MOLECULAR MECHANISM(S) OF HYPOXIA/ISCHEMIA - REPERFUSION INDUCED INJURY: BENEFICIAL EFFECTS OF PICROLIV

Thesis

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DOCTOR OF PHILOSOPHY

By

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Under the supervision of

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The Thesis "Molecular Mechanism(s) of Hypoxia/Ischemia – Reperfusion Induced Injury : Beneficial Effects of Picroliv" submitted by Subhashree Madhavan, is the result of her research work done under the supervision of Prof. Radha K. Maheshwari, Professor, Department of Pathology, Uniformed Services University of Health Sciences (USUHS), Bethesda, Maryland, USA, at USUHS which is an off campus thesis station of Birla Institute of Technology and Science, Pilani - 333031, (Rajasthan) India.

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ABSTRACT

Molecular oxygen is the central fulcrum upon which life processes depend. Hypoxia/Ischemia, which is prolonged exposure to low oxygen tension, leads to impaired cellular physiology which ultimately leads to cell death. In this study we have investigated the protective efficacy of picroliv, an extract from the roots of a rhizome, *Picrorhiza kurrooa* in three different models involving hypoxia. In an *in vitro* model, cells from different origins, HUVEC, Hep 3B and Glioma were subjected to 1% oxygen in a modular incubator chamber and various regulatory molecules were analyzed upon picroliv treatment. Picroliv reduced the cellular damage caused by hypoxia as revealed by a significant reduction in LDH release compared to untreated control. Cellular adaptation to hypoxia involves regulation of specific genes such as vascular endothelial growth factor (VEGF) and hypoxia inducible factor (HIF)-1. Increasing attention has been paid to the hypoxic induction of VEGF, since it is pivotal in physiological and pathological angiogenesis, chronic ischemic disorders and wound healing. The expression of VEGF and HIF-1 subunits, α and β, was enhanced by treatment with picroliv during normoxia and hypoxia in HUVEC and Hep 3B cells and upon reoxygenation the mRNA levels of these genes were significantly reduced as revealed by RT-PCR analysis. A significant inhibition was observed in VEGF and HIF-1 mRNA levels in picroliv treated Glioma cells, as shown by northern blots and RT-PCR. Immunofluorescence studies on VEGF expression at the post-transcriptional level were in agreement with the modulations observed at the mRNA level. To explore the involvement of signaling cascades triggered by picroliv during hypoxia, we examined the kinase activity of tyrosine phosphorylated proteins and protein kinase C (PKC) expression in Glioma cells that received picroliv

treatment during hypoxia/reoxygenation. A selective inhibition of protein tyrosine kinase activity leading to tyrosine dephosphorylation of several proteins including 80 kd protein, and a reduction in PKC was seen in cells treated with picroliv and hypoxia. These findings suggest that picroliv may act as a protective agent against hypoxia/reoxygenation induced injuries, and the underlying mechanism may involve a novel signal transduction pathway.

Since VEGF, growth factor that regulates angiogenesis, is induced by picrioliv and to place these molecular advances in a physiological context, we directed our studies to understand the effect of picroliv on angiogenesis, as this process is an important patho-physiological response to hypoxia. Angiogenesis, the process of generating new blood vessels leading to neovascularization, is essential during reproduction, embryonic development, tissue and organ growth and wound healing. Matrigel tube and ex-vivo rat aorta assays were performed for evaluating the efficacy of picroliv to enhance angiogenesis. Picroliv promoted tube formation in endothelial cells grown on matrigel. To elucidate the mechanism of angiogenesis, genes involved in this process such as, VEGF and matrix metalloproteinases were analyzed. Picroliv pre-treatment of HUVEC cells for 24 h resulted in an increase in transcription of VEGF and MMP-9 at the dose of 1 μg/ml of picroliv, which was consistent with the increase in angiogenic differentiation of HUVEC on matrigel. Aortic rings, embedded in a matrigel gave rise to microvessels and enhanced capillary formation in the presence of picroliv as compared to untreated control. Histology was also analyzed from the sections obtained from cutaneous wound healing models in rats upon treatment with picroliv. Picroliv treated wounds displayed enhanced fibrogenesis and neovascularization as compared to untreated controls. Our data suggest that picroliv enhances angiogenesis in both *in vitro* and *in vivo* models and therefore has the potential to be developed as a proangiogenic drug.

A third model of rat renal ischemia-reperfusion injury was employed to gauge the ability of picroliv to abate hypoxic injury. Male Sprague Dawley rats were fed

with 12 mg/kg oral dose of picroliv once daily for 7 days prior to renal ischemia reperfusion injury (IRI), which was induced by clamping the renal artