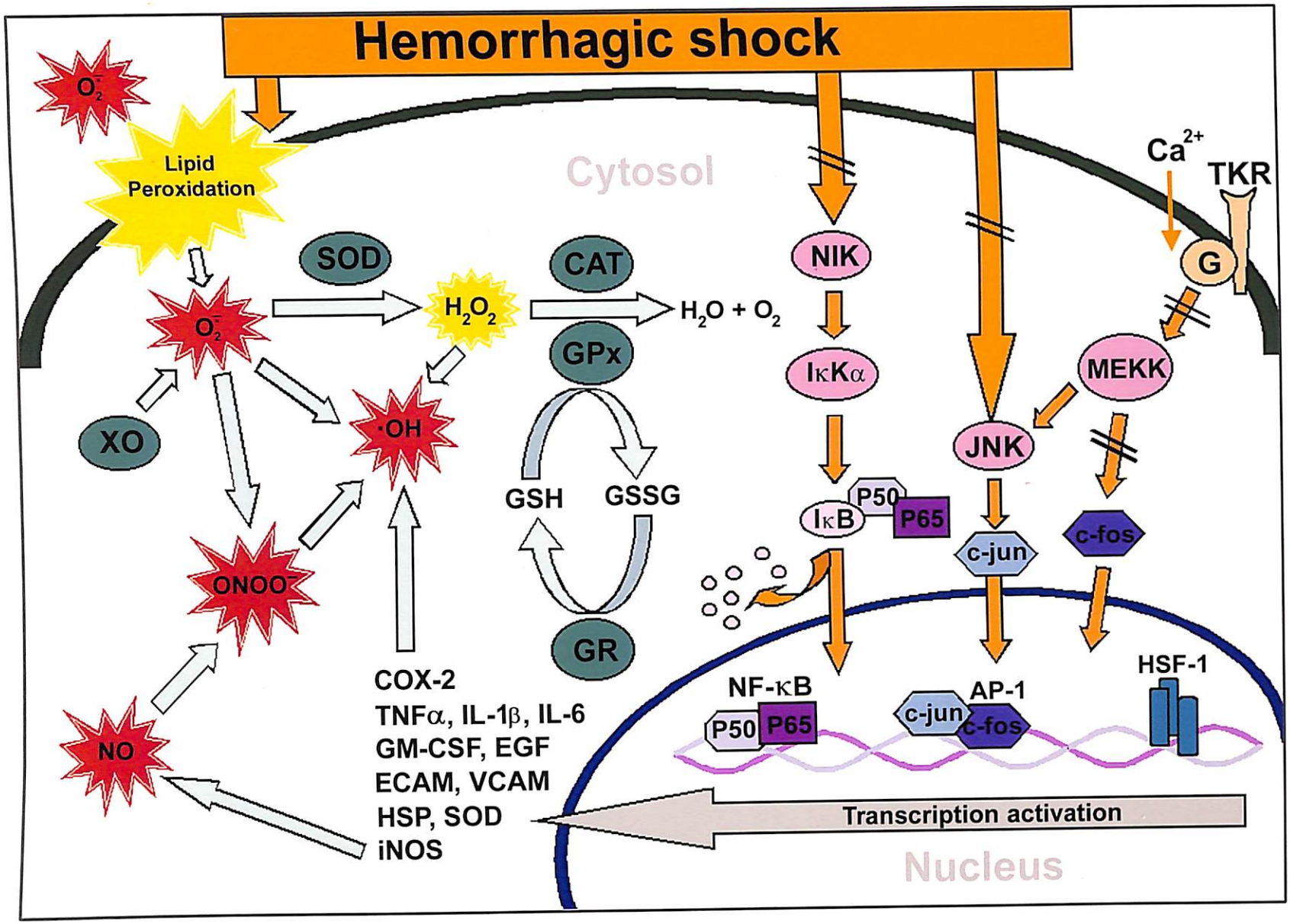
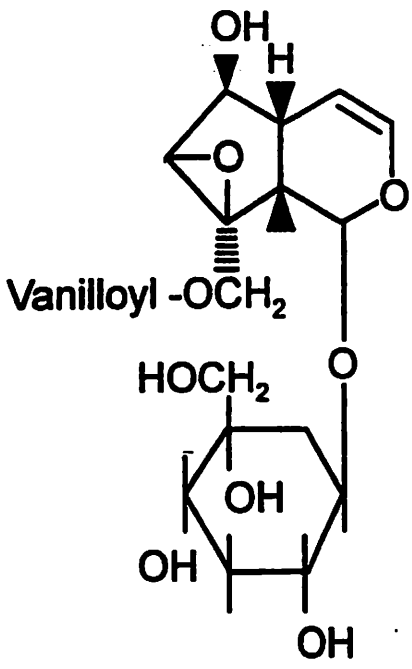
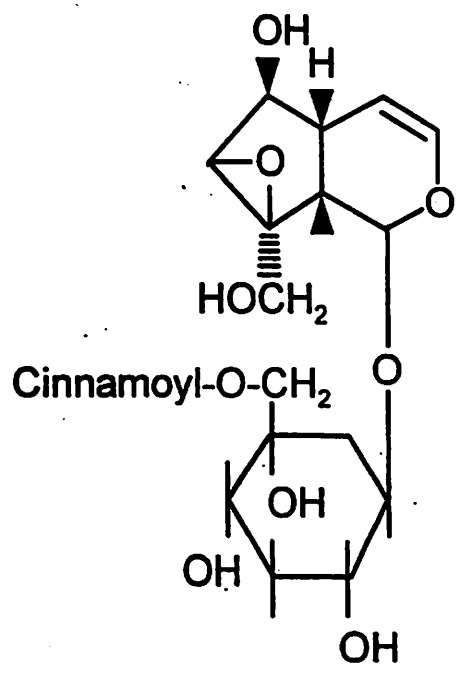

Hemorrhagic shock
O_2^-
Lipid Peroxidation
Cytosol
Ca^{2+}
TKR
G
SOD
CAT
H_2O_2
$H_2O + O_2$
GPx
NIK
IκKα
MEKK
JNK
XO
·OH
GSH
GSSG
IκB
P50
P65
c-jun
c-fos
ONOO⁻
GR
COX-2
TNFα, IL-1β, IL-6
GM-CSF, EGF
ECAM, VCAM
HSP, SOD
iNOS
NO
NF-κB
AP-1
HSF-1
Transcription activation
Nucleus

Fig. 2: Structure of the constituents of picroliv, picroside-1 and kutkoside.

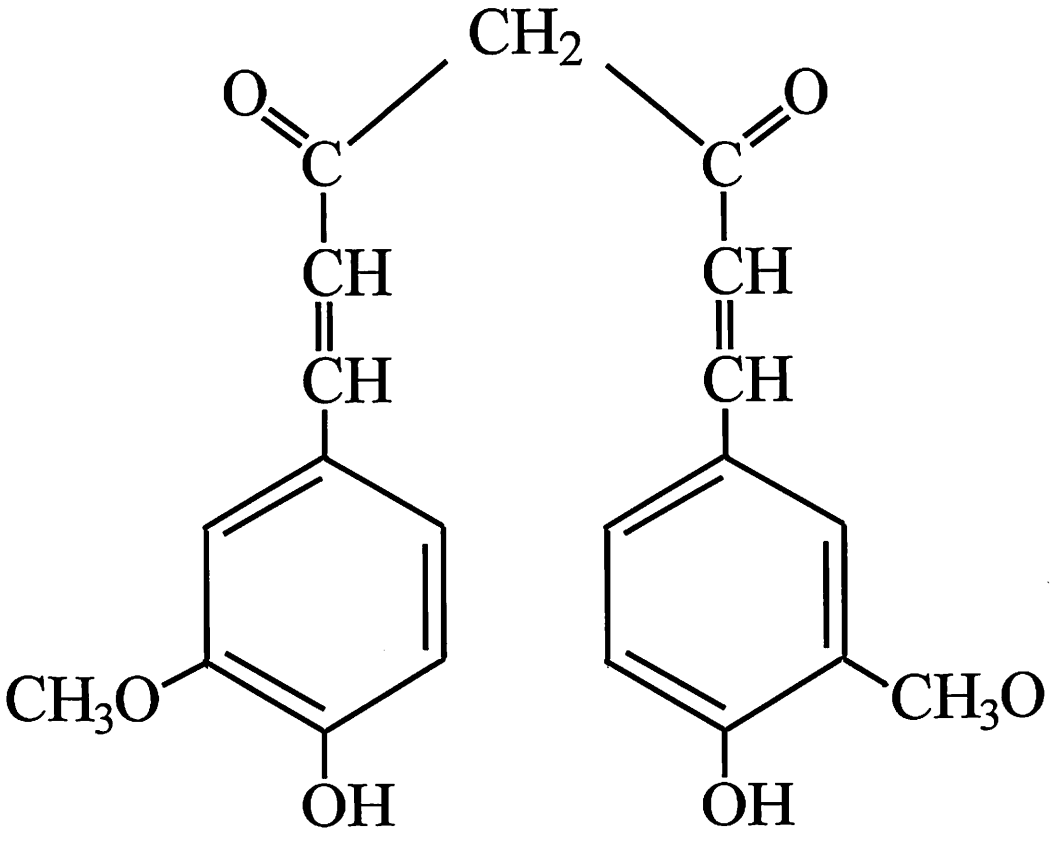


Kutkoside



Picroside-I

Fig. 3: Structure of curcumin (diferuloyl methane).


CH2
O
C
CH
CH
CH3O
OH
O
C
CH
CH
CH3O
OH

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (350-400 g) were purchased from Charles River Laboratories, Wilmington, MA. Female BALB/c mice (18-20 g) were purchased from the National Cancer Institute, Frederick, MD. Animals were housed in a 12 h light-dark cycle and were allowed to acclimatize to the housing environment for a week prior to the study. They had free access to food and water. The study was approved by the Laboratory Animal Review Board of the Uniformed Services University of the Health Sciences (USUHS), Bethesda, MD. It was conducted in compliance with the Animal Welfare Act and the National Institutes of Health's "Guide for the Care and Use of Laboratory Animals".

Treatments

Picroliv and curcumin were obtained from Central Drug Research Institute, Lucknow, India. Rats were fed picroliv (12 mg/kg) or curcumin (40 mg/kg) by oral gavage once daily for seven days prior to experimental procedures. All animals were handled similarly. Control animals were fed equivalent volumes of water.

Hemorrhagic shock model (Mouse)

Mice were randomly divided into three groups of five each (n = 5). Normal animals in the first group (Norm) were without any manipulation. Animals of the sham group (Sham) underwent anesthesia, groin dissection and ligation of the left femoral artery, but no hemorrhage. Animals in the hemorrhage group (Hem) underwent surgery under anesthesia followed by hemorrhage. They were anesthetized by isoflurane (Abbott Laboratories, Chicago, IL) inhalation and placed on a thermostatically controlled heating pad. With minimal dissection and under sterile conditions, the left femoral artery was isolated and cannulated with a pre-heparinized micro-cannula (Biotime, Berkeley, CA). Animals were bled ~37% blood volume over 5 min. The cannula was removed following hemorrhage, the wound sutured and the animals were allowed to recover from anesthesia. Mice were returned to cages and provided food and water and were sacrificed by an overdose of anesthetic 1, 4 and 24 h following hemorrhage. Blood was collected from the orbital sinus for liver function tests. Vital organs were perfused with normal saline through the heart. Liver was excised and stored at -70° C for gene and protein expression studies.

Hemorrhage/resuscitation model (Rat)

Different groups were fed picroliv or curcumin prior to the experiment. Picroliv and curcumin control groups (Pic; n=4 or Cur; n=4) did not undergo any surgical manipulation. Sham controls (Sham; n=8) underwent surgery without hemorrhage. Picroliv or curcumin treated (Pic + H/R: n=8 or Cur + H/R; n= 8) as

well as hemorrhage control animals (n=8) underwent surgery under anesthesia. They were anesthetized by Pentothal (40 mg/kg ip; Abbot Laboratories, North Chicago, IL) and Ketamine (60 mg/kg im; Fort Dodge Animal Health, Fort Dodge, IA) and placed on a thermostatically controlled heating pad. With minimal dissection and under aseptic conditions, both femoral arteries were catheterized with polyethylene-50 tubing (Clay-Adams, Parsippany, NJ). Heart rate and mean arterial pressure (MAP) were constantly monitored using a commercially available Digi-Med arterial blood pressure analyzer (Micro-Med Inc, Louisville, KY) through one of the catheters. Animals were bled rapidly through the other catheter (30 ml of blood/kg body weight). After retaining the animals in hypovolemic shock for 1 h, they were resuscitated with lactated Ringer's solution (2 times the shed blood volume for 20 minutes). Harvard '33' Double Syringe pump (Harvard Apparatus Holliston, MA) was used for bleeding and resuscitating the animals. After resuscitation, catheters were removed, blood vessels were ligated, and the incisions were closed. Surviving animals (n=27) were returned to cages and provided free access to food and water. They were euthanized 2 and 24 h after resuscitation. Before sacrifice, blood was collected by cardiac puncture in order to assay the liver injury markers in serum. Rats were sacrificed by an overdose of anesthetic and vital organs were perfused with normal saline. Liver was excised and stored at –70°C and in buffered formalin for biochemical assays and gene expression studies.

Biochemical Parameters

Liver function tests

Blood was centrifuged at 1600g for 20 min to separate serum. To assess liver injury following hemorrhagic shock, biochemical assays were performed for AST and ALT enzyme activity in serum, at the Clinical Pathology Lab, Laboratory Animal Medicine, Uniformed Services University (USUHS, Bethesda, MD), using the Vitros 250 Chemistry System (Ortho-Clinical Diagnostics, Rochester, NY) following the manufacturer's protocol. Briefly, serum samples were applied to Vitros AST and ALT slides and were monitored by reflectance spectrophotometry at 37° C. The rate of change in reflectance density was measured over a linear region and then converted to enzyme activity.

Measurement of free radical-mediated lipid peroxidation

Malondialdehyde (MDA) levels were measured in liver tissue homogenates. Frozen livers were homogenized in ice-cold 20 mM Tris-HCl buffer (pH 7.4) to prepare 10% w/v homogenates. Homogenates were centrifuged at 3000 g for 10 min at 4°C to remove cell debris. Free radical-mediated lipid peroxidation was assessed by spectrophotometry at 586 nm in the supernatant in terms of nanomoles of MDA/mg protein using a colorimetric biochemical kit (Calbiochem-Novabiochem Corporation, San Diego, CA) following manufacturer's protocol. MDA contents were calculated by comparison with the standard provided with the kit as described before (Seth, Kumari et al., 2000).

Measurement of GSH, GPx and GR

Reduced glutathione was estimated colorimetrically using a Glutathione assay kit (Calbiochem-Novabiochem Corporation, San Diego, CA) according to manufacturer's protocol. Briefly, 5% w/v liver homogenates were prepared in ice cold 5 % metaphosphoric acid and centrifuged at 3000 g for 10 min at 4°C to remove cell debris. Potassium phosphate buffer, pH 7.8 (containing 0.2mM diethylene triamine pentaacetic acid and 0.025% LUBROL), was added to 100 μl of the supernatant. 50 μl of chromogenic agent was added to this mixture to form thioesters, which were measured at 400 nm. GPx and GR activities were assayed using biochemical kits obtained from Oxis International Inc., Portland, OR. For GR activity, 10% tissue homogenates prepared in ice cold 50 mM Tris HCl pH 7.5 containing 1 mM EDTA were centrifuged to remove cell debris. Samples were processed as per the kit insert and reaction kinetics was observed at 25° C for 6 min at 340 nm spectrophotometrically. For GPx activity, 5 mM EDTA and 1 mM mercaptoethanol were added to homogenizing buffer and enzyme activity was estimated by studying the oxidation of NADPH to NADP+ for 3 min at 340 nm spectrophotometrically (Seth, Kumari et al., 2000).

Estimation of NO levels

Tissue homogenates prepared for MDA levels were filtered through 10K molecular weight cut-off NANOSEP microcentrifuge filters (Pall Filtron Corporation, Northborough, MA). Filtrates were used for the measurement of total

NO levels as its stable form, nitrite. Nitrite assay was carried out following protocol suggested for biochemical kits procured from Assay Designs Inc., Ann Arbor, MI. Briefly, samples were incubated in a 96-well plate with reaction buffer, reduced-βNADH and diluted nitrate reductase (provided in the kit) for 30 min at 37° C, to allow conversion of total nitrate into nitrite. Reaction mixtures were then added to Griess reagent, also provided in the kit, and allowed to stand at room temperature for 10 min. Optical densities of the samples were read at 570 nm and total nitrite contents were calculated by comparison with sodium nitrate standard.

Cell-Death Detection

In situ cell death was detected using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Serologicals Co., Norcross, GA) following the manufacturer's instructions. Formalin-fixed, paraffin-embedded tissues were treated with proteinase K, and the endogenous peroxidase activity was blocked by hydrogen peroxide. The slides were then processed according to kit instructions; DNA fragments generated by apoptosis were labeled with digoxygenin-conjugted nucleotides by the action of terminal deoxynucleotidyl transferase (TdT) enzyme. Apoptotic nuclei were visualized by immunoperoxidase detection of the labeled DNA. Slides were counterstained using methyl green (according to kit instructions), mounted on cover-slips and apoptotic nuclei were counted under light microscope.

Gene Expression Studies

Total RNA isolation

DNA-free total cellular RNA was isolated from ~100 mg frozen liver tissue using the QIAGEN RNeasy Midi Kit and RNase-Free DNase Set (QIAGEN Inc., Valencia, CA). Manufacturer's instructions were followed with minor modifications to increase RNA yield. RNA integrity was checked on 1% formaldehyde-agarose gels and quantitated by spectrophotometry.

cDNA array analysis

GEArray™ pathway – specific expression array kits (mouse stress and toxicity I) were obtained from Superarray Inc., Bethesda, MD. Each kit contained two nylon cDNA arrays, hybridization buffer, and reagents for labeling the probe. MMLV reverse transciptase and RNase inhibitor were from Promega Co., Madison, WI. [α-^{32}P]-dCTP (10 mCi/ml) was bought from Amersham Biosciences Co., Piscataway, NJ. Each array had 23 genes specific for genes known to be regulated during cellular stress, along with the housekeeping genes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). pUC18 bacterial plasmid spots allowed the determination of specificity of hybridization. The list of genes is provided in Table 1, along with their location on the array corresponding GenBank numbers.

Total RNA (10 μg) was used as the template for synthesis of a radioactive cDNA probe according to the manufacturer's protocol. The array was hybridized overnight in GEAhyb buffer to which labeled cDNA probe and heat-denatured salmon sperm DNA (Invitrogen, Carlsbad, CA) had been added. The array membrane was washed according to kit instructions. The wet membrane was sealed in a hybridization bag and exposed to X-ray film with an intensifying screen at –70°C. The films were scanned into the image analysis program Scanalyze (Eisen, 1999) and spot intensities were quantitated. The results were analyzed using the GEArray Analyzer program (Superarray Inc., Bethesda, MD). The relative abundance of each cDNA transcript was estimated by comparing its signal intensity to the signal derived from GAPDH on the membrane. To confirm the results of the hybridization experiment, each membrane was stipped and reprobed with RNA from three different mice.

Northern Blotting

Total RNA (15 μg) from individual mouse livers was run on a NorthernMax™-Gly gel (Ambion Inc., Austin, TX) and transferred to BrightStar™ Plus nylon membrane (Ambion Inc., Austin, TX) by downward capillary transfer. RNA was bound to the membrane by UV-crosslinking. Double-stranded DNA probes were produced using the Strip-EZ™ PCR kit (Ambion Inc., Austin, TX) following the manufacturer's instructions. The probes were purified by passing them through ProbeQuant G-50 microcolumns (Amersham Biosciences Co., Piscataway, NJ). PCR primers for Strip-EZ PCR were designed using the

Primer3 software (Rozen, Skaletsky, 1998) and mouse cDNA sequences published in the GenBank database and were synthesized by QIAGEN Operon, Alameda, CA (Table 2). Hybridization and washing procedures were carried out following Ambion's protocol for the NorthernMax™ Gly kit. The membrane was then treated for chemiluminescence detection using the BrightStar™ Bio-detect™ kit (Ambion Inc., Austin, TX) and exposed to X-ray film. The images on the film were scanned and quantitated using the public domain NIH Image program (version 1.62, developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

RT-PCR

Equal amounts of total RNA (3-5 μg) were reverse-transcribed using the SUPERSCRIPT™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Primers for rat c-jun, c-fos, IL-1α, IL-1β, IL-2, IL-6, IL-10, and TNF-α were obtained from BIOGNOSTIK, Gottingen, Germany. PCR primers for c-fos, Egr-1, HSF-1, NF-κB, iNOS, L28 and GAPDH (Table 2) were designed using the Primer3 software (Rozen, Skaletsky, 1998) and rat and mouse cDNA sequences published in the GenBank database. The primers were synthesized by QIAGEN Operon, Alameda, CA or the in-house DNA synthesizing facility at USUHS, Bethesda, MD. Linear range of amplification for each gene was determined by doing 20-35 cycles in increments of 3 cycles each. Based on these results, the reactions were performed at 94°C for 30 seconds, 55-60°C for 45 seconds and 72°C for 60 seconds for the appropriate

number of cycles using 10% of cDNA and 10 pmol of each prim er. GAPDH and L28 (ribosomal protein) genes were the internal controls. Each set of PCR reactions included control samples without RNA and without RT enzyme to ensure that the products resulted from amplification of the target mRNAs and not contaminating or genomic DNA. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining and images captured by Eagle Eye II Still Video System (Stratagene, La Jolla, CA). Densitometric analysis of PCR bands was done using NIH Image 1.62 program and normalized with respect to GAPDH or L28.

Nuclear extract preparation

The procedure of Schreiber et al. was followed with minor modifications for the preparation of nuclear extracts (Schreiber, Matthias et al., 1989). Briefly, 50-100 mg of frozen liver tissue was weighed quickly and placed in 10µl homogenizing buffer/mg tissue containing protease inhibitors (1 mM dithio-threitol (DTT), 0.5 mM phenyl methyl sulfonyl fluoride (PMSF), 1 µg/ml pepstatin A, 10 µg/ml leupeptin and 10 µg/ml aprotinin added fresh). After incubation for 5-10 min. on ice, the tissue was homogenized 15 times in a Dounce B homogenizer and spun at 1200 g for 10 min. at 4°C. The pellet was gently resuspended in an equal volume of high salt buffer containing protease inhibitors (1mM DTT, 1 mM PMSF, 1µg/ml pepstatin A, 10 µg/ml leupeptin, 10µg/ml aprotinin, 25 mM sodium fluoride and 100 µM sodium orthovanadate added fresh). The suspension was incubated for 15 min at 4°C and centrifuged at 14,000 g for 10 min at 4°C. The

supernatant was stored at –70°C after removing a small aliquot for protein estimation.

Protein extract preparation

Frozen liver tissue (50-80 mg) was homogenized on ice at a ratio of 1 mg per 5µl of ice-cold RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM sodium chloride, 1% Triton X 100, 1% sodium deoxycholate, 0.1% SDS to which 1mM DTT, 1 mM PMSF, 1µg/ml pepstatin A, 10 µg/ml leupeptin, 10µg/ml aprotinin, 25 mM sodium fluoride and 100 µM sodium orthovanadate were added fresh). The homogenate was passed through a 25-gauge needle 10 times and centrifuged at 14000 g for 20 min at 4°C. The supernatant was transferred to fresh tubes and re-centrifuged at 14000 g for 10 min at 4°C. The supernatant was stored at –70°C after removing a small aliquot for protein estimation.

Electrophoretic mobility shift assay (EMSA)

Gel Shift Assay System (Promega Corporation, Madison, WI) was used. The oligonucleotide probes (double stranded) for NF-κB and AP1 were provided in the kit. The consensus sequence for HSF-1 and Egr-1 was purchased from Geneka Biotechnology Inc., Quebec, Canada.

Activated transcription factors bound to consensus sequences were detected in nuclear protein extracts by electrophoretic mobility shift assay. Probes were labeled with [γ-^{32}P] ATP (3000 Ci/mmol) (Amersham Biosciences

Co., Piscataway, NJ) using T4 polynucleotide kinase and were purified by passing through ProbeQuant™ G-50 micro columns (Amersham Biosciences Co., Piscataway, NJ). Probe binding was carried out according to the Gel Shift Assay kit instructions using 10 μg nuclear protein and ~100,000 dpm of the labeled probe. Radioactive count was determined on a Beckman LS6000TA liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA). After incubation at room temperature for 20 min, the DNA-protein complex was separated from unbound oligonucleotide on a 4% non-denaturing polyacrylamide gel. After electrophoresis, gels were dried and analyzed using a phosphor-imager and the data expressed in terms of gross counts per min (cpm) of radioactivity. Dried gels were also exposed to X-ray film and quantitated using NIH Image 1.62 software. HeLa extract (supplied by the company) was used as positive control. Specificity of binding was checked by adding cold probe to the reaction mixture ~20 times in excess of the hot probe. Water instead of nuclear extract was the negative control. For supershift assay, 4μg of anti NF-κB p65, anti c-jun or anti c-fos antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was added to the binding reaction and incubated for 30 min. at room temperature before adding the probe.

Western blotting

Frozen nuclear (20 μg) and whole cell extracts (50 μg) were thawed on ice and mixed with 4X sample buffer (Invitrogen, Carlsbad, CA) and heated at 70°C for 10 min, spun down and loaded on 4-12 % NuPage Bis-Tris gel

(Invitrogen, Carlsbad, CA) at 200 V. Predetermined molecular weight standards (Invitrogen, Carlsbad, CA) were used as markers. Protein on the gel was blotted onto nitrocellulose membranes at 30 V for 60 min. After transfer, the membranes were incubated with blocking buffer (5% skim milk in wash buffer (1X TBS, 0.05 % Tween 20)) for one hour at room temperature. Target proteins were detected by incubating with 1:1000 dilution of primary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) in blocking buffer. The membranes were washed three times with wash buffer and then incubated with the appropriate secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at dilutions of 1:2000 to 1:3000 in blocking buffer. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) according to the specifications of the manufacturer (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Blots were scanned and optical densities of the protein bands were quantitated with NIH image 1.62 software.

Protein estimation

Protein contents of the extracts were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) according to manufacturer's instructions. The assay was carried out in 96 well microtitre plates in 220 µl reaction mixtures. The optical density at 570 nm was measured on a CERES 900 scanning autoreader (Bio-Tek Instruments Inc., Winooski, VT).

Statistical Analysis

Data are expressed as mean ± standard error of mean (SEM) of seven or eight animals unless otherwise stated. Statistical significance was measured by one-way analysis of variance (ANOVA). Tukey-HSD multiple comparisons test was used for *post hoc* analysis (SPSS Version 10.1, SPSS Inc., Chicago, IL). $p < 0.05$ was considered significant.

RESULTS

Hemorrhagic Shock Causes Differential Expression of Stress-Induced Genes

Gene expression profile

To understand the global effect of hemorrhagic shock on stress-induced genes, total RNA was reverse-transcribed, ^{32}P-labeled and hybridized to a 23-gene macroarray. The effect of hemorrhagic shock on mouse liver at various time points was examined by comparing the relative intensities of cDNA spots of Norm, Sham and Hem groups. Representative cDNA arrays showing differential gene expression are given in Fig. 4. A typical gene expression profile obtained after array-hybridization at the 1 h time point is shown in Fig. 5. Only those genes whose hybridization signals were greater than 1.5 fold of normal or sham samples were included in data analysis. The significant gene expression changes in mice following hemorrhagic shock at 1, 4 and 24 h compared with Sham are shown in Table 3.

1 h following hemorrhage, 13 out of the 23 genes listed in Table 1 were differentially expressed in Hem compared with Sham. They were bax, c-fos, αB-crystallin, Egr-1, GADD45, GADD45β, HSF-1, hsp86, IκBα, mdm2, mkk4, NF-κB and p53. More than 2-fold change was observed in αB-crystallin, Egr-1, GADD45, GADD45β, HSF-1 and IκBα. The highest magnitudes of difference were observed for Egr-1 (23.7 fold) and GADD45 (7.8 fold). p53 was the only

gene downregulated in Hem at this time point. Activating transcription factor-2 (ATF-2) was not expressed. There was no difference in the expression of glucose regulated protein 78 (Grp78), p21Waf1, p38 MAPK, SOD1 and testosterone repressed prostate message-2 (TRPM-2) of Hem compared with Sham.

4 h after shock, fewer genes showed changes (9/23); bax, c-fos, HSF-1, hsp86, IκBα, mdm2, p21Waf1, p38 MAPK and p53. More than 2-fold changes were observed in bax, c-fos, hsp86, IκBα, p38 MAPK and p53. p21Waf1, p38 MAPK and p53 were newly upregulated in Hem. IκBα showed the highest fold-difference (8.2 fold). c-fos was downregulated ~ 4 fold. ATF-2 transcription factor was expressed in both Sham and Hem groups, but there was no difference between them. Egr-1 mRNA was not expressed at this time point.

At 24h, only four genes showed significant changes- bax, GADD45, IκBα and p38 MAPK, all more than 2-fold. GADD45β signal was not detectable on the blots and there was no difference in the expression of the other genes.

A majority of the stress-genes were expressed in normal mouse liver at the 1 h time point including IEGs ATF-2, c-fos and Egr-1. Norm values were comparable to that of Hem for most of the genes. p53 gene was highly expressed in Norm group. Highly inducible by sham surgery and hemorrhage at all time points was the p53-associated protein p21Waf1. The changes in Sham and Hem animals compared with Norm are shown in Table 4. More than 2-fold downregulation of αB crystallin, Egr-1, GADD45, HSF-1, hsp86, mkk4, p38 MAPK, p53 and TRPM-2 was observed in Sham compared with Norm. Stress-induced downregulation of p38 MAPK and p53 was seen in the Hem group. GADD45β, IκBα and p21Waf1 were consistently upregulated in Sham and Hem.

Greatest differences were observed in the case of p21Waf1: there was a 50 to 100-fold increase in p21Waf1 levels after sham surgery and hemorrhage during the first four hours compared with Norm. The fold difference decreased to ~ 25 times that of Norm at 24 h.

At the 4 h time point, ATF-2, bax, αB-crystallin, Egr-1, GADD45 and p53 were highly expressed in Norm compared with the treatment groups. Very few genes were differentially regulated at 24 h following shock. There was minimal gene expression in all three groups, except for TRPM-2, which was high in both Sham and Hem.

We observed that most of the stress-induced genes included in the array were expressed under all experimental conditions except c-jun, c-myc, hsp25 and iNOS, which were not detectable in any experimental group. In addition to these genes, Norm did not express GADD45β and p21Waf1. SOD1 and TRPM-2 genes were the most highly expressed in all samples. We have identified nine genes previously unreported as being regulated during hemorrhagic shock: activating transcription factor-2 (ATF-2), αB-crystallin, GADD45, GADD45β, Hsp86, Mdm2, p21Waf1, p53 and TRPM-2.

mRNA analysis by Northern blot and RT-PCR

We chose SOD1 and TRPM-2 for further analysis by Northern blotting since they were over-expressed in all experimental groups. Genes coding for the IEGs c-fos, Egr-1 and the transcription factors HSF-1 and NF-κB were assessed by RT-PCR. Representative Northern blots confirming the cDNA array results are

shown in Fig. 6. SOD1 gene was significantly lower in Hem compared with Sham at the 1 h time point. This difference was not observed in the corresponding array spot for SOD1 (Table 3). At all other times, SOD1 levels in Sham and Hem were comparable to those of Norm. There was a significant decrease in TRPM-2 in Sham and Hem compared with Norm at 4h following shock but this difference was undetectable in the 4 h array blot. Significant induction of TRPM-2 ($p < 0.01$) was seen in both Sham and Hem groups at the 24 h time point compared with Norm, confirming the results of the array blot at 24 h.

RT-PCR showed differential regulation of the various transcription factors (Fig. 7). Normal mouse liver expressed a high level of c-fos, Egr-1 and HSF-1 mRNAs but not NF-κB. 1 h following hemorrhage all four genes were significantly upregulated ($p < 0.05$) compared with Sham. At the 4 h time point, c-fos mRNA was significantly downregulated in Hem. There was no difference in Egr-1 and HSF-1 mRNA levels between Norm, Sham and Hem. Both Sham and Hem groups showed high levels of NF-κB mRNA compared with Norm. This IEG response subsided by 24 h; there were no differences in Norm, Sham and Hem for any of the four genes at this time point. In general, the PCR results correlated well with the pattern of gene expression observed using cDNA arrays.

Effect of hemorrhage on transcription factor activity

To examine the effect of sham surgery and hemorrhage on the protein activity of c-fos, Egr-1, HSF-1 and NF-κB genes, we estimated the DNA-binding activity of the corresponding transcription factors (AP-1, Egr-1, HSF-1 and NF-

κB) in liver nuclear extracts at 1, 4 and 24 h following hemorrhage. Typical autoradiograms are shown in Fig. 8. Norm group showed significantly low binding activity except for HSF-1, which was highly transactivated in Norm.

AP-1 was significantly induced at 1 h compared with Sham ($p < 0.01$). Both Sham and Hem values were high compared with Norm ($p < 0.01$). The pattern at 4 h was the same as at 1 h, with consistent upregulation compared with Sham ($p < 0.01$). AP-1 binding decreased by 24 h, but even at that late time point, Sham and Hem showed significantly higher binding activity compared with Norm.

For Egr-1, Both Sham and Hem were highly upregulated at all time points compared with Norm. There was no difference between Sham and Hem at 1 h, Hem values were slightly but significantly lower at 4 h and were higher at 24 h.

HSF-1 binding activity in Sham and Hem groups were significantly lower compared with Norm throughout the time course of study ($p < 0.01$). Differences between Sham and Hem for HSF-1 were discernible only at 24 h, when hemorrhagic shock induced HSF-1 binding.

NF-κB was highly induced 1 h following Hem ($p < 0.001$). The transactivation of NF-κB persisted at 4 h and 24 h, though it was of a lower magnitude than at 1h. Only NF-κB remained persistently activated through the 24 h following hemorrhage, indicating the prolonged activation of pro-inflammatory gene expression.

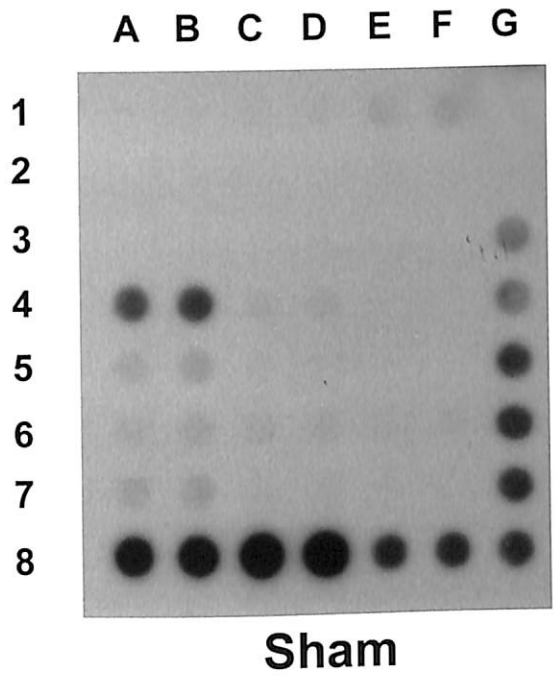
Hemorrhagic shock causes liver injury

Effect of hemorrhage on liver function: Serum levels of enzymes AST and ALT were assessed to determine the severity of hemorrhagic shock. The results, presented in Table 5 show that hemorrhagic shock causes liver injury as evidenced by increased AST and ALT levels in serum, significantly higher than Sham. Normal mice had significantly lower levels of both enzymes compared with the treated groups ($p < 0.01$). Alterations in AST were observed as early as I h following shock and increased ALT levels were observed at the 4 h time point. There were no liver injury markers present in the serum collected at 24 h.

Effect of hemorrhage on lipid peroxidation in liver: We chose MDA as a marker to determine the extent of membrane damage in the liver due to lipid peroxidation. There were no significant changes in MDA levels of the three groups during the early hours after hemorrhage. But at 24 h, increased lipid peroxidation was observed in the Hem group compared with Sham ($p < 0.05$). These results are shown in Fig. 9.

Apoptotis-induction by hemorrhage: Cell death due to apoptosis was detected by labeling the DNA fragments generated within apoptotic nuclei and visualizing them by immunoperoxidase detection. As shown in Fig. 10, increased numbers of apoptotic nuclei were observed in liver 24 h following hemorrhage ($p < 0.01$ compared with Sham). There was no apoptosis at other time points. Sham was comparable to normal mouse liver.

Fig. 4: Representative cDNA arrays showing differential gene expression. Mice were subjected to sham surgery (Sham) or hemorrhagic shock (Hem) and gene expression in liver was analyzed 1, 4 and 24 h following experimental procedure. Total RNA was reverse transcribed, ^{32}P-labeled and hybridized to nylon membranes spotted with duplicate copies of complementary cDNAs corresponding to 23 mouse genes known to be activated during stress. Signals were visualized by autoradiography. This representative figure compares Sham and Hem at 4 h time point. (1A, 1B): ATF-2, (1C, 1D): bax, (1E, 1F): c-fos, (2A, 2B): c-jun, (2C, 2D): c-myc, (2E, 2F): αB-crystallin, (3A, 3B): Egr-1, (3C, 3D): GADD45, (3E, 3F): GADD45β, (4A, 4B): grp78, (4C, 4D): HSF-1, (4E, 4F): hsp25, (5A, 5B): hsp86, (5C, 5D): IkBα, (5E, 5F): iNOS, (6A, 6B): mdm2, (6C, 6D): mkk4, (6E, 6F): NF-κB, (7A, 7B): p21Waf1, (7C, 7D): p38, (7E, 7F): p53, (8A, 8B): SOD1, (8C, 8D): TRPM-2, (3G, 4G): β-actin, (5G, 6G), (7G, 8E), (8F, 8G): GAPDH, (1G, 2G): pUC18. For a description of individual genes, see Table 1.


A B C D E F G
1
2
3
4
5
6
7
8
Sham

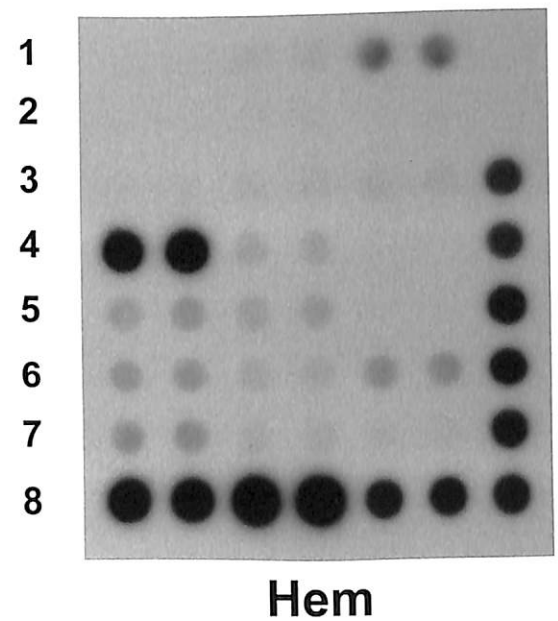

1
2
3
4
5
6
7
8
Hem

Fig. 5: Gene expression profile in mouse liver following hemorrhagic shock. Mice were subjected to sham surgery (Sham) or hemorrhagic shock (Hem) and gene expression in liver was analyzed 1, 4 and 24 h following experimental procedure. Total RNA was reverse transcribed, ^{32}P-labeled and hybridized to nylon membranes spotted with duplicate copies of complementary cDNAs corresponding to 23 mouse genes known to be activated during stress. Signals were visualized by autoradiography. This representative figure compares Sham and Hem at the 1 h time point for the genes c-fos, Egr-1, Hsf1, NF-κB, SOD1 and TRPM-2. Gene expression was quantitated based on the analysis of three replicate blots using software provided by SuperArray Inc. Signal intensities were normalized with respect to the housekeeping gene GAPDH. Fold differences are: c-fos – 1.8, Egr-1 – 23.7, Hsf1 – 7.6, NF-κB – 1.9, SOD1 – no difference, TRPM-2 – no difference.

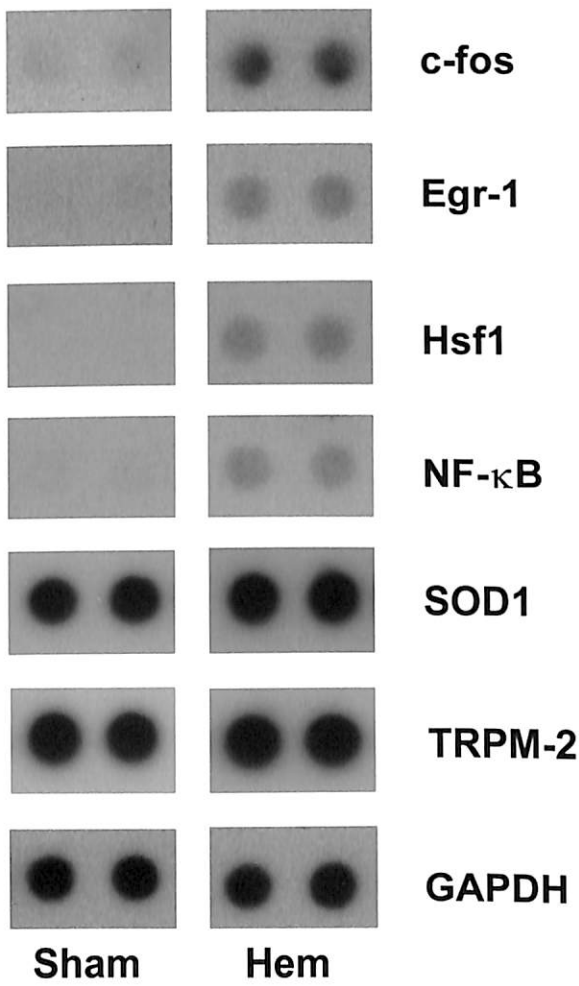

c-fos
Egr-1
Hsf1
NF-κB
SOD1
TRPM-2
GAPDH
Sham
Hem

Fig. 6: mRNA analysis by Northern blotting. Gene expression in the liver of normal mice (Norm: N), those subjected to sham surgery (Sham: S) and hemorrhagic shock (Hem: H) was analyzed 1, 4 and 24 h following experimental procedure. Total RNA was separated on a glyoxal-DMSO gel and transferred to nylon membrane. The membrane was then probed with denatured 200 base biotin-labeled DNA probes against SOD1 and TRPM-2 and visualized by chemiluminescence detection. Blots were stripped and rehybridized with a 255 base probe against GAPDH. Values were normalized as a percentage of the corresponding bands for GAPDH to account for variations in the amount of RNA between samples. Data are represented as mean ± SEM of five animals. * signifies $p < 0.05$ compared with Sham and # signifies $p < 0.05$ compared with Norm.

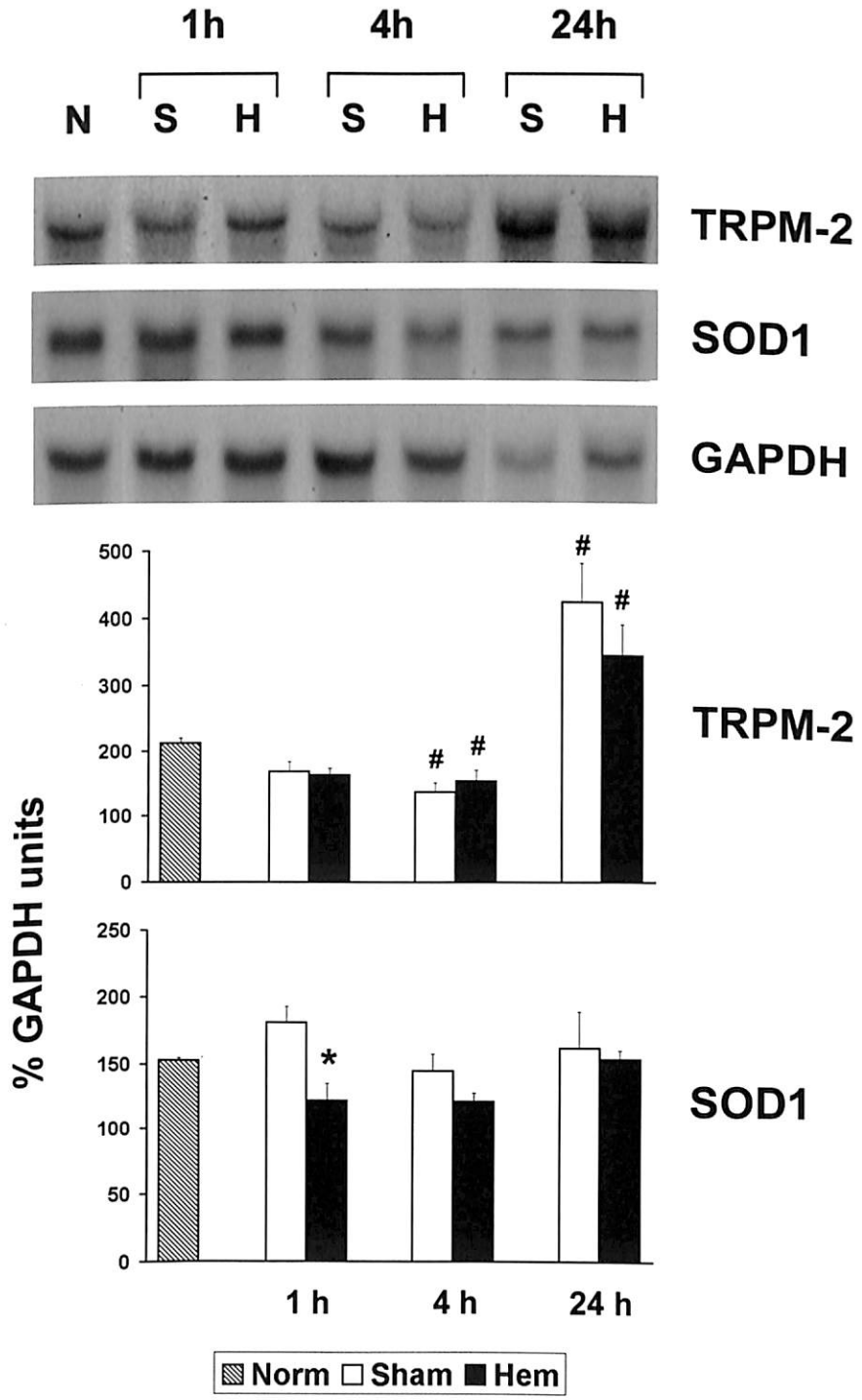

1h
4h
24h
N
S
H
S
H
S
H
TRPM-2
SOD1
GAPDH
% GAPDH units
500
400
300
200
100
0
#
#
#
#
TRPM-2
250
200
150
100
50
0
*
SOD1
1 h
4 h
24 h
Norm
Sham
Hem

Fig. 7: mRNA analysis by RT-PCR. Gene expression in the liver of normal mice (Norm: N), those subjected to sham surgery (Sham: S) and hemorrhagic shock (Hem: H) was analyzed 1, 4 and 24 h following experimental procedure. Total RNA was reverse transcribed and subjected to PCR. Products were visualized on 2% agarose gels stained by ethidium bromide. Representative PCR bands for 1, 4 and 24 h time points are shown in the figure. Densitometric values are expressed as a percentage of the GAPDH gene. Data are represented as mean ± SEM of five animals. * signifies $p < 0.05$ compared with Sham and # signifies $p < 0.05$ compared with Norm.

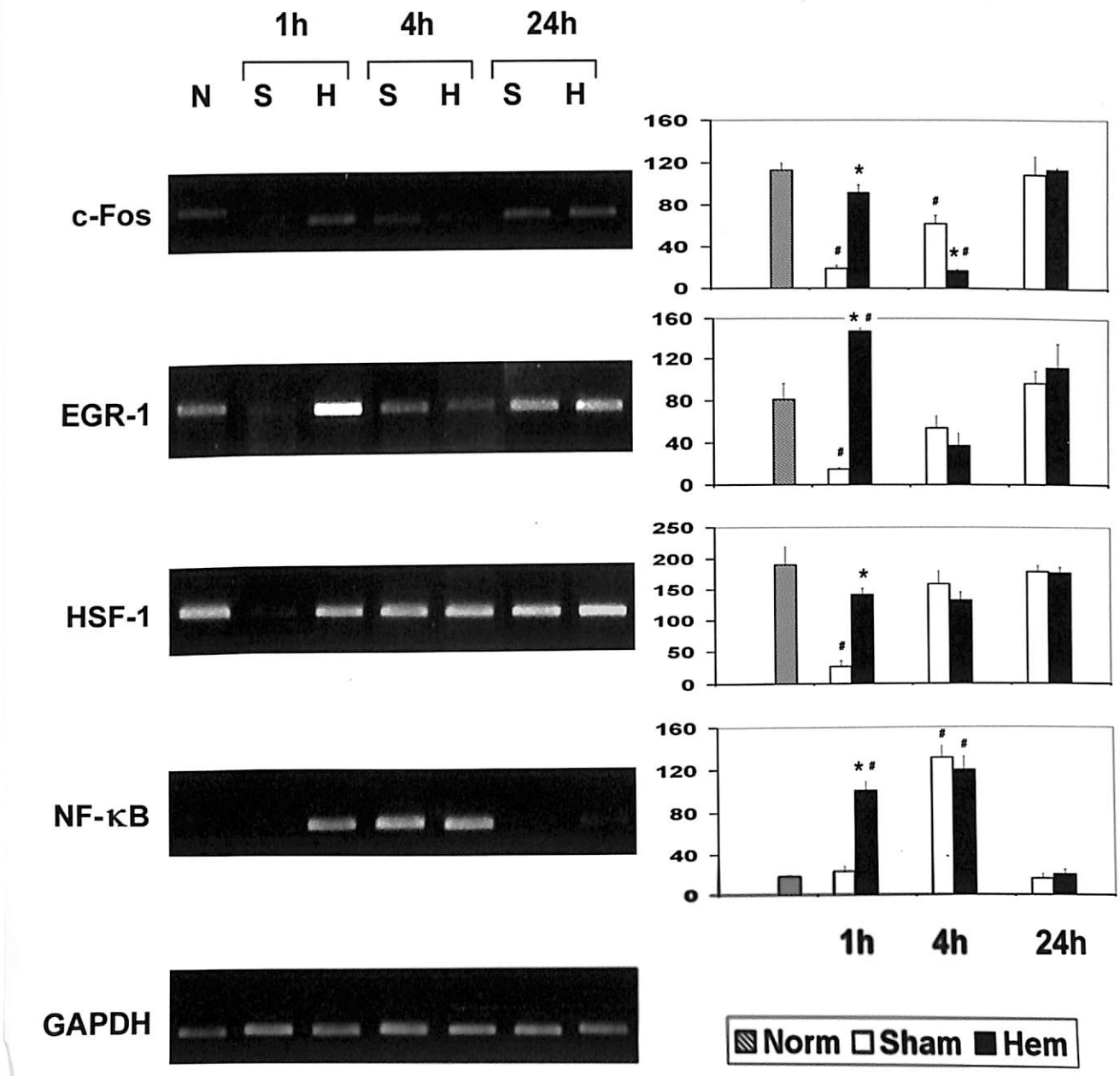

1h
4h
24h
N
S
H
S
H
S
H
c-Fos
EGR-1
HSF-1
NF-κB
GAPDH
160
120
80
40
0
250
200
150
100
50
1h
4h
24h
Norm
Sham
Hem

Fig. 8: Effect of hemorrhage on transcription factor activity. Nuclear extracts were prepared from the liver of normal mice (Norm: N), those subjected to sham surgery (Sham: S) and hemorrhagic shock (Hem: H). Transcription factor binding was analyzed at 1, 4 and 24 h following experimental procedure by EMSA. ^{32}P-labeled oligonucleotide probes were incubated with the nuclear extracts and analyzed on 4% non-denaturing polyacrylamide gels. Dried gels were exposed to X-ray film for visualization and analyzed using a phosphor-imager. Data are represented as gross cpm of radioactivity (mean ± SEM of five animals). * signifies $p < 0.01$ compared with Sham and # signifies $p < 0.01$ compared with Norm.

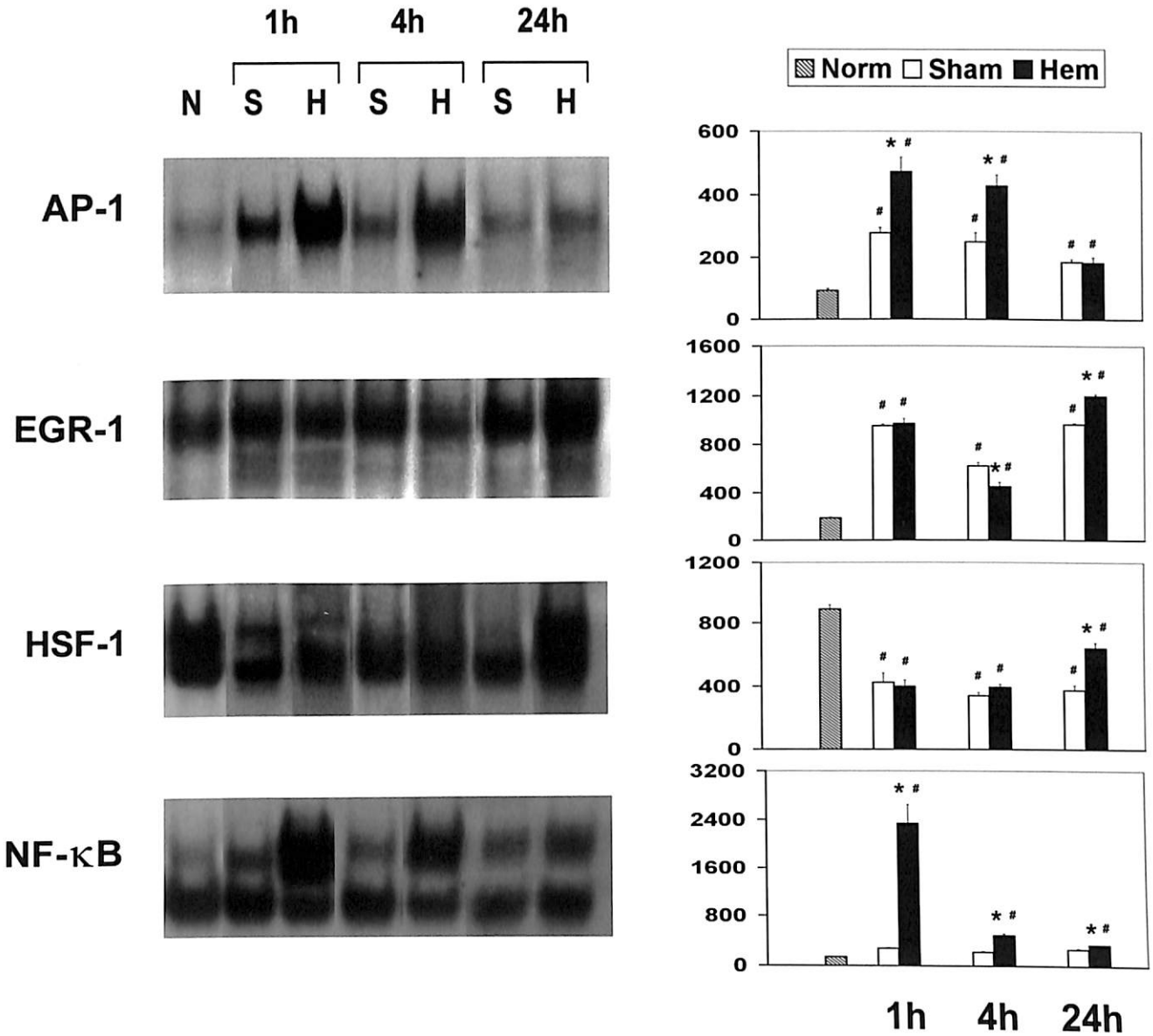

1h
4h
24h
N
S
H
S
H
S
H
AP-1
EGR-1
HSF-1
NF-κB
Norm
Sham
Hem
600
400
200
0
1600
1200
800
400
0
1200
800
400
0
3200
2400
1600
800
0
1h
4h
24h

Fig. 9: Effect of hemorrhage on lipid peroxidation in mouse liver. MDA was measured as the cellular marker for lipid peroxidation. Liver homogenates were incubated with a chromogenic reagent and the absorbance measured using a spectrophotometer at 586 nm. MDA values were calculated based on the standards provided by the manufacturer and are expressed in terms of tissue protein content. Values are mean ± SEM of five mice. * $p < 0.05$ compared with Sham.

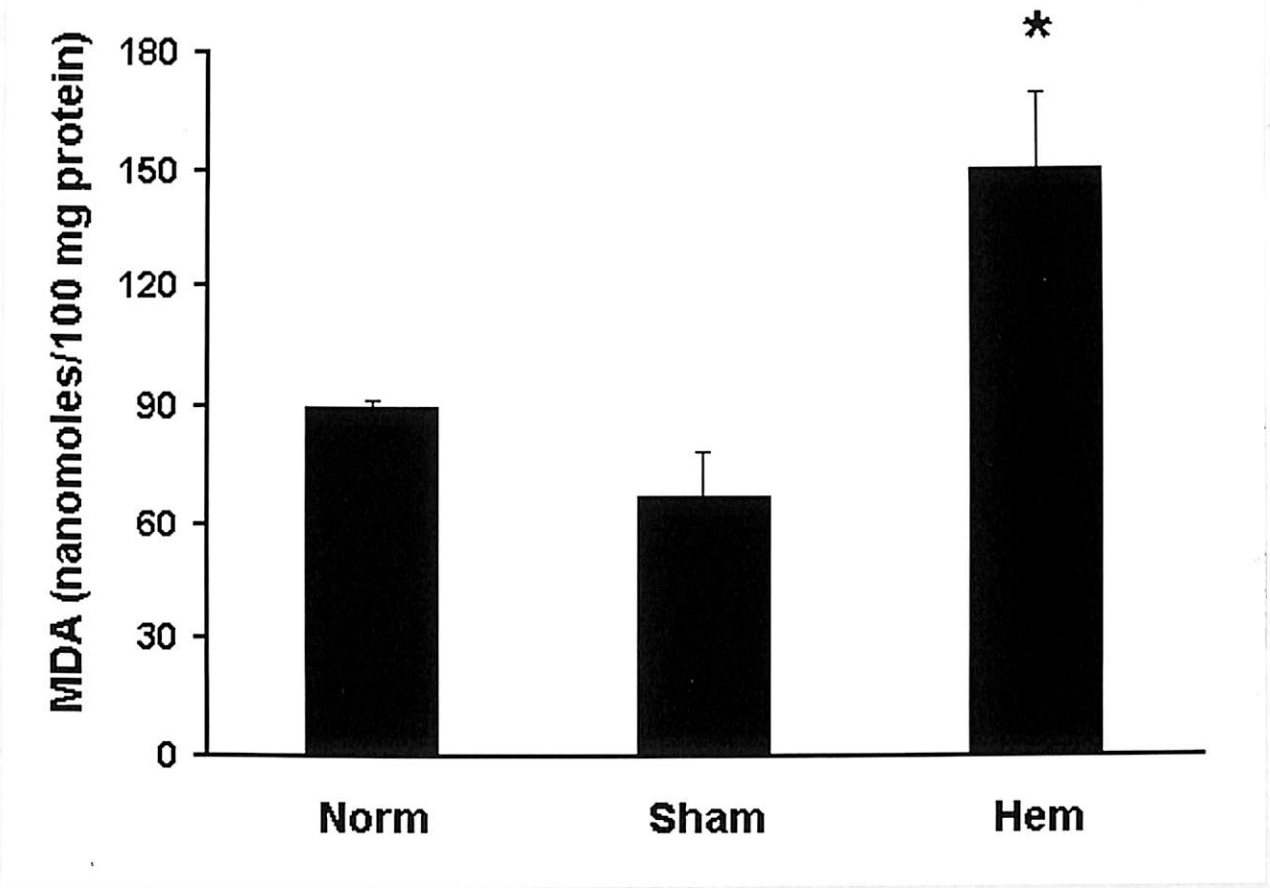
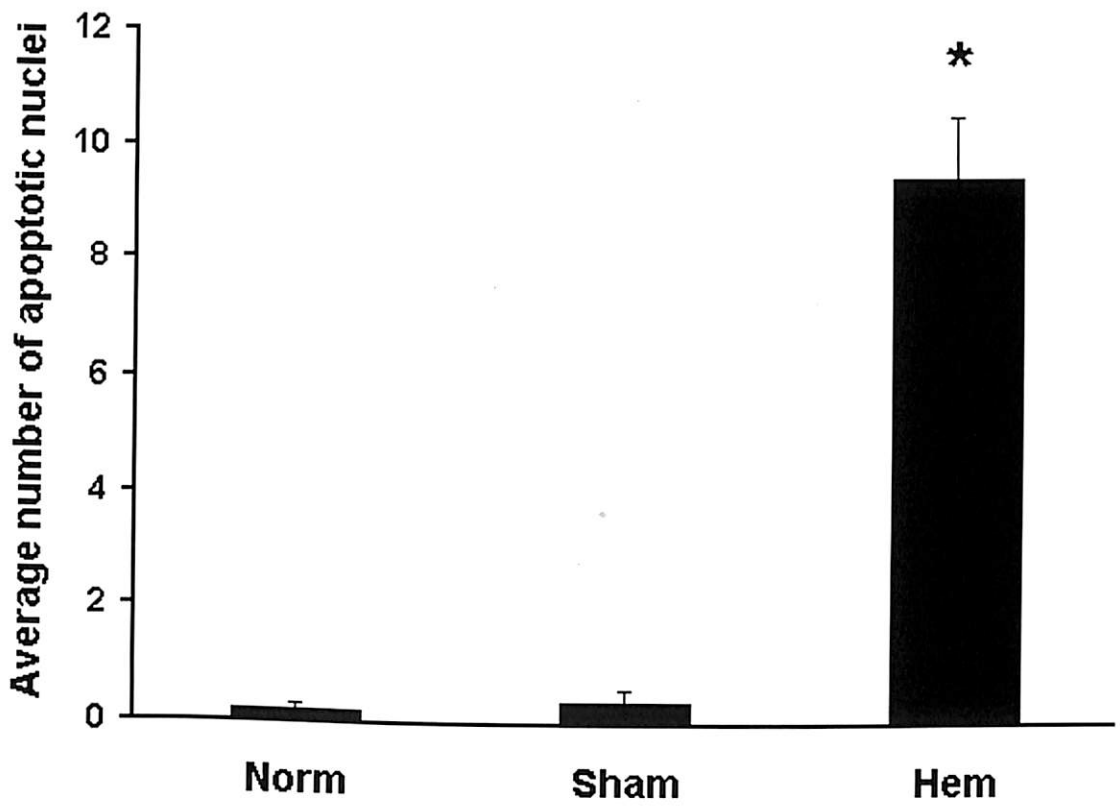

MDA (nanomoles/100 mg protein)
180
150
120
90
60
30
0
*
Norm
Sham
Hem

Fig. 10: Apoptotis-induction by hemorrhage. The mean ± SEM of apoptotic nuclei observed in ten different microscopic fields (40X magnification) was plotted. The degree of liver damage was significantly increased in hemorrhaged animals. * $p < 0.01$ compared with Sham.


Average number of apoptotic nuclei
12
10
8
6
4
2
0
*
Norm
Sham
Hem

Picroliv Improves Antioxidant Status and Downregulates Inflammatory Genes

Effect of picroliv on MAP

The change in blood pressure was monitored throughout hemorrhage and resuscitation in the rat H/R model (Fig. 11). At the onset of hemorrhage, blood pressure decreased to about 25% of normal MAP. During shock, MAP stabilized at approximately 50 mmHg and recovered to near-initial values at the end of resuscitation. Picroliv treatment did not cause any appreciable change in MAP during the experiment.

Effect of picroliv on serum AST

We studied the effect of picroliv on liver injury by estimating an important liver injury marker, AST in serum. Our observations indicate that following H/R, both the enzymes are significantly augmented compared with sham and picroliv control rats, indicating substantial injury. AST levels significantly increased from 158.8 ± 8.8 U/L in the sham group to 203.5 ± 28.1 U/L in the H/R group ($p < 0.05$). Picroliv pretreated hemorrhaged rats exhibited a remarkable decrease ($p<0.05$) of AST levels (127.8 ± 6.8 U/L), indicating improved liver function. Picroliv-fed control samples showed slightly lower enzyme levels than sham (136.2 ± 14.7 U/L).

Effect of picroliv on lipid peroxidation

MDA levels in liver tissue were measured to assess the effect of picroliv on lipid peroxidation. Following H/R injury we observed an increase in the free radical mediated lipid peroxidation as compared with sham animals ($p < 0.05$). Picroliv pre-fed rats were protected against lipid peroxidation as indicated by a significant ($p < 0.05$) decrease in their MDA levels post H/R, comparable with that of the picroliv control group (Fig. 12). Sham surgery caused a slight increase in lipid peroxidation compared with picroliv control animals that were not otherwise manipulated.

Effect of picroliv on the GSH redox system

Reduced glutathione (GSH) is part of the cellular antioxidant pool that defends against free radical insult. We studied the effect of H/R injury on GSH levels in liver homogenates of rats pretreated with picroliv. We observed that control H/R rats had a substantial decrease in GSH levels ($p<0.05$) compared with sham-operated rats, indicating a depletion of the GSH pool. Picroliv did not affect GSH levels after H/R (Table 6).

Glutathione reductase (GR) and glutathione peroxidase (GPx) contribute to the maintenance of the antioxidant status of the cell by redox regulation of GSH. Hence, we studied the alterations in the activity of GR and GPx enzymes in liver tissue following H/R. There was a significant increase ($p < 0.05$) in the GR activity in liver following H/R, which was further increased in picroliv pre-fed hemorrhaged rats, suggesting a better anti-oxidant status. GR enzyme activity in

picroliv control rats was significantly lower than the other groups. We observed similar GPx activities in all groups (Table 6).

Effect of picroliv on NO and iNOS

We studied the alterations in NO levels using a colorimetric assay to assess the extent of reactive-species mediated injury in liver after H/R. We observed a significant increase of NO in liver homogenates following H/R, which was significantly abated in the picroliv-pretreated H/R group ($p < 0.05$). The NO levels in the picroliv control group were below detectable limits (Fig. 13).

Alterations in NO could be the result of iNOS activity; hence mRNA expression of this enzyme was studied using RT-PCR. The pattern of iNOS expression was similar to that of NO. Control H/R samples had significantly higher iNOS mRNA compared with sham ($p < 0.05$). There was a significant decrease ($p < 0.05$) in iNOS expression in samples from picroliv-pretreated H/R rats compared with the control H/R group (Fig. 14). There was minimal expression of iNOS in the picroliv control group.

Effect of picroliv on AP-1 and NF-κB

We used gel shift assay to compare the transactivation of AP-1 and NF-κB across picroliv-treated and untreated animals. The AP-1 binding site was used as oligonucleotide probe and mixed with nuclear extracts prepared from liver tissue. As illustrated in Fig. 15, there was minimal AP-1 expression in picroliv control

animals (lane 4). AP-1 was strongly induced by H/R (lane 6) compared with sham (lane 5). There was approximately six-fold increase in the band intensity of H/R group compared with Sham. Picroliv substantially inhibited the transactivation of AP-1 (lane 7). Transactivation of AP-1 in the picroliv control group was similar to the Sham animals. Positive, negative and competitor controls were used to assess the specificity of binding (lanes 1 - 3). Supershift assay revealed AP-1 to be a homodimer of c-jun or a heterodimer of c-jun and c-fos (lanes 8 - 10). The band-intensities are shown in the legend of Fig. 15. Though H/R activated NF-κB, picroliv treatment did not have any effect on its transactivation (figure not shown).

Effect of picroliv on IEGs

Since the AP-1 complex downregulated by picroliv was found to consist of c-jun and c-fos, we assessed the amount of c-jun and c-fos mRNA and protein in liver tissue by RT-PCR and Western blotting respectively. c-jun gene expression was not significantly affected by H/R or picroliv treatment at the 2 hr time point (Fig. 16). The c-fos mRNA levels were significantly upregulated in H/R controls compared with sham animals ($p < 0.05$) (Fig. 16). Pretreatment with picroliv reduced c-fos mRNA level significantly ($p < 0.05$). Picroliv control animals had similar level of c-fos expression as the sham group.

Western blotting showed that picroliv treatment *per se* had no effect on the expression of c-jun and c-fos proteins (Fig. 17). Sham surgery increased c-fos expression substantially compared with the picroliv control group. There was an increase in c-jun and c-fos protein in whole cell extracts of H/R controls

Fig. 11: Effect of picroliv on MAP. Blood was withdrawn from anaesthetized rats through the femoral artery. Hemorrhage was initiated at 0 min. After 1 h of shock, animals were resuscitated over a period of 20 min till MAP stabilized at 90 min. MAP was recorded every 5 min in terms of mm Hg. and each data point is mean ± SEM of six animals.

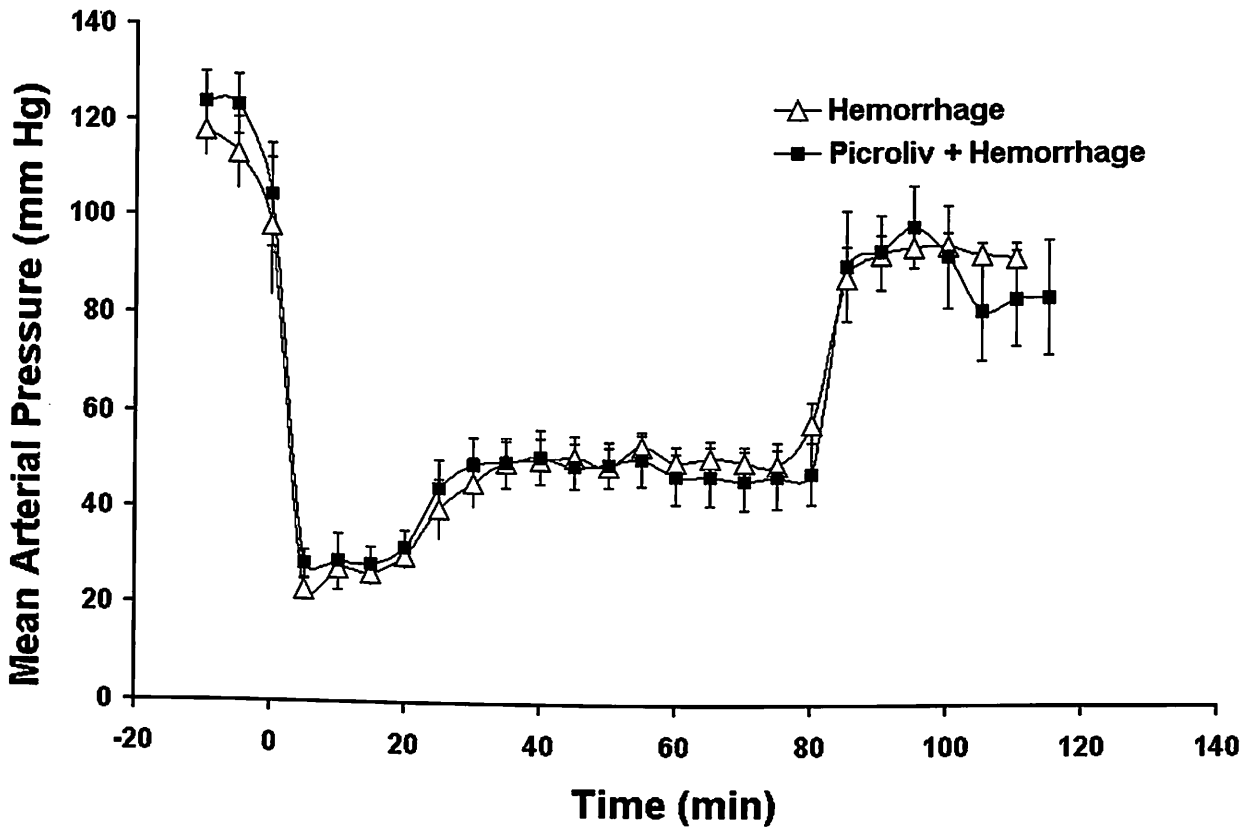

Mean Arterial Pressure (mm Hg)
140
120
100
80
60
40
20
0
-20
0
20
40
60
80
100
120
140
Time (min)
Hemorrhage
Picroliv + Hemorrhage

Fig. 12: Picroliv decreases liver MDA levels after H/R. Lipid peroxidation was measured using MDA as marker. Liver homogenates were prepared in 20 mM Tris-HCl buffer, incubated with a chromogenic reagent at 45°C and the absorbance of the resulting stable chromophore was measured at 586 nm. Values were calculated using MDA standards provided by the manufacturer and are expressed as mean ± SEM of four animals for picroliv control (Pic) and seven to eight animals for the other groups. * $p < 0.05$ vs. Sham, ** $p < 0.05$ vs. H/R.

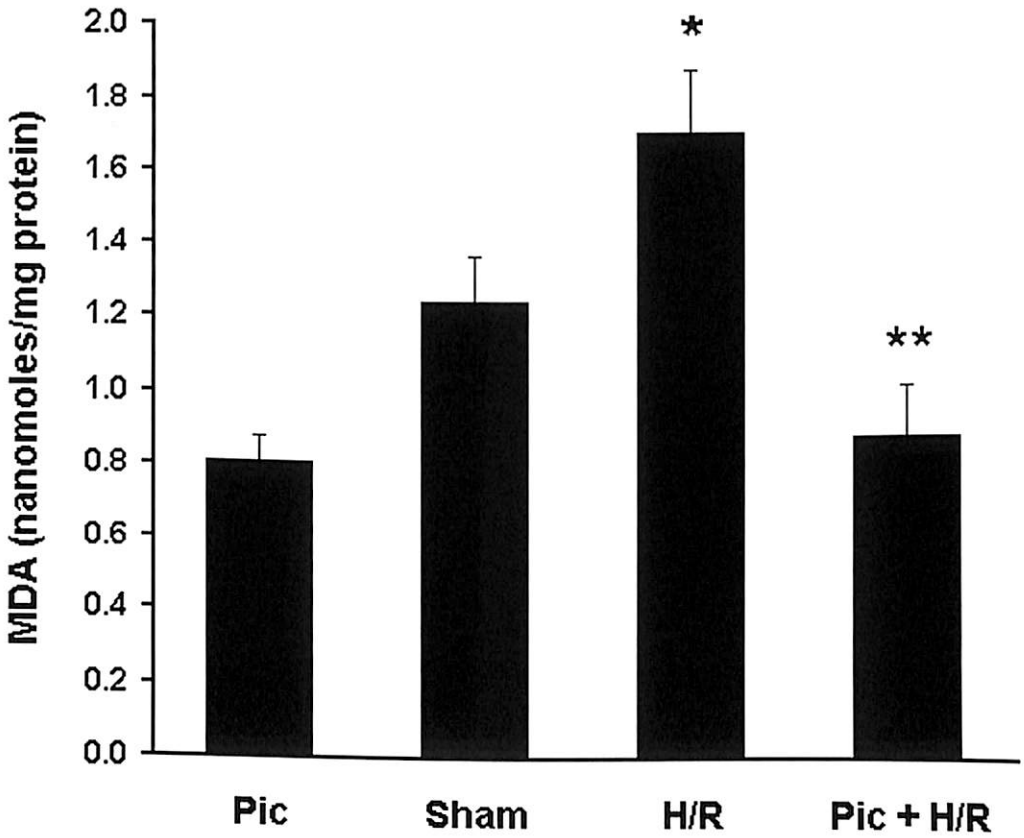
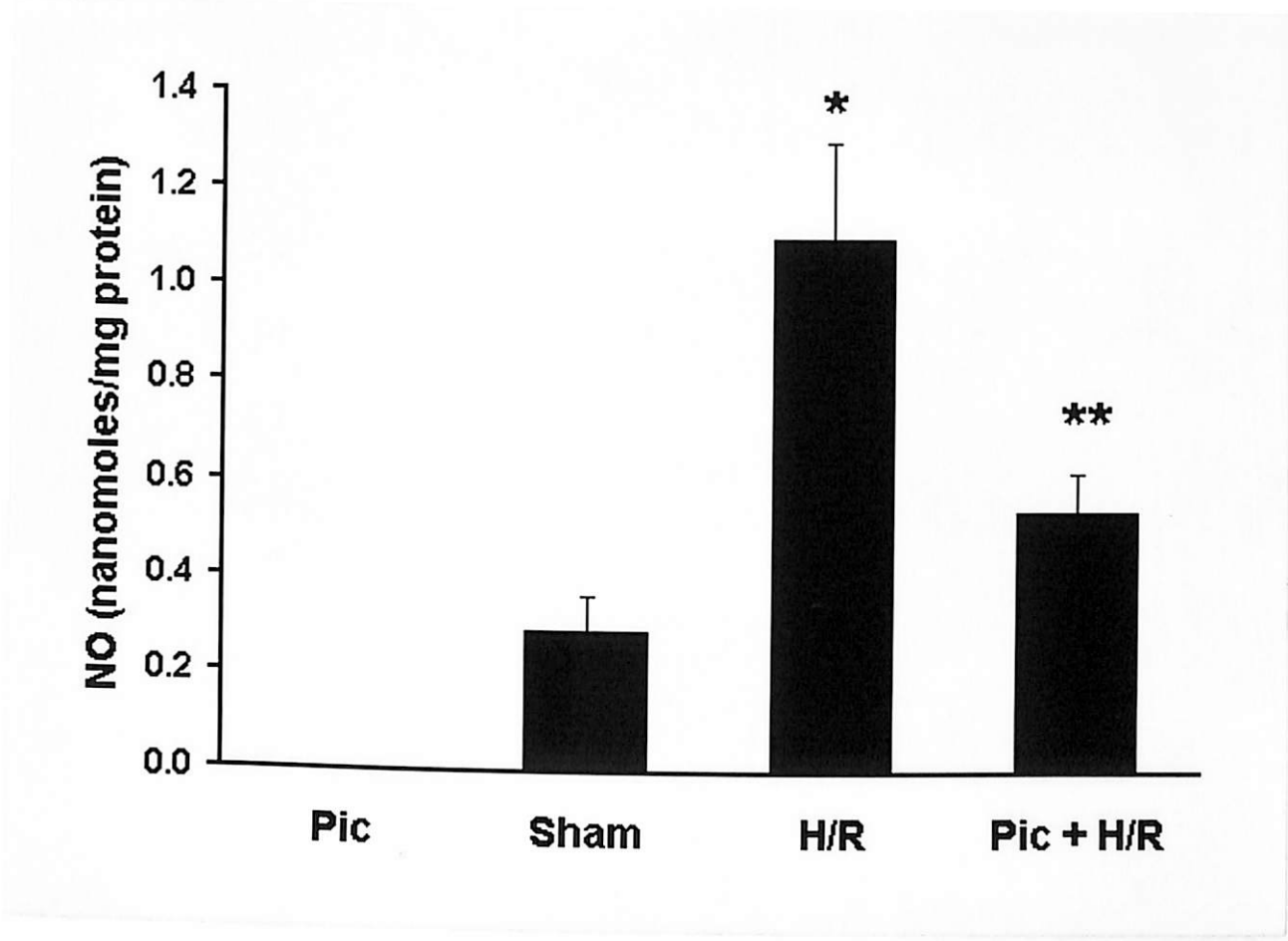

MDA (nanomoles/mg protein)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
Pic
Sham
H/R
Pic + H/R

Fig. 13: Picroliv decreases NO in liver tissue following H/R. Nitric oxide levels in liver tissue were assessed in terms of total nitrite. Liver homogenates were incubated with reaction buffer, reduced NADH and nitrate reductase. Greiss reagent was then added to the reaction mix and absorbance was measured at 570 nm. Sodium nitrite, provided in the kit, was used as standard to calculate NO concentration. NO levels in Pic group were below detectable limits. Values are expressed as mean ± SEM of four animals for picroliv control (Pic) and seven to eight animals for the other groups. * $p < 0.05$ vs. Sham, ** $p < 0.05$ vs. H/R.


NO (nanomoles/mg protein)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
Pic
Sham
H/R
Pic + H/R

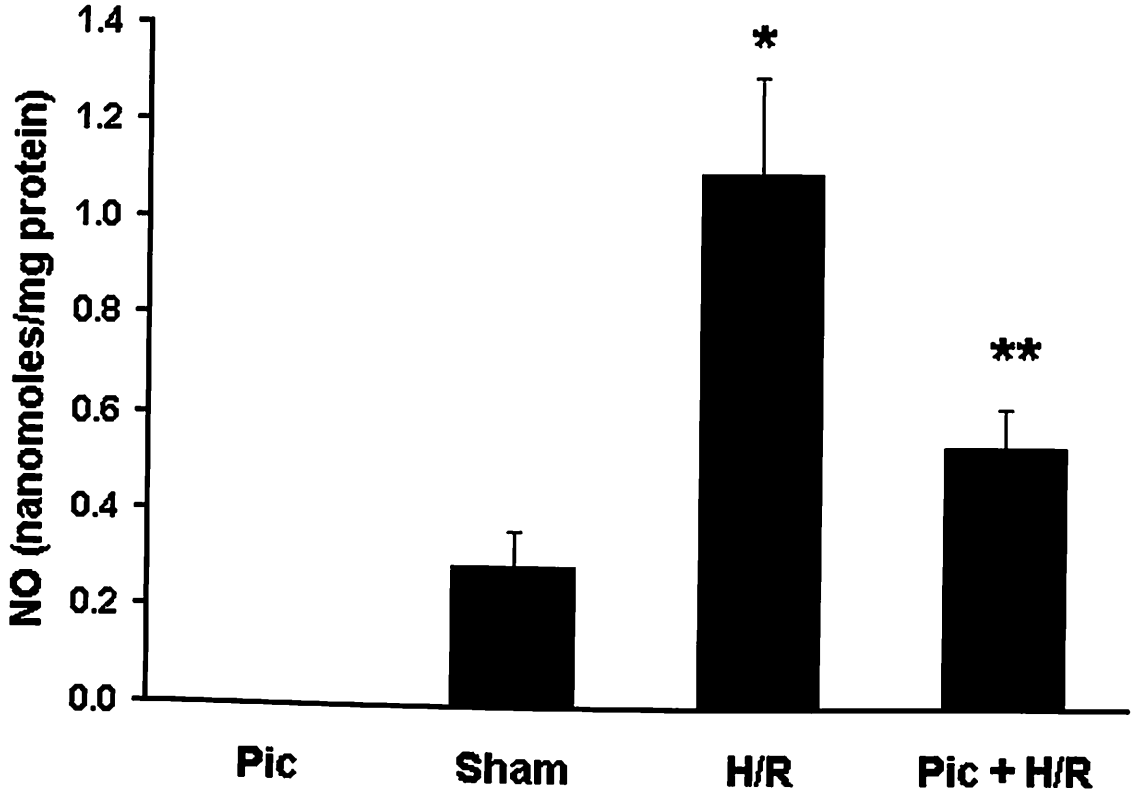

NO (nanomoles/mg protein)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
Pic
Sham
H/R
Pic + H/R

Fig. 14: Picroliv inhibits iNOS gene expression following H/R. Total RNA was isolated from liver tissue and reverse transcribed. cDNA was amplified by PCR and analyzed on ethidium bromide stained 2% agarose gels. GAPDH was used as the internal control for RNA quality. Densitometry units were normalized with respect to GAPDH. Values are expressed as mean ± SEM of four animals. * $p < 0.05$ vs. Sham, ** $p < 0.05$ vs. H/R

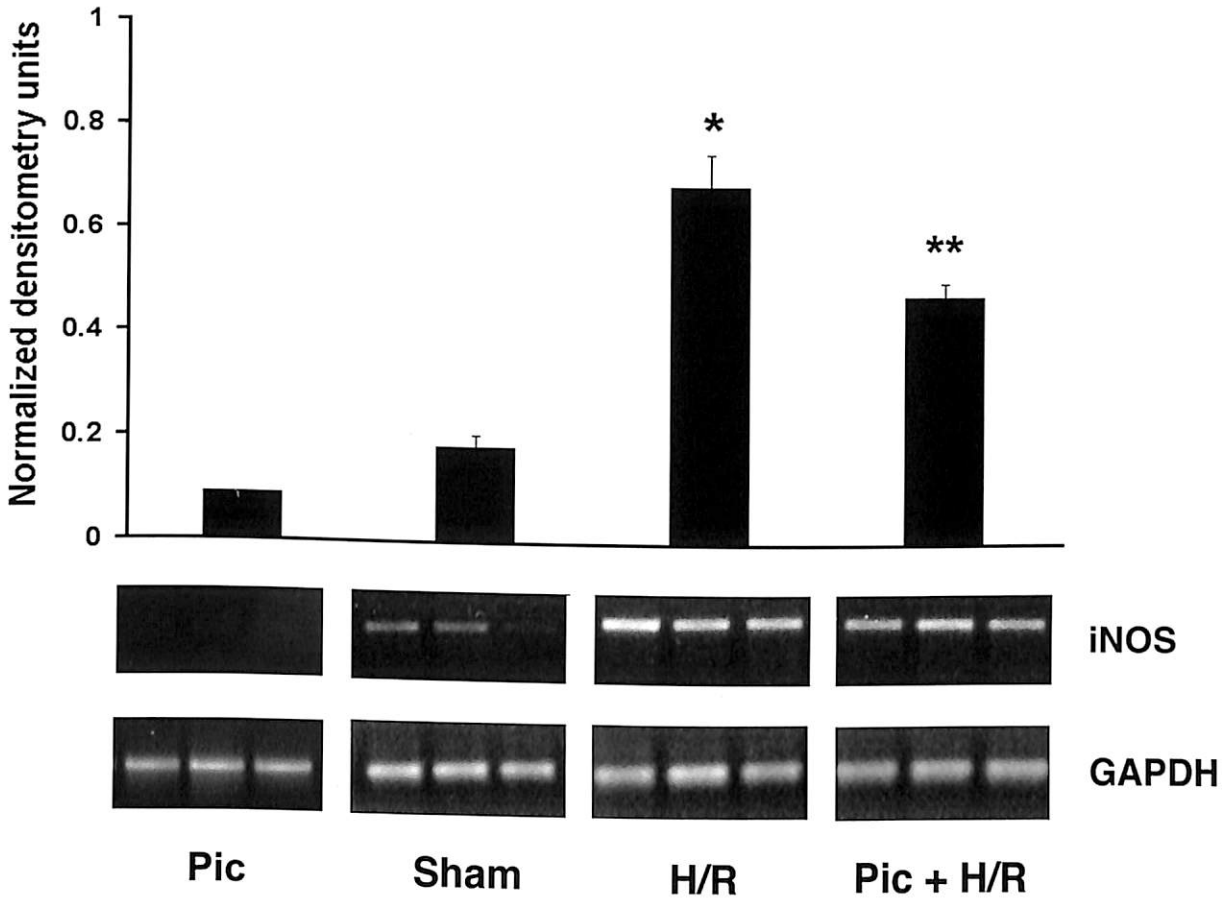

Normalized densitometry units
1
0.8
0.6
0.4
0.2
0
*
**
iNOS
GAPDH
Pic
Sham
H/R
Pic + H/R

Fig. 15: Picroliv inhibits the transactivation of AP-1 in liver following H/R. ^{32}P-labeled AP-1 probe was incubated with liver nuclear extracts and analyzed on 4% non-denaturing polyacrylamide gels. Dried gels were exposed to X-ray film. Supershift assay was carried out by incubating the nuclear extracts with anti c-jun or anti c-fos antibodies before probe binding. Arrow indicates the relevant AP-1 band. Lane 1: positive control (HeLa extract), lane 2: negative control (no nuclear extract), lane 3: specific competitor (cold probe in excess), lane 4: picroliv control, lane 5: sham, lane 6: hemorrhage, lane 7: picroliv + hemorrhage, lane 8: hemorrhage, lane 9: c-jun antibody, lane 10: c-fos antibody. The intensities of the bands in lanes 4-7 were 4014 ± 312, 5261 ± 289, 30125 ± 586 and 6996 ± 104 arbitrary units respectively. Figure is representative of 4 animals for picroliv control and 6 animals for the other groups.

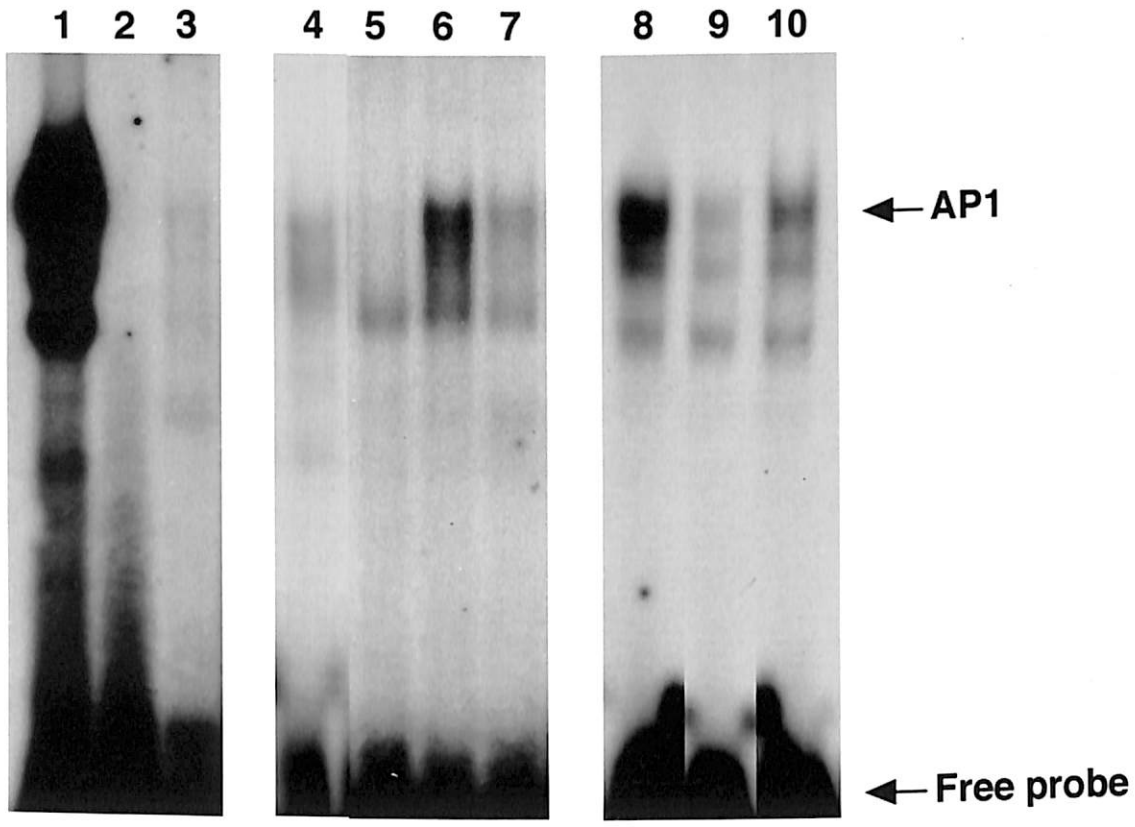

1 2 3
4 5 6 7
8 9 10
AP1
Free probe

Fig. 16: Effect of picroliv on c-jun and c-fos mRNA levels. Total RNA was isolated from liver tissue and reverse transcribed. cDNA was amplified by PCR and analyzed on ethidium bromide stained 2% agarose gels. Ribosomal protein L28 was used as internal control for RNA quality. Densitometry units were normalized with respect to L28. Values are expressed as mean ± SEM of 4 animals for picroliv control (Pic) and 5-6 animals in the other groups. * $p < 0.05$ vs. Sham. ** $p < 0.05$ vs. H/R.

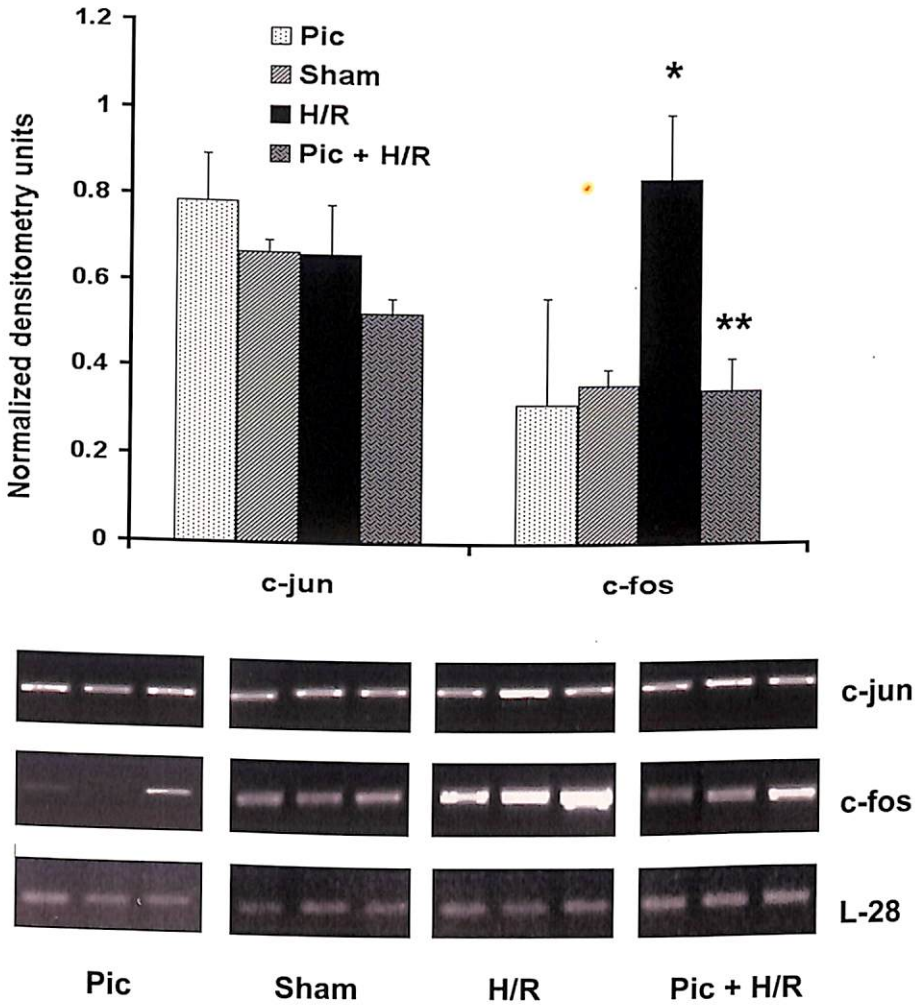

Normalized densitometry units
1.2
1
0.8
0.6
0.4
0.2
0
Pic
Sham
H/R
Pic + H/R
*
**
c-jun
c-fos
c-jun
c-fos
L-28
Pic
Sham
H/R
Pic + H/R

Fig. 17: Effect of picroliv on c-jun and c-fos protein. Protein extracts were prepared by homogenizing liver tissue in lysis buffer containing protease inhibitors and analyzed by SDS-PAGE and immunoblotting with polyclonal anti c-jun or anti c-fos antibodies. Specific 39 kD (c-jun) and 62 kD (c-fos) bands were visualized by enhanced chemiluminescence. Figure is representative of four rats for the Pic group and six rats for the other groups. The band intensities are 3870 ± 110, 3766 ± 53, 9792 ± 230, 3965 ± 148 (c-jun) and 2764 ± 325, 7459 ± 85, 8088 ± 93, 3532 ± 27 (c-fos).

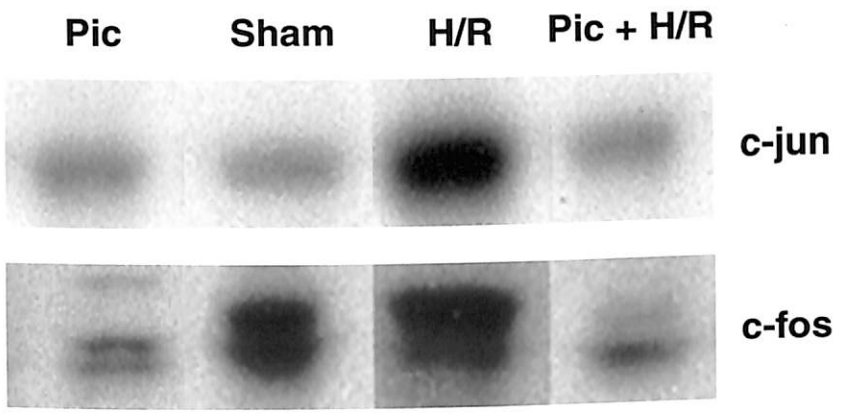
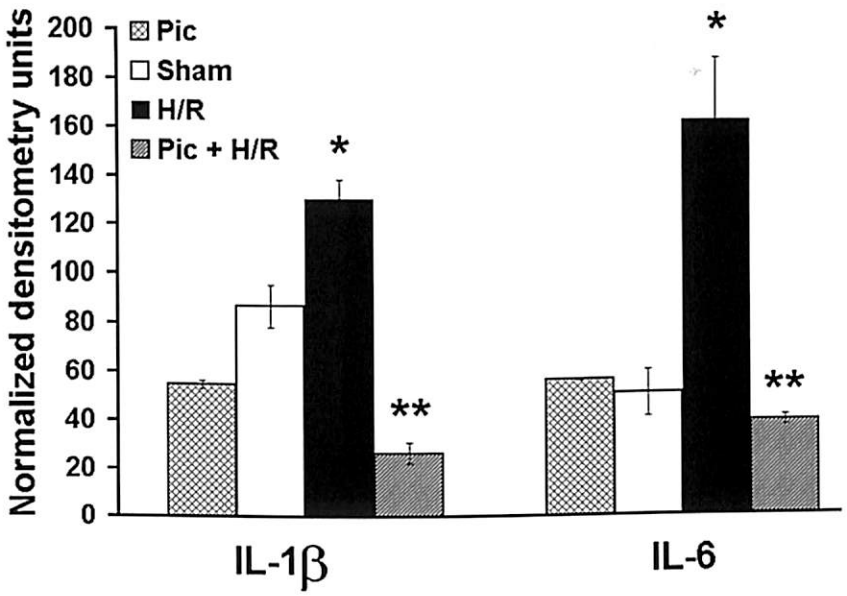
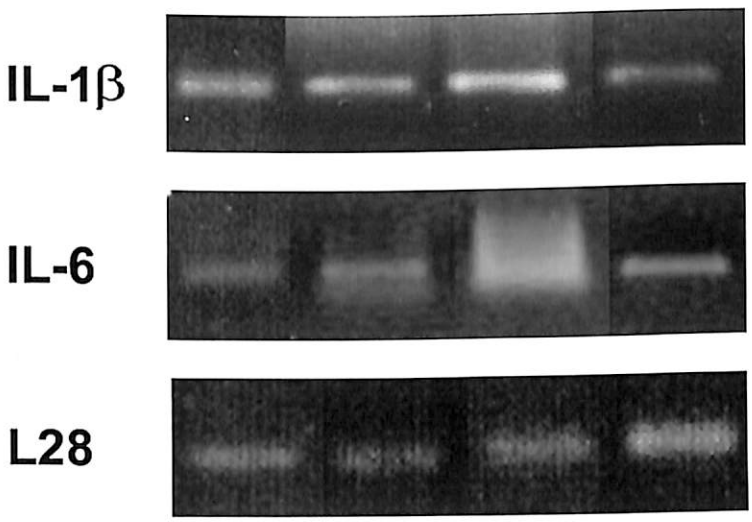

Pic
Sham
H/R
Pic + H/R
c-jun
c-fos

Fig. 18: Effect of picroliv on pro-inflammatory cytokines. IL-1β and IL-6 were assessed in liver samples by RT-PCR and visualized by ethidium bromide staining. Densitometric units were normalized with respect to L28. Figure is representative of four animals for the picroliv control (Pic) group and five to six animals for the other groups. * $p < 0.05$ compared with Sham, ** $p < 0.05$ compared with H/R group.


Normalized densitometry units
200
180
160
140
120
100
80
60
40
20
0
Pic
Sham
H/R
Pic + H/R
*
**
*
**
IL-1β
IL-6


IL-1β
IL-6
L28

Curcumin Modulates Cytokines and Transcription Factors

Effect of curcumin on MAP

We assessed the changes in arterial blood pressure during hemorrhage and resuscitation in both curcumin-treated and untreated groups. Average pre-hemorrhage mean arterial pressures (MAP) were similar for both groups at approximately 130 mm Hg as shown in Fig 19 (values given as average MAP ± SEM). The mean blood pressure in all groups decreased to 30-50 mm Hg during hemorrhagic shock. Upon resuscitation the MAP reached 90-100 mm Hg. Blood pressures for both hemorrhage and curcumin treated hemorrhage groups showed similar patterns during the entire experiment.

Effect of curcumin on serum AST

Liver injury was assessed by measuring the concentration of AST in serum. There were no significant differences in AST levels between different treatment groups 2 h post-resuscitation (data not shown). However, AST levels 24 h post-resuscitation increased from 273 ± 40 U/L in sham to 1660 ± 295 U/L in hemorrhage group ($p < 0.05$). AST level in curcumin pretreated hemorrhage group of animals was 1010 ± 89 U/L, significantly lower compared with hemorrhage group ($p < 0.05$). Curcumin-fed controls showed much lower AST activity compared with all other groups (130 ± 21 U/L).

Effect of curcumin on the expression of cytokines in liver

We investigated the cytokine mRNA profiles in curcumin-fed rats subjected to hemorrhagic shock and resuscitation. Fig. 20 shows the RT-PCR results along with quatitative densitometric analyses represented graphically. GAPDH gene expression indicates equal amounts of target RNAs in RT-PCR reactions. In the curcumin control group, there was little or no mRNA expression for most cytokines except IL-1β and IL-2. Marked differences were observed between the cytokine mRNA levels of curcumin treated hemorrhage, untreated hemorrhage and sham groups.

Gene expression analysis of 2 h post resuscitation groups showed increased production of IL-1α, IL-1β, IL-2, IL-6 and IL-10 in the hemorrhage group compared with sham animals ($p < 0.05$). There was no change in TNF-α between sham and hemorrhage groups. Curcumin pretreatment inhibited the expression of IL-1α, IL-1β, IL-2 and IL-6 significantly but did not affectL -10 or TNF-α mRNA levels ($p < 0.05$).

24 h post-resuscitation, the pattern of expression of these inflammatory cytokines was different from that at 2 h, except for IL-6 (Fig. 20). The pro-inflammatory cytokine response subsided at this late time point. H/R did not cause any changes in IL-1α, IL-1β and IL-2 levels compared with the sham group. Curcumin pretreatment resulted in slightly reduced IL-1β, which is significant only in comparison with sham. Unlike in the 2 h groups, curcumin treatment resulted in significantly higher levels for IL-2 at 24 h post-resuscitation in comparison with sham. IL-6 levels were similar to that of 2 h groups with a

significant increase after H/R and significant reduction in curcumin treated hemorrhage group. In case of IL-10 and TNF-α mRNAs, H/R caused significant decrease in mRNA levels. Curcumin treatment had no effect on IL-10 compared with the control H/R group whereas TNF-α levels were reversed to those observed in sham group.

Effect of curcumin on the activity of NF-κB and AP-1

To explore the effect of curcumin on the transactivation of NF-κB and AP-1 following H/R, we examined the DNA binding activity of NF-κB and AP-1 transcription factors in nuclear extracts obtained from rats at 2 h and 24 h post-resuscitation. A typical autoradiogram for NF-κB is shown in Fig 21. The relevant DNA-protein complex is the upper band, indicated by an arrow. The intensities of the bands (arbitrary units) for NF-κB are shown in the legend of Fig 21. The relative intensity of the bands of the curcumin control group was much lower than that of the other groups. Relative intensities increased 1.5 fold in hemorrhage group (lane 3) compared with sham (lane 2) at 2 h post-resuscitation. No significant change was observed between sham and hemorrhage controls (lanes 5 and 6) of 24 h post resuscitation. Curcumin treatment followed by H/R strongly inhibited the binding of NF-κB (lanes 4 and 7). The NF-κB band was supershifted by binding with NF-κB p65 antibody (lane 11), indicating activation of the classic NF-κB complex.

Likewise, typical autoradiograms of DNA-protein complexes for AP-1 obtained from curcumin-fed, sham, untreated H/R and curcumin treated H/R

significant increase after H/R and significant reduction in curcumin treated hemorrhage group. In case of IL-10 and TNF-α mRNAs, H/R caused significant decrease in mRNA levels. Curcumin treatment had no effect on IL-10 compared with the control H/R group whereas TNF-α levels were reversed to those observed in sham group.

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Likewise, typical autoradiograms of DNA-protein complexes for AP-1 obtained from curcumin-fed, sham, untreated H/R and curcumin treated H/R

curcumin treated hemorrhage group. The control curcumin group had the least expression of p65 protein. This expression pattern of p65 was similar to the pattern of regulation of NF-κB in the nucleus (Fig. 21). The band intensities of p65 from whole cell extracts is similar to those of nuclear extracts in the 2 h H/R groups but in 24 h groups the intensities were not significantly different across the three groups. These observations revealed that transcription factor NF-κB protein is significantly decreased by curcumin treatment confirming the gel shift assay results.

IκBα protein was decreased in the cytoplasm after H/R, indicating its degradation to release NF-κB. In the curcumin + H/R group, IκBα levels were increased. Western immunoblot experiments with nuclear as well as whole cell extracts for c-jun and c-fos showed no significant differences in their protein expression between curcumin treated animals, hemorrhage control group and sham animals (data not shown).

Fig. 19: Effect of curcumin pretreatment on MAP. Blood withdrawal was initiated at 0 min and lasted for 10 min. After 1 h of shock, animals were resuscitated over a period of 20 min and were observed until the mean arterial pressure stabilized around 90 mm Hg. MAP was recorded every 5 min and each data point is mean $\pm$ SEM of six animals.

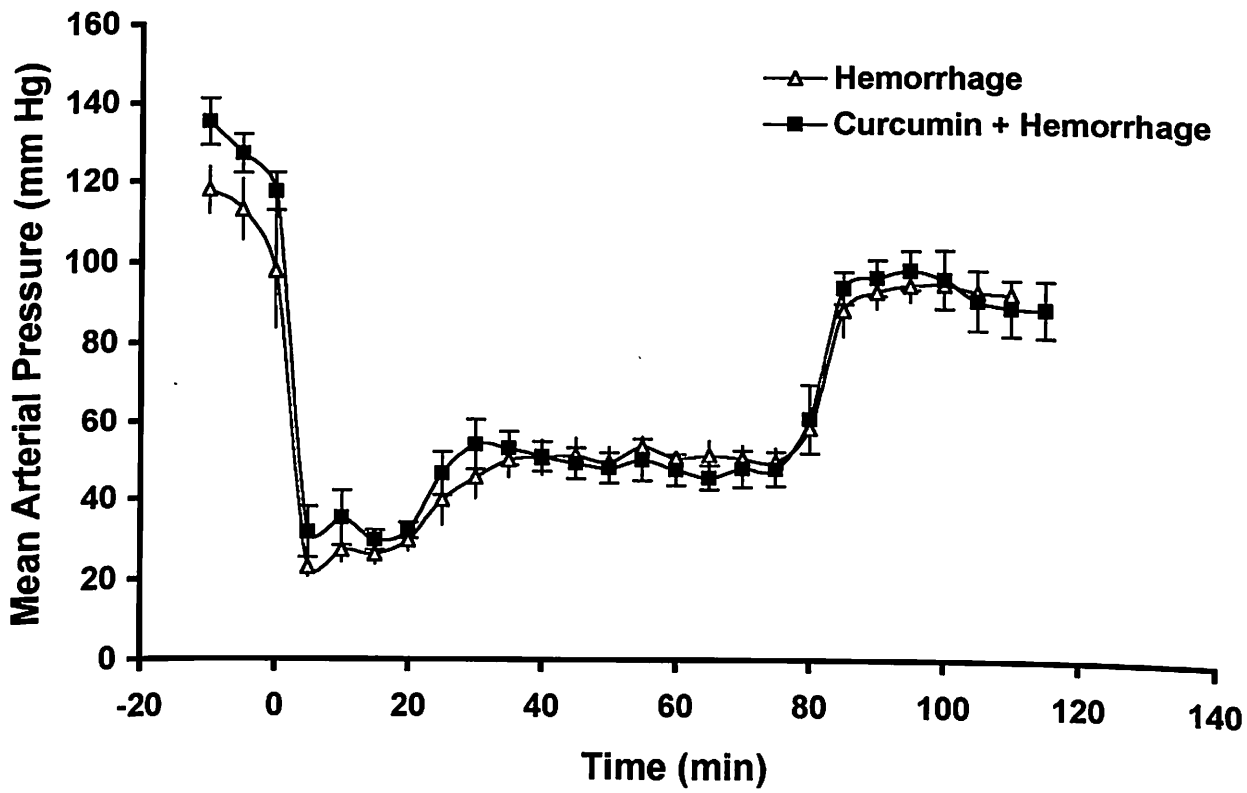

Mean Arterial Pressure (mm Hg)
160
140
120
100
80
60
40
20
0
-20
0
20
40
60
80
100
120
140
Time (min)
Hemorrhage
Curcumin + Hemorrhage

Fig. 20: Differential regulation of cytokines by curcumin. Hemorrhage resulted in increased levels of IL-1α (1.4 fold), IL-1β (1.4 fold), IL-2 (4.5 fold), IL-6 (3.2 fold) and IL-10 (1.3 fold) mRNAs 2 h post-resuscitation. Pretreatment with curcumin prevented the upregulation caused by H/R. 24 h groups show a different pattern of change in cytokine mRNA levels. Hemorrhage caused 1.7 fold increase of IL-6 and 2 fold decrease of TNF-α compared with sham controls. Curcumin treatment resulted in increased expression of IL-1α (1.3 fold), IL-2 (1.7 fold) TNF-α (1.8 fold) and decreased levels for IL-6 (1.9 fold) compared with the hemorrhage group. Two animals representing highest and lowest expression are shown from each group. Equal amount of RNA in the RT-PCR reactions was confirmed by analyzing GAPDH expression. Values are mean ± SEM of four animals for the curcumin control group and six animals for the other groups. * $p<0.05$ hemorrhage compared with sham; ** $p<0.05$ curcumin + hemorrhage compared with hemorrhage; *** $p<0.05$ curcumin + hemorrhage compared with sham.

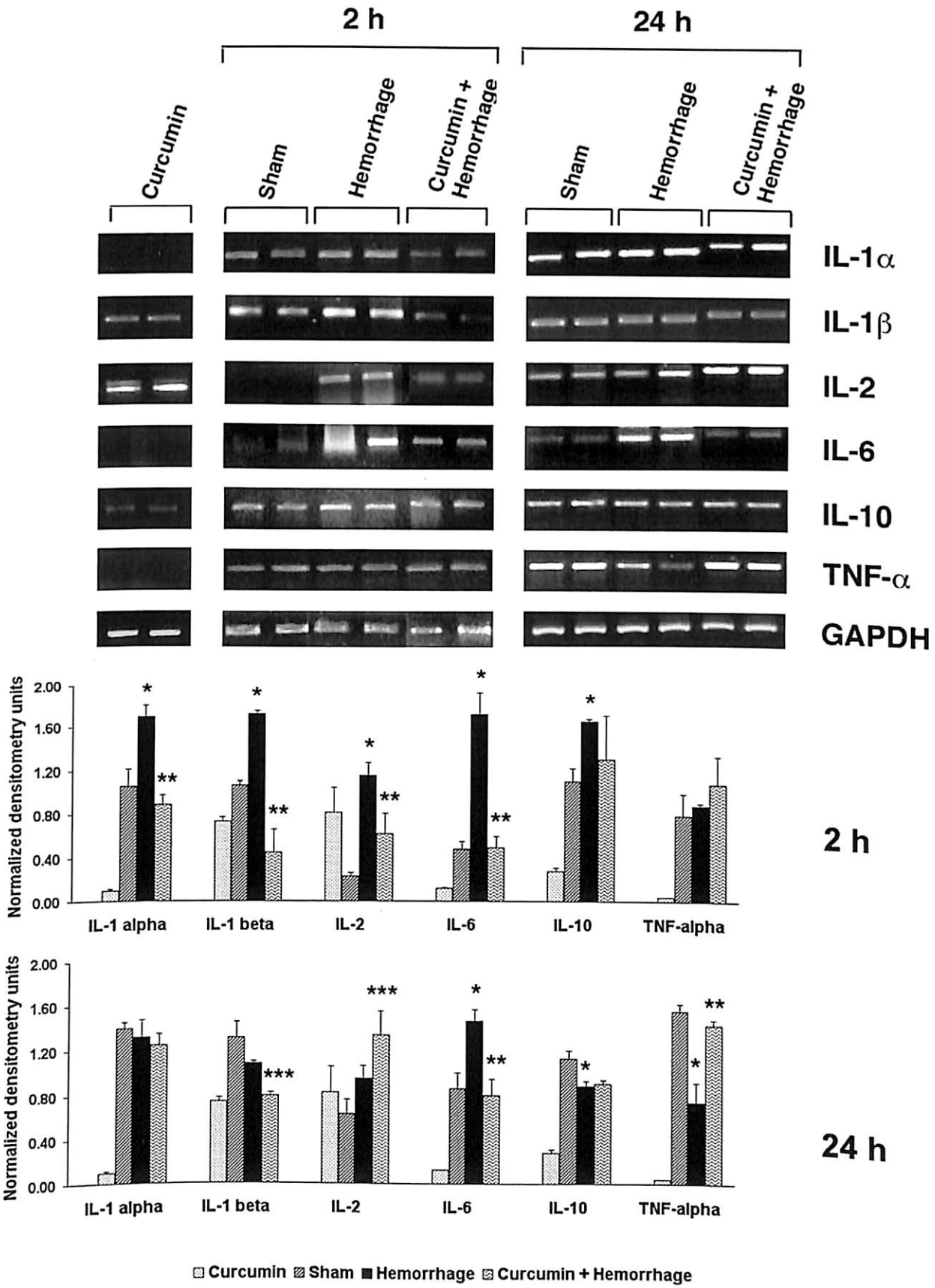

2 h
24 h
Curcumin
Sham
Hemorrhage
Curcumin + Hemorrhage
Sham
Hemorrhage
Curcumin + Hemorrhage
IL-1α
IL-1β
IL-2
IL-6
IL-10
TNF-α
GAPDH
Normalized densitometry units
2.00
1.60
1.20
0.80
0.40
0.00
IL-1 alpha
IL-1 beta
IL-2
IL-6
IL-10
TNF-alpha
2 h
24 h
Curcumin
Sham
Hemorrhage
Curcumin + Hemorrhage

Fig. 21: Curcumin inhibits the transactivation of NF-κB in liver following H/R. Gel mobility shift assays were performed using labeled NF-κB probe added to liver nuclear extracts from 2 h and 24 h groups, with curcumin-fed control (lane 1), sham (lanes 2 and 5), hemorrhage (lanes 3 and 6) and curcumin-treated hemorrhage (lanes 4 and 7) groups. Other controls are no nuclear extract (lane 8), HeLa nuclear extract (lane 9) and cold probe in excess (lane 11). Lane 11 represents the supershift observed with the addition of NF-κB p65 antibody. The intensities of the bands were 2828 ± 330, 3727 ± 215, 5606 ± 556, 3743 ± 389, 5101 ± 218, 4592 ± 183 and 2921 ± 301 arbitrary units for lanes 1-7 respectively. Values are mean ± SEM of four animals for curcumin control group and six animals for the other groups.

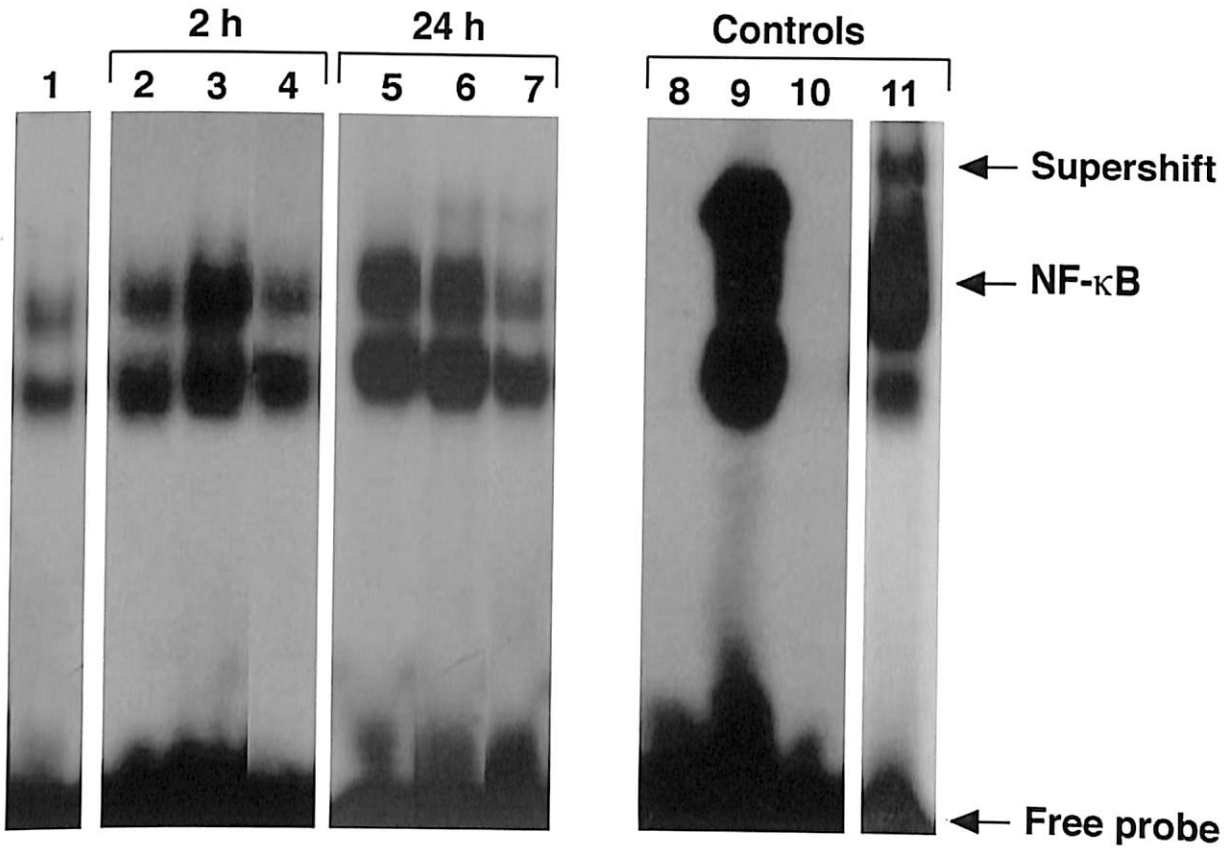

2 h
24 h
Controls
1
2
3
4
5
6
7
8
9
10
11
Supershift
NF-κB
Free probe

Fig. 22: Effect of curcumin on the transactivation of AP-1. Gel mobility shift assays were performed using labeled AP-1 probe added to liver nuclear extracts from 2 h and 24 h groups, with curcumin-fed control (lane 1), sham (lanes 2 and 5), hemorrhage (lanes 3 and 6) and curcumin treated hemorrhage (lanes 4 and 7) and with controls representing HeLa extract (lane 8) and no nuclear extract (lane 9). Supershift assay is shown for AP-1 probe without antibody (lane 10), with c-jun antibody (lane 11) and c- fos antibody (lane 12). Band intensities were 1956 ± 223, 1736 ± 93, 5796 ± 549, 4699 ± 777, 3141 ± 396, 1692 ± 344 and 1812 ± 135 arbitrary units for lanes 1-7 respectively. Values are mean ± SEM of four animals for the curcumin control group and six animals for the other groups.

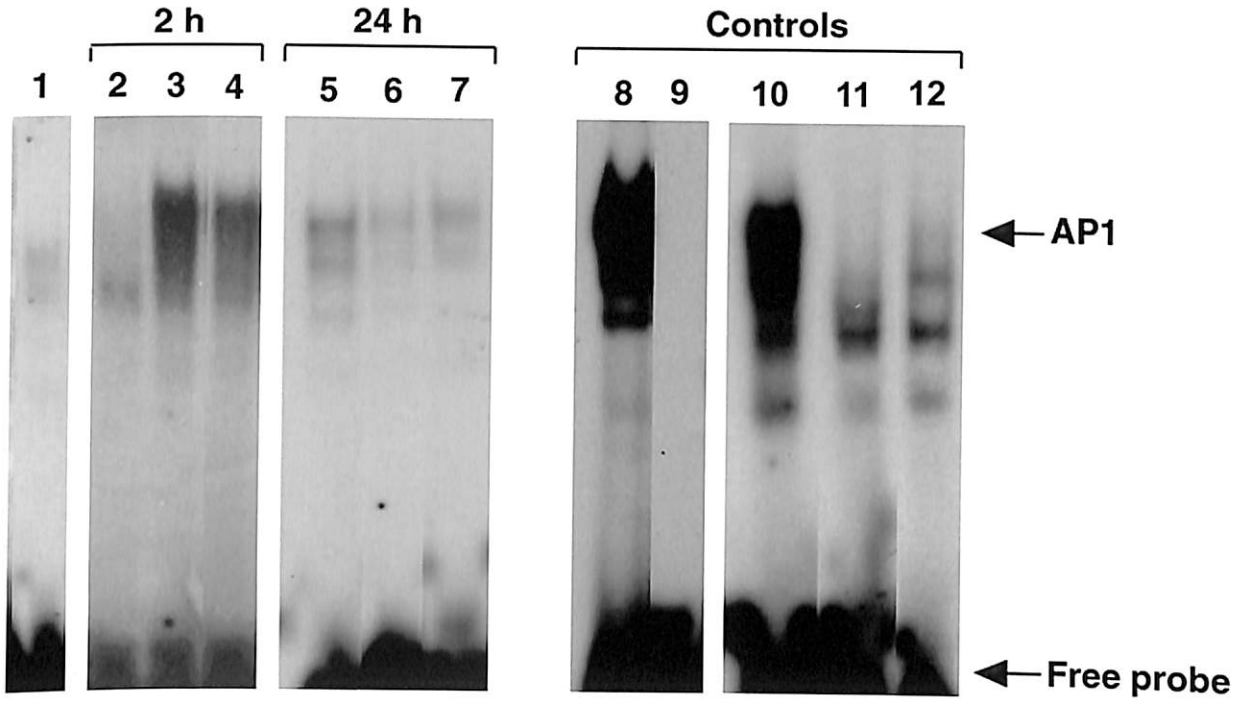

2 h
24 h
Controls
1
2
3
4
5
6
7
8
9
10
11
12
AP1
Free probe

Fig. 23: Analysis of NF-κB (p65) and IκBα in nuclear and whole cell extracts. Western blot analysis was done using anti-p65 antibody for nuclear and whole cell extracts obtained from livers of rats 2 h and 24 h post-resuscitation. IκBα levels were determined in cytoplasmic protein extracts. Specific bands were visualized by enhanced chemiluminescence (p65 – 65 kD, IκBα - 40 kD). Figure is representative of four rats for the curcumin control group and six rats for the other groups. Band intensities are 408 ± 18, 1885 ± 124, 2907 ± 195, 1046 ± 133, 2245 ± 200, 1200 ± 740, 641 ± 55 (p65 Nuclear extract); 1003 ± 52, 2763 ± 228, 6551 ± 308, 5106 ± 187, 2019 ± 175, 1692 ± 70, 3420 ± 264 (p65 Whole cell extract); 1071 ± 103, 926 ± 68, 619 ± 40, 1355 ± 164 (IκBα).

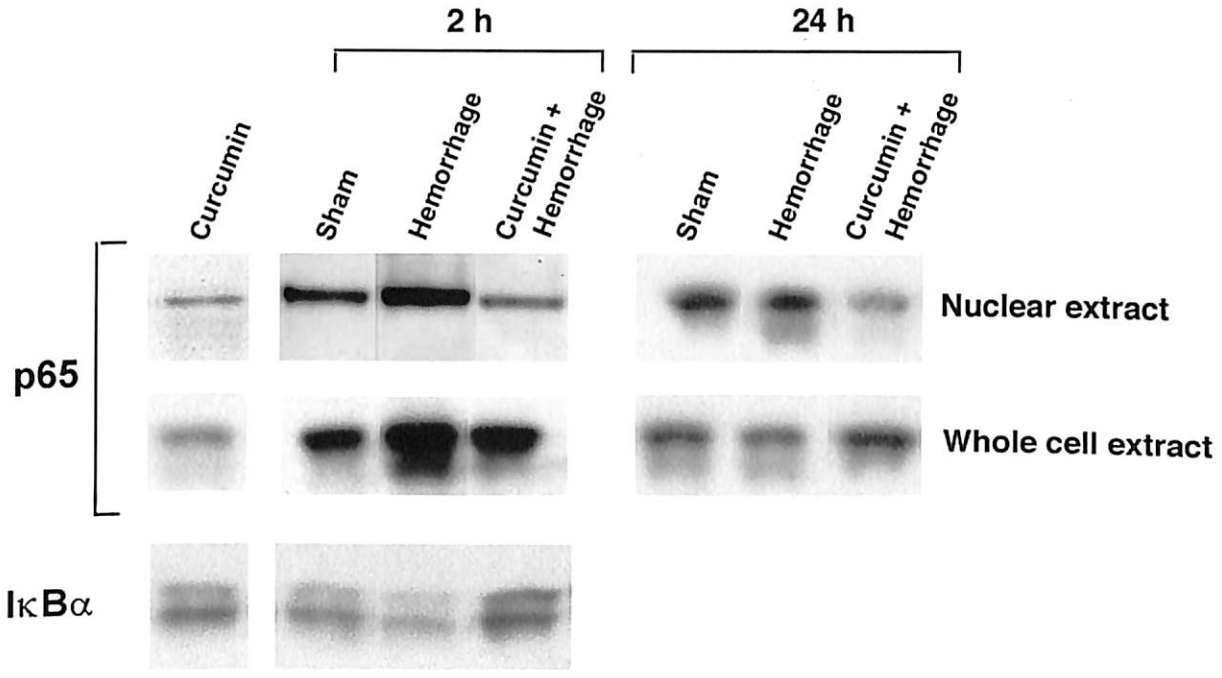

2 h
24 h
Curcumin
Sham
Hemorrhage
Curcumin + Hemorrhage
Sham
Hemorrhage
Curcumin + Hemorrhage
p65
Nuclear extract
Whole cell extract
IκBα

Table 1: List of genes spotted on the mouse stress and toxicity cDNA array.

Gene Name	Genbank	Location on the array	Description / Alternative names
ATF-2	U46026	(1A, 1B)	Activating transcription factor 2 (CRE-BP1 transcription factor)
bax	L22472	(1C, 1D)	Bcl2-associated X protein (pro-apoptotic gene)
c-fos	V00727	(1E, 1F)	Mouse c-fos oncogene (FBJ osteosarcoma oncogene)
c-jun	J04115	(2A, 2B)	Jun oncogene
c-myc	X01023	(2C, 2D)	Mouse normal c-myc gene and translocated homologue from J558 plasmocytoma cells
αB-crystallin	M63170	(2E, 2F)	HSPB1, small heat shock protein
Egr-1	M20157	(3A, 3B)	Early growth response-1 transcription factor
GADD45	L28177	(3C, 3D)	Growth arrest and DNA-damage inducible transcript 1
GADD45β	NM_008655	(3E, 3F)	Growth arrest and DNA-damage inducible protein β
grp78	NM_022310	(4A, 4B)	78 KD glucose regulated protein
Hsf-1	X61753	(4C, 4D)	heat shock transcription factor 1
hsp25	U03560	(4E, 4F)	heat shock protein (HSP27)
hsp86	J04633	(5A, 5B)	heat shock protein 86, hsp90
IkBα	U36277	(5C, 5D)	I-kappa B alpha chain (NF-κB inhibitor)
iNOS	M92649	(5E, 5F)	Inducible nitric oxide synthase
Mdm2	U40145	(6A, 6B)	Transformed mouse 3T3 cell double minute 2
mkk4	U18310	(6C, 6D)	SAPK/Erk kinase 1, MAP kinase kinase 4, JNKK1
NF-κB	M57999	(6E, 6F)	Nuclear factor of kappa light chain gene enhancer in B-cells, p105
p21Waf1	U24173	(7A, 7B)	Cyclin-dependent kinase inhibitor p21Waf1 (p21Cip1)
p38	D83073	(7C, 7D)	p38 mitogen activated protein (MAP) kinase
p53	K01700	(7E, 7F)	Transformation related protein 53, Tumor antigen
SOD1	M35725	(8A, 8B)	Cu/Zn superoxide dismutase
TRPM-2	D14077	(8C, 8D)	Clusterin, complement lysis inhibitor, SP-40, apoJ
β-actin	M12481	(3G, 4G)	Cytoplasmic β-actin (housekeeping gene)
GAPDH	M32599	(5G, 6G) (7G, 8E) (8F, 8G)	Glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene)
pUC18	PUC18	(1G, 2G)	Bacterial plasmid (negative control)

Table 2: Primer sequences used for PCR amplification.

	Gene	Sense primer	Antisense primer	Product size (bp)
Mouse primers	c-fos	accatctccgaaatcctaca	accatctccgaaatcctaca	257
	Egr-1	ctgcttcatcgtcttcctct	cctgttgttgtggaaacaga	255
	hsf-1	gcttccgaaaagtagtccac	aggagcttggagtccataca	247
	NF-κB	tcctacccacaggtcaaaat	ccctaatacacgcctctgtc	253
	SOD1*	gattaactgaaggccagcat	tcacacgatcttcaatggac	201
	TRPM-2*	atgctctggaggacactagg	gggtgagctctggtttagaa	200
	GAPDH*	tgacatcaagaaggtggtga	tggtccagggtttcttactc	255
Rat primers	iNOS	tgggtcttgttagcctagtc	gtgcagtcccagtgaggaac	264
	L28	gcatctgcaatggatggtcg	gatgcactcctggtggtagtt	240
	GAPDH	ccatggagaaggctgggg	aaagttgtcatggatgacc	195

* These PCR products were used as probes for Northern blotting.

Table 3: Hemorrhagic shock-induced changes in gene expression in mouse liver compared to sham animals.

Gene name	Time course		
	1 h	4 h	24 h
ATF-2	NE	ND	ND
bax	1.6	2.5	-2.7
c-fos	1.8	-3.9	ND
c-jun	NE	NE	NE
c-myc	NE	NE	NE
αβ-crystallin	4.4	ND	ND
Egr-1	23.7	NE	ND
GADD45	7.8	ND	-3.3
GADD45β	2.0	ND	NE
grp78	ND	ND	ND
Hsf-1	7.6	1.6	ND
hsp25	NE	NE	NE
hsp86	1.5	2.1	ND
IκBα	5.1	8.2	2.0
iNOS	NE	NE	NE
Mdm2	1.5	1.6	ND
mkk4	1.9	ND	ND
NF-κB	1.9	-1.9	ND
p21Waf1	ND	1.8	ND
p38	ND	3.4	2.2
p53	-1.7	2.2	ND
SOD1	ND	ND	ND
TRPM-2	ND	ND	ND

Genes whose expression changed by more than 1.5 fold compared to Sham are shown. Negative values indicate downregulation. ND: No Difference, NE: Not Expressed.

Table 4: Differential gene expression in Sham and Hem groups compared to normal mouse liver.

Gene name	Time course					
	1 h		4 h		24 h	
	Sham	Hem	Sham	Hem	Sham	Hem
ATF-2	-1.7	-2.7	-2.4	-3.8	ND	ND
bax	ND	ND	-2.3	-1.8	4.7	ND
c-fos	-1.8	ND	ND	-2.3	ND	ND
c-jun	NE	NE	NE	NE	NE	NE
c-myc	NE	NE	NE	NE	NE	NE
αB-crystallin	-3.4	ND	-3.5	-7.1	ND	ND
Egr-1	-7.4	3.2	-5.0	-9.5	ND	ND
GADD45	-13.1	-1.7	-8.4	-1.8	ND	-3.3
GADD45β	2.7	5.4	2.2	3.9	-2.4	1.5
grp78	ND	ND	ND	ND	ND	ND
Hsf-1	-6.2	ND	ND	ND	ND	ND
hsp25	NE	NE	NE	NE	NE	NE
hsp86	-2.1	ND	ND	1.8	ND	ND
IκBα	3.1	5.2	-5.2	1.6	ND	2.1
iNOS	NE	NE	NE	NE	NE	NE
Mdm2	ND	1.8	ND	ND	-2.0	-2.0
mkk4	-2.3	ND	-3.2	ND	-1.7	ND
NF-κB	ND	-1.6	-2.7	-4.9	ND	ND
p21Waf1	83.4	121.3	57.3	103.2	25.3	26.8
p38	-2.3	-4.7	-2.5	ND	-2.7	ND
p53	-5.4	-9.3	-1.9	-1.9	-2.7	-3.1
SOD1	ND	ND	ND	ND	ND	1.6
TRPM-2	-2.0	ND	ND	ND	3.5	2.5

Genes whose expression changed by more than 1.5 fold compared to Norm are shown. Negative values indicate downregulation. ND: No Difference, NE: Not Expressed.

Table 5: Alterations in serum AST and ALT enzymes following hemorrhagic shock in mice.

Time after hemorrhage	Parameters	Norm	Sham	Hem
1 h	AST (U/L)	91.3 ± 7.2 §	131.5 ± 7	174.5 ± 4 *
4 h	ALT (U/L)	39 ± 1.2 §	56 ± 4	74 ± 1.7 *

All values are represented as mean ± SEM of 5 animals.

* $p < 0.05$ compared with Sham

§ $p < 0.05$ compared with Sham and Hem

Table 6: Alterations in the glutathione redox system in rat liver caused by picroliv following H/R injury.

Parameters	Pic	Sham	H/R	Pic + H/R
GSH (μmoles/mg protein)	43.73 ± 0.67	49.62 ± 8.97	35.35 ± 4.39 *	38.53 ± 2.37
GR (mU/min/mg protein)	272.1 ± 21.80 §	510.7 ± 25.84	583.25 ± 13.47 *	667.6 ± 31.10 **
GPx (U/min/mg protein)	9.70 ± 0.51	11.98 ± 1.21	11.47 ± 0.49	13.72 ± 1.36

All values are represented as mean ± SEM of 5-6 animals.

* $p < 0.05$ compared to Sham.

** $p < 0.05$ compared to H/R.

§ $p < 0.05$ compared to the other groups.

DISCUSSION

Gene expression following hemorrhagic shock

Recent advances in technology and whole-genome knowledge has led to an increased awareness of the complex interconnections in gene activity. Functional genomics provides a global view of cellular processes, providing insight into this complexity (Chung, Laramie et al., 2002). In the study of cellular events following hemorrhagic shock, this approach is very relevant since hemorrhage activates a large number of genes that interact in a complex, interrelated and redundant manner. Though microarray techniques have been widely used to detect changes in gene expression associated with disease conditions such as cancer (Arcellana-Panlilio, Robbins, 2002), fewer studies have been carried out to analyze the gene expression profile under oxidative stress or ischemic conditions in vivo (Huang J., Qi et al., 2001; Yoshida, Kurella et al., 2002; Yoshida, Tang et al., 2002). The only study that has used a "global approach" in the context of hemorrhagic shock emphasizes the differences between various resuscitation strategies (Alam, Stegalkina et al., 2002). Alterations in genes not previously implicated in hemorrhage-resuscitation were found in this study, with the largest number of organ-specific changes (63/167 genes) noted in the liver.

Only a limited number of genes have thus far been extensively studied in terms of the effects of non-resuscitated hemorrhagic shock on liver, including transcription factors such as NF-κB and genes of the inflammatory cascade like

IL-6, TNF-α, COX-2 and iNOS (Altavilla, Saitta et al., 2002; Rajnik, Salkowski et al., 2002). We used a targeted approach to understand the pattern of stress-related gene activation in liver at various time points following non-resuscitated hypovolemic hemorrhagic shock, to help elucidate the underlying mechanisms of gene activation under these conditions. Our study shows that sham surgery as well as hemorrhagic shock induces profound changes in cellular gene expression pattern of IEGs, heat shock response genes, genes of the NF-κB pathway, genes coding for signal transducers and cell growth/death related genes. We have identified nine genes previously unreported as being regulated during hemorrhagic shock: ATF-2, αB-crystallin, GADD45, GADD45β, Hsp86, Mdm2, p21Waf1, p53 and TRPM-2.

IEGs

The protein products of the IEGs c-jun and c-fos act as transcriptional regulators by forming dimers with each other and with other members of the Fos/Jun family and binding to the AP-1 site in a number of pro-apoptotic and anti-apoptotic genes (Wisdom, 1999; Shaulian, Karin, 2002). c-fos, which cannot homodimerize, has to bind with a Jun family member for transcriptional activation. We assessed the mRNA levels of c-fos in liver and its DNA-binding activity by observing the transactivation of the associated transcription factor AP-1. The pattern of induction of c-fos and AP-1 was transient; there was no significant difference between Sham and Hem at the late time point. mRNA levels of c-fos show differential regulation with a steady increase in Sham which was

significantly higher than Hem at 4 h, indicating that sham surgery without hemorrhage is also capable of IEG induction in mouse liver. Earlier studies on IEG expression following resuscitated hemorrhage have shown little or no c-fos induction in liver while c-jun was significantly upregulated (Saito, Matsumoto et al., 1998; Alam, Stegalkina et al., 2002). In contrast, c-fos is highly induced in ischemic liver (Schiaffonati, Rappocciolo et al., 1990) and myocardium (Brand, Sharma et al., 1992) while there is little or no change in c-myc and c-jun expression. This correlates with our observation that c-fos is induced following Hem but not c-jun and c-myc. Hence it appears that the pattern of c-jun and c-fos mRNA expression is sensitive to resuscitation of tissue and the duration and magnitude of the insult.

Yan S-F et al (Yan, Fujita et al., 2000) identified a central role for Egr-1 in the pathogenesis of ischemic tissue damage in mouse lung. Deletion of this gene strongly decreased vascular injury and increased animal survival in a murine model of lung ischemia/reperfusion. Egr-1 is also activated in response to cultured monocytes exposed to hypoxic conditions (Yan, Lu et al., 1999). We observed a significant increase in Egr-1 mRNA 1 h following hemorrhage. Stress caused by sham surgery was sufficient to activate Egr-1 DNA-binding in our study; only by 24 h did Hem show a significant increase compared with Sham. This might be due to induction of apoptotic cell death at this later time point.

Heat shock response genes

The expression of αB-crystallin as early as 1 h in our study could be indicative of the protective effect of this small heat shock protein. The enhanced expression of αB-crystallin protects myocardium against ischemia reperfusion injury (Ray, Martin et al., 2001). In this study, transgenic mice overexpressing αB-crystallin developed less oxidative stress, and decreased the effects of both necrosis and apoptosis. The persistent expression of this heat shock protein in our study in all three groups could indicate the triggering of cellular protective mechanisms in liver.

Ischemia (without reperfusion) rapidly triggers the DNA binding activity of HSF in rat liver (Tacchini, Radice et al., 1999). We observed significant increase in HSF-1 mRNA at the 1 h time point but increase in DNA binding was significant only at 24 h. The targets of this transcription factor include various heat shock proteins (HSPs) such as Grp78, Hsp25 and Hsp86 as well as SOD1 (Christians, Yan et al., 2002). In our study, there were no significant differences in Grp78 gene expression between groups at any time point, while Hsp25 was not expressed in any of the treatment groups. Hsp86 (also known as Hsp90), is activated in liver following ischemic injury (Gasbarrini, Esposti et al., 1998). Another study showed that enhanced induction of HSP90 might be beneficial to liver following ischemia/reperfusion (Fudaba, Ohdan et al., 2001). The early induction of Hsp86 gene in our study could possibly reflect the transactivation of HSF-1 and the activation of the protective heat shock response.

NF-κB pathway and signal transduction molecules

The link between the heat shock response pathway and NF-κB pathway is by now well established (Malhotra, Eaves-Pyles et al., 2002). Although both HSF and NF-κB are activated by ischemia-reperfusion, there are differences in time course and possible mechanisms of activation (Tacchini, Radice et al., 1997). NF-κB DNA binding activity is induced in rat liver as early as 10 min. following acute blood loss, triggering the inflammatory cascade and leading to death (Altavilla, Saitta et al., 2001b; Altavilla, Saitta et al., 2002). In a hemorrhage model very similar to ours, Rajnik and colleagues have demonstrated the upregulation of NF-κB target genes, which induce the inflammatory cascade within few hours of hemorrhage (Rajnik, Salkowski et al., 2002). We observed a similar pattern of NF-κB mRNA levels but activation of the transcription factor was more protracted, possibly due to sustained inflammatory process. NF-κB activation is regulated by IκB, which binds to NF-κB and prevents its translocation into the nucleus (Senftleben, Karin, 2002). In our study, the observed increase in IκBα mRNA does not necessarily indicate an increase in protein levels since there is significantly higher NF-κB in the nucleus at the later time points.

The lack of expression of iNOS gene in our study is remarkable since iNOS is an NF-κB target and is known to be upregulated following fixed-volume hemorrhage without resuscitation (Rajnik, Salkowski et al., 2002). Our results are supported by the observations of Alam et al., who found no difference in iNOS gene expression in liver following hemorrhage-resuscitation, though other studies have shown that iNOS gene expression correlates with the inflammatory process

and organ damage following hemorrhage-resuscitation (Hierholzer, Harbrecht et al., 1998; Alam, Stegalkina et al., 2002).

Stress-induced AP-1 and NF-κB activation is controlled by mitogen activated protein kinases (MAPKs) especially p38 MAPK and JNK, which may converge pathways through MAPK kinase 4 (MKK4) activation (Arbabi, Maier, 2002). Though we saw induction of p38 MAPK and Mkk4 genes caused by hemorrhagic shock, which could indicate such activation of signaling pathways, we did not find any changes in the amount of p38 protein present in liver homogenates (data not shown). Hence the changes in expression at mRNA level need not necessarily reflect a corresponding change at the protein level.

Cell growth/death related genes

Studies on mice genetically deficient in poly (ADP-ribose) polymerase have shown that apoptosis plays a major role in liver dysfunction after shock (Liaudet, Soriano et al., 2000). Activation of NF-κB is known to offer cytoprotection by downregulating the JNK cascade, possibly through GADD45β (De Smaele, Zazzeroni et al., 2001). The upregulation of GADD45 and GADD45β mRNAs observed in our study could be part of this protective regulatory mechanism. It is also known that the GADD45 proteins interact with each other as well as other proteins to modulate cell survival or apoptosis depending on the stress-stimulus encountered (Liebermann, Hoffman, 2002). The GADD45 genes are targets of p53, which was also induced by hemorrhage at later time points. Suppression of p53 gene expression at 1 h correlates with the induction of Mdm2

which binds to p53 and inactivates it (el-Deiry, 1998). The strong induction of p21Waf1 (another p53 target and mediator of G1 cell-cycle arrest) might be indicative of the efforts to modulate apoptotic cell death in Sham and Hem groups. (el-Deiry, 1998).

We found decreased SOD1 mRNA expression in Hem compared with Sham at 1 h following shock. This finding correlates with the known effects of hemorrhagic shock on SOD1 enzyme activity (Maruyama, 1996; Mauriz, Matilla et al., 2001). We also observed a significant induction of TRPM-2 gene in both Sham and Hem 24 h after treatment. TRPM-2 (clusterin) gene confers protection against heat shock and oxidative stress (Viard, Wehrli et al., 1999). TRPM-2 has AP-1 and HSF-1 binding sites; these transcription factors might cause its differential regulation depending on the type of stress (Wong, Pineault et al., 1993; Michel, Chatelain et al., 1997). Prolonged late expression of TRPM-2 (24-96 h) was observed following ischemic renal injury, which could indicate a role for this gene in tissue repair (Rosenberg, Paller, 1991). High constitutive levels of TRPM-2 have also been reported in liver (Jordan-Starck, Lund et al., 1994), which substantiates the high expression of this gene in all experimental groups of the present study.

The induction of Hsf1 and the immediate early genes ATF-2, c-fos and Egr-1 in normal mouse liver was surprising. ATF-2 is a constitutive transcription factor but is known to be induced by ischemic stress and may modulate the early genetic response to such injury (Morooka, Bonventre et al., 1995). However, this induction was not observed at the protein level for c-fos and Egr-1. A possible explanation lies in our use of an overdose of methoxyflurane to sacrifice all animals. Though liver was excised and stored within five minutes of death, this

time period is sufficient for the expression of immediate early genes. A variety of general anesthetics are known to cause the upregulation of c-fos and other IEGs in liver (Hamaya, Takeda et al., 2000). Hence it is possible that our experimental conditions are responsible for the induction of certain immediate early genes in the normal animals, which could be a transient transcriptional response to anesthetic overdose.

The pattern of gene expression observed following sham surgery or hemorrhage (Table 3 and Table 4) indicates that hemorrhagic shock induces genes promoting both cell death and cell survival and a tilt in this balance could determine the ultimate fate of the tissue. Depre et al put forward the hypothesis that myocardial ischemia followed by reperfusion induces antagonistic sets of genes, forcing cells toward either death or survival (Depre, Tomlinson et al., 2001). The induction of pro-apoptotic genes p53 and its target genes bax, c-fos, GADD45, GADD45β and p21Waf1 may lead to viable growth arrest or apoptotic cell death (el-Deiry, 1998; Gottlieb, Oren, 1998) while the small heat shock protein αB-crystallin, transcription factor Hsf1, the p53-inhibitor Mdm2 and TRPM-2 could actively promote cell survival (el-Deiry, 1998; Viard, Wehrli et al., 1999; Ray, Martin et al., 2001; Christians, Yan et al., 2002). The other differentially regulated genes ATF-2, Egr-1, IκBα, MKK4, NF-κB and p38 MAPK take part in a variety of signaling cascades, whose role in cell death or survival is mediated through interconnected pathways. The balance between the opposing target genes of the transcription factor AP-1 could also determine the final fate of the cell – survival or death, also depending on the transcription factors of other regulatory pathways. AP-1 could also act as a homeostatic regulator, inducing

apoptosis in extensively damaged cells and proliferation in cells that are still able to do so (Shaulian, Karin, 2002).

Thus the gene expression changes following severe hemorrhagic shock involve a complex and delicate balance between cell survival and death. This is also confirmed by our studies on liver injury following hemorrhagic shock. We found that liver injury markers were elevated in the serum of mice subjected to hemorrhagic shock. 24 h following shock, increased membrane damage and apoptotic cell death was noted in the liver. This correlates well with the induction of pro-apoptotic genes p53, bax, c-fos, p21 and GADD45 in liver. Recent studies have shown the effectiveness of alternative therapeutic strategies in decreasing apoptosis indicators and maintaining the redox environment (Mongan, Capacchione et al., 2002). The detailed elucidation of the synergistic and interconnected pathways contributing to the balance between cell death and survival and understanding the manner in which therapeutic drugs affect this balance could result in better treatment strategies for shock and other critical injuries. Thus picroliv and curcumin, due to their multifarious beneficial properties, are ideal pharmacological agents to be used against H/R injury.

Picroliv

The pathophysiology of acute hemorrhagic shock in relation to cellular and organ responses, the hematological abnormalities associated with it and treatment procedures available have been studied extensively (Tremblay, Rizoli et al., 2001). Standard protocols of fluid resuscitation following hemorrhage often prove to be inadequate. Typical fluids like Ringer's lactate, though widely used,

have been shown to cause apoptosis (Deb, Martin et al., 1999) and activation of neutrophils, the primary mediators of reperfusion-induced tissue injury (Rhee, Burris et al., 1998). Numerous alternatives have been under evaluation to replace or modify the existing resuscitation fluids. Some of the newer treatments include peroxynitrite decomposition catalysts, selective superoxide dismutase mimetics and blood substitutes (Cuzzocrea, Riley et al., 2001; Tremblay, Rizoli et al., 2001). There exists a need to search or develop more such novel agents that would potentially reduce tissue injury by inhibiting free radical mediated lipid peroxidation and enhancing the activity of intracellular antioxidants. Therefore, we undertook this study on the prophylactic effect of the natural product picroliv, a well-known antioxidant and hepatoprotective agent, on the outcome of hemorrhagic shock and resuscitation. We also aimed to provide a possible molecular mechanism for the cytoprotective effect of picroliv.

Serum AST was assessed, as this enzyme is a reliable marker of liver damage. Liver injury markers such as AST, ALT and γ-glutamyl transpeptidase (GGT) are known to be elevated in serum following hepatic insult and previous studies on picroliv have proven its hepatoprotective efficacy by lowering the level of these enzymes (Saraswat, Visen et al., 1999; Rajeshkumar, Kuttan, 2000). In a mice model of carbon tetrachloride-induced liver damage, picroliv decreased AST and ALT levels significantly (Santra, Das et al., 1998), which is in agreement with our observations.

We measured lipid peroxidation as a cellular marker for oxidative stress. Previous studies conducted by our laboratory and others have demonstrated that picroliv is very effective against lipid peroxidation caused by ischemic tissue

injury (Seth, Kumari et al., 2000; Singh A. K., Mani et al., 2000) as well as in the hypoxia-reoxygenation scenario. Picroliv reduces the cellular damage caused by hypoxia as revealed by a significant reduction in lactate dehydrogenase release compared with untreated control in Hep 3B and Glioma cell lines (Gaddipati, Madhavan et al., 1999). In an aflatoxin model of hepatotoxicity, picroliv was comparable with the drug silymarin in its ability to abate lipid peroxidation and increase antioxidant enzyme levels (Rastogi, Srivastava et al., 2001b). MDA generation in rat liver microsomes is inhibited by picroliv (Chander, Kapoor et al., 1992b). In our study, picroliv pretreatment significantly reduced lipid peroxidation caused by hemorrhage and resuscitation and resulted in an overall improved anti-oxidant status.

Nitric oxide molecule has been implicated in various ischemia-reperfusion and H/R injuries. Simultaneous production of nitric oxide and superoxide radicals favors the generation of a toxic reaction product, peroxynitrite anion, which has been shown to be deleterious to cellular microenvironments, and more harmful than its precursors (Beckman, Koppenol, 1996; Szabo, Billiar, 1999). Studies with iNOS knockout mice have shown that iNOS contributes to local hepatic injury following ischemia-reperfusion (Lee, Johnson et al., 2001), suggesting that iNOS expression is deleterious in ischemic conditions. Hence, reduced levels of NO and downregulation of iNOS expression would be expected to ameliorate H/R injury. We observed an increase in NO release and iNOS gene expression following H/R, which has been documented to be pathophysiological in H/R settings. Picroliv pretreatment reduced both the nitrite levels as well as iNOS mRNA in liver tissue, suggesting a better antioxidant outcome. A decrease in the production of oxidative free radicals, as reflected in the reduced MDA levels in

liver tissue, further lessens the risk of NO interacting with other reactive species to form peroxynitrite radicals.

The substantial decrease in GSH levels in the H/R group correlates with increased sensitivity to NO toxicity (Luperchio, Tamir et al., 1996). It has been shown that different cell types cope with nitrosative stress by using different pathways to maintain critical amounts of glutathione (Berendji, Kolb-Bachofen et al., 1999). In our study, though picroliv decreased the NO levels, GSH levels remained unchanged. Increases in GR levels seen in the H/R group in our study may be due to the cellular response to H/R injury and may account for the less than expected decrease of the GSH levels in H/R group. GR levels increased further with picroliv treatment as shown earlier (Chander, Kapoor et al., 1992a; Rastogi, Srivastava et al., 2001a). However, in picroliv control rats, GR levels were significantly reduced compared with the other groups, perhaps due to unavailability of GSSG, the substrate of this enzyme. We believe that in the absence of H/R, the levels of GSSG may be limiting and may account for decreased GR activity. Following H/R, GSSG levels would increase, thus increasing the GR activity. We found no change in GPx following picroliv treatment and H/R, contrary to the results observed in rat kidney after picroliv treatment and ischemia-reperfusion (Seth, Kumari et al., 2000). This could be due to the differences in the capability of hepatocytes and renal cells to maintain critical GSH levels.

The stress-sensitive transcription factor AP-1 and its components c-jun and c-fos, have been implicated in various hypoxic conditions such as ischemia-reperfusion and hemorrhage-resuscitation injury (Saito, Matsumoto et al., 1998; Wieland, Oellerich et al., 2000; Hoetzel, Vagts et al., 2001; Leu, Crissey et al.,

2001; Rensing, Jaeschke et al., 2001). Marked activation of AP-1 has also been shown to result from aldehydic lipid peroxidation products (Camandola, Poli et al., 2000). Increase in AP-1 has also been correlated with a decrease in cellular GSH (Domenicotti, Paola et al., 2000). Rensing et al. have shown that the activation of AP-1 but not NF-kB following H/R is dependent on reactive oxygen species (Rensing, Jaeschke et al., 2001). In agreement with these studies, we found that AP-1 is highly upregulated following H/R. Since picroliv decreased lipid peroxidation and improved the antioxidant status after hemorrhagic shock, it is reasonable to assume that it would deactivate AP-1. In the present study, picroliv treatment did cause a significant inhibition of AP-1 following H/R. This result conforms to other studies, which have shown improved antioxidant status upon inhibition of AP-1. Treatment with the antioxidant trolox inhibits AP-1 transactivation (Rensing, Jaeschke et al., 2001) and liver injury following ischemia-reperfusion is reduced by the inhibition of AP-1 (Zhou, Zhang et al., 2001). Activation of AP-1 in liver after hemorrhagic shock is dependent on oxygen free radicals and is substantially attenuated by antioxidants trolox and tempol (Paxian, Bauer et al., 2002). We also perceived that differences in iNOS expression correlate with those of AP-1. Our observations on AP-1 and iNOS are similar to another study where reactive oxygen species induced iNOS expression in rat alveolar macrophages is AP-1-dependent and is decreased by antioxidants (Pepperl, Dorger et al., 2001). Regulation of iNOS could be through the activation of the AP-1 binding site in the promoter region of this gene (Xie, Whisnant et al., 1993). The fact that picroliv did not have any effect on NF-κB levels further

confirms the hypothesis that its effects are mediated through the cellular antioxidant defense system.

Rapid and transient induction of c-jun and c-fos mRNAs has been observed in rat liver following H/R and the pattern of induction depended on the duration of shock and pattern of resuscitation used (Saito, Matsumoto et al., 1998). This could explain our observation that c-jun and c-fos follow different patterns of expression after H/R. Picroliv did not affect the expression of c-jun mRNA but significantly reduced c-jun protein in whole cell extracts as seen by Western blotting. We believe that the decrease in c-jun protein, but not mRNA, could be due to picroliv affecting the phosphorylation and/or ubiquitination of c-jun protein in a mechanism similar to that of curcumin (Huang T. S., Kuo et al., 1995). The significant decrease of c-fos mRNA and protein following picroliv treatment indicates that it might be involved in the regulation of c-fos at the transcriptional level.

Picroliv is known to possess immunomodulatory properties affecting both cell-mediated and humoral immunity (Atal, Sharma et al., 1986; Dhawan, 1995). Earlier studies from our laboratory have shown that picroliv treatment reduces mRNA levels of pro-inflammatory cytokines IL-1α and IL-1β in rat liver following ischemia-reperfusion (Singh A. K., Mani et al., 2000). In the present study, we observed a similar decrease in IL-1β and IL-6 in picroliv pre-treated rats. This anti-inflammatory effect of picroliv could be mediated through the downregulation of AP-1 transcription factor, present in the promoter region of both IL-1β and IL-6 (Wisdom, 1999; Shaulian, Karin, 2002).

In summary, our studies suggest that picroliv pretreatment prevents hemorrhage-resuscitation injury by modulating free-radical scavenging mechanisms. Picroliv reduces membrane damage and differentially regulates the glutathione redox system. It inhibits NO production by downregulating iNOS at the mRNA level and inhibiting the release of inflammatory cytokines. These physiological protective effects appear to be mediated by downregulation of the transcription factor AP-1. Further pharmacological and molecular studies are required to develop picroliv as a therapeutic agent against H/R injury in the clinical setting.

Curcumin

In recent years, interest in complementary and alternative medicines for treatment of various diseases has helped in recognizing the underlying pleiotropic biological activities of some indigenous medicines such as curcumin. Curcumin has long been used for the treatment of inflammatory diseases and conditions (Satoskar, Shah et al., 1986; Ammon, Wahl, 1991) but the underlying mechanism of action has not yet been elucidated. This study demonstrates that pretreatment with curcumin can decrease liver injury following H/R as inferred from significant decrease in AST values 24 h post-H/R. This coincides with dramatic alterations in the inflammatory cytokine release observed in response to H/R. Interestingly, the effect of curcumin on transcription factor NF-κB is more profound compared with AP-1. There were no significant differences in blood pressure between curcumin treated animals and controls.

Although the expression and release of cytokines during hemorrhagic injury is well characterized, variations have been observed in the cytokine expression profiles between different studies. These probably reflect the variations in the protocols adopted. For example, no significant changes were observed in liver TNF-α and IL-1α content in a rat model of fixed-pressure hemorrhage (Molina, Abumrad, 2000). In another study with endotoxin-tolerant and normal rats, highest mRNA levels were detected in liver biopsies for TNF-α, IL-6 and IL-10 immediately after reinfusion of shed blood; the differences were lost 2 h after reperfusion (Ackermann, Reuter et al., 2001). These results are in contrast to the findings in a mouse hemorrhagic shock model in which Kupffer cells showed enhanced and prolonged IL-1 and TNF-α production following trauma-hemorrhage-resuscitation (Ayala, Perrin et al., 1991). In another study, the spontaneous secretion of IL-1α, TNF-α and IL-6 by Kupffer cells remained high for more than 16 h after ischemia/reperfusion (Wanner, Muller et al., 1998). The results of the present study show an increase in IL-1α due to hemorrhage at both 2 and 24 h time points. The other pro-inflammatory cytokines IL-1β and IL-6 also show similar expression patterns as IL-1α. In similar studies, where animals were sacrificed at 4 or 24 h after hemorrhage and resuscitation significantly increased IL-6 mRNA levels were observed (Omert, Tsukada et al., 1998). Curcumin pretreatment in the present study markedly decreased the levels of IL-1α, IL-1β and IL-6 to near-basal sham levels. In case of IL-2, significant decreases have been reported following hemorrhage in murine splenocytes (Meldrum, Ayala et al., 1991; Knoferl, Diodato et al., 2000) whereas cells from Peyer's patches have shown significant increase in IL-2 after hemorrhage

(Shenkar, Abraham, 1993). The considerable increase in IL-2 mRNA observed in the present study at the 2 h time point may be the result of accumulation and activation of liver-associated alternative T cells (Crispe, Mehal, 1996). Also, the reason for significant upregulation of IL-2 by curcumin in the 24 h group is not clear. More detailed analysis is required to answer the exact nature of modulation of IL-2 by hemorrhagic shock and curcumin.

Multifunctional pro-inflammatory cytokine TNF-α has been shown to play a major role in organ injury following hemorrhage and resuscitation (Ackermann, Reuter et al., 2001). It is among the first cytokines to be released by cells under oxidative stress. However, our results indicate no significant alterations in the levels of TNF-α at 2 h. But the low levels of TNF-α mRNA observed in our study at 2 h time point are in agreement with the results of Colletti and Green who showed that ischemia-reperfusion injury of rat liver resulted in the increased release of TNF-α as early as 30 min .By 2 h the TNF-α levels had dropped down significantly (Colletti, Green, 2001). Hence early time point analysis for this cytokine might be helpful in better understanding its modulation. We observed a significant downregulation of TNF-α mRNA 24 h post-resuscitation compared with sham. However, TNF-α mRNA expression inhibited by hemorrhage was restored by curcumin to levels comparable to that of sham. Our observations on TNF are in contrast to the direct and indirect inhibition of TNF by curcumin observed in other studies (Chan, 1995; Grandjean-Laquerriere, Gangloff et al., 2002; Nanji, Jokelainen et al., 2003) but are in agreement with similar studies on the effect of ibuprofen on TNF where increased TNF release was associated with improved cellular immune responses (Ertel, Morrison et al., 1992). Inhibition of

PGE2 production by ibuprofen enhanced the production of TNF-α in rat liver after ischemic injury (Wanner, Muller et al., 1998). These contradictory observations could be due to inherent variations in the models and the time kinetics for the expression of this cytokine and also the difference between *in vitro* and *in vivo* experimental conditions. The above-mentioned studies on curcumin have been conducted in cell lines where the metabolism and pharmacokinetics of the drug is much less complicated than in the whole body or organ system.

The potent anti-inflammatory cytokine IL-10, which counteracts the effects of IL-1, IL-6 and TNF-α (Mosmann, 1994) was induced by hemorrhagic shock. Increased IL-10 following hemorrhagic shock has been observed previously (Ackermann, Reuter et al., 2001; Molina, 2001) and could be part of the beneficial cytokine response to counter shock-related injury. IL-10 administration suppresses the release of TNF following hemorrhagic shock (Karakozis, Hinds et al., 2000) and is associated with enhanced survival (Kahlke, Dohm et al., 2002) Even though curcumin showed no profound effect on the mRNA expression of IL-10 at the given time points the effect observed on other cytokines may be contributing towards amelioration of injury.

Curcumin-induced regulation of cytokine production might result from several mechanisms including inactivation of redox-sensitive nuclear transcriptional factors NF-κB and AP-1. As early as 10 min after hypovolemic shock, NF-κB binding activity increases in rat liver (Altavilla, Saitta et al., 2001b). A four fold increase in the activation of NF-κB was seen 4 h after resuscitation from hemorrhagic shock compared with sham animals (Hierholzer, Harbrecht et al., 1998). Similarly, our data indicate a significant increase in NF-κB activation

in the hemorrhage group compared with sham. Earlier reports also show that curcumin inhibits NF-κB activation in human myelomonoblastic leukemia cells (Singh S., Aggarwal, 1995), intestinal epithelial cells (Jobin, Bradham et al., 1999) and monocytic macrophage cell line (Chan, 1995). Both NF-κB and AP-1 activation was shown to be inhibited by curcumin in cultured human promyelocytic leukemia (HL-60) cells stimulated with TPA (Surh, Han et al., 2000). Curcumin prevents alcohol-induced liver injury in rats by the inhibition of NF-κB (Nanji, Jokelainen et al., 2003). Prevention of hemorrhage-induced activation of NF-κB by curcumin as reported in the present study is in agreement with the other published reports. We also observed the accumulation of IκBα in the cytoplasm, caused by curcumin. Other studies have demonstrated that curcumin-mediated NF-κB inhibition is due to the blocking of IκBα degradation in the cytoplasm (Chan, 1995; Jobin, Bradham et al., 1999; Surh, Han et al., 2000). The fact that curcumin-treated nuclear extracts showed decreased p65 compared with whole cell extracts further substantiates this mechanism of action of curcumin.

In contrast to NF-κB, activation of AP-1 due to hemorrhage was observed only in the early time group of 2 h post-resuscitation and treatment with curcumin decreased the activation slightly. Animals in sham group from 24 h post-resuscitation showed minimal activation and curcumin treated and untreated hemorrhage groups showed little or no activation. This variation could be due to the transient nature of c-jun and c-fos expression and further analysis with earlier time points may be helpful. In a time kinetics study using various hemorrhage models, c-jun and c-fos expression was found to be maximum at 0-2 h following

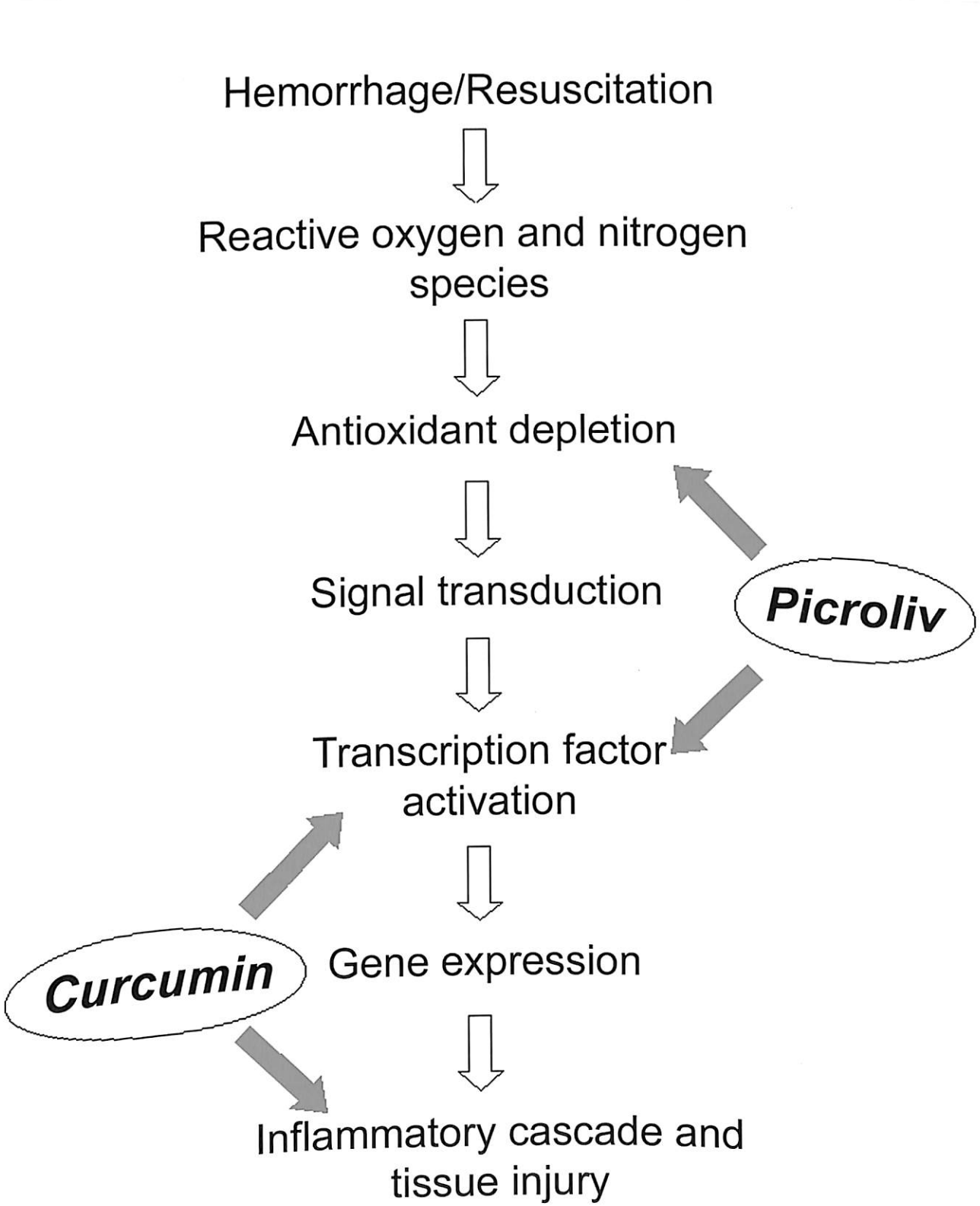
H/R and was reduced to basal levels after 10 h, which is similar to our observations (Saito, Matsumoto et al., 1998). Findings from a time kinetic study of the activation patterns of NF-κB and AP-1 after H/R are different from our observations (Rensing, Jaeschke et al., 2001). In that study, a weak NF-κB activation and a significant AP-1 activation is reported. We suspect that these significant differences could be due to variations in the protocols adopted. Our protocol involves resuscitation with 2 times the shed blood volume of LR for 20 min where as the protocol of Rensing and colleagues involved resuscitation with 3 volumes of LR for 2 h followed by constant infusion of 10 ml/kg/h LR for another 4 h. Our findings are in accordance with another study on the effect of curcumin on UVB-induced cytokine expression in keratinocytes. Curcumin selectively inhibited NF-κB; AP-1 was unaffected (Grandjean-Laquerriere, Gangloff et al., 2002). Previous reports demonstrate the suppression of AP-1 (Bierhaus, Zhang et al., 1997; Park, Lee et al., 1998) and phorbol ester-induced c-fos expression by curcumin (Huang T. S., Kuo et al., 1995), whereas we found no such affect of curcumin on the expression of c-jun and c-fos proteins. This discrepancy may have arisen due to the intrinsic differences between *in vitro* and *in vivo* systems as hypothesized by Chuang et al. based on their studies of rat liver tissue in which curcumin inhibited NF-κB but did not affect c-jun and c-fos after diethylnitrosamine-induced inflammation (Chuang, Cheng et al., 2000).

Our results indicate that curcumin modulates cytokines and transcription factors leading to the attenuation of liver injury following H/R. Other studies involving systemic administration of curcumin have shown its beneficial effects by the enhancement of muscle regeneration after trauma in vivo by modulating NF-

κB activity (Thaloor, Miller et al., 1999). In patients with post-operative inflammation, curcumin produced a much better anti-inflammatory response compared with placebo (Satoskar, Shah et al., 1986). Phase 1 clinical trials for curcumin in high-risk patients suggest a role for it in cancer therapy (Cheng, Hsu et al., 2001). These properties, along with the non-toxic nature of curcumin observed in both animals (Wahlstrom, Blennow, 1978) and humans (Cheng, Hsu et al., 2001) make it an attractive agent for development as a therapeutic against various traumatic injuries and disease conditions.

In conclusion, this study demonstrates that H/R initiates a series of chemical reactions and biological events, generating harmful free-radicals and initiating signaling events and inflammatory cascades leading to tissue injury and ultimately to cell death. Based on the results of this study, we propose that picroliv replenishes the depleted antioxidant pool of the cell and reduces injury by downregulation of the redox-sensitive transcription factor AP-1 and associated inflammatory mediators. Curcumin directly inhibits the inflammatory cascade by downregulating the pleiotropic transcription factor NF-κB, thus minimizing tissue injury (Fig. 24). It is evident that picroliv and curcumin have potent protective effects against liver injury and death caused by H/R. They might be developed as prophylactic agents or nutritional supplements to boost the body's antioxidant defense system and suppress inflammatory events that cause tissue damage.

Fig. 24: Hypothesis for the mechanism of action of picroliv and curcumin. Open arrows indicate the sequence of events following H/R. Solid arrows indicate positive intervention by picroliv and curcumin.


Hemorrhage/Resuscitation
Reactive oxygen and nitrogen species
Antioxidant depletion
Signal transduction
Picroliv
Transcription factor activation
Curcumin
Gene expression
Inflammatory cascade and tissue injury

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LIST OF PUBLICATIONS

1. Manuscripts in preparation:

Sundar SV, Li Y-Y, Rollwagen FM, Maheshwari RK. Differential regulation of stress-induced genes and transcription factors in mouse liver following hemorrhagic shock.

2. Papers published:

Seth P, **Sundar SV**, Seth RK, Sidhu GS, Sharma SC, Kulshreshtha DK, Maheshwari RK. Picroliv modulates antioxidant status and downregulates AP1 transcription factor following hemorrhage and resuscitation. *SHOCK* 19(2): 150-6, 2003.

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4. Papers presented at national and international conferences:

Sundar SV, Li YY, Seth P, Singh AK, Gaddipati JP, Rollwagen FM, Maheshwari RK. Picroliv and curcumin reduce liver injury in mouse hemorrhage model. 41st American Society for Cell Biology Annual Meeting, Washington, DC, USA (2001).

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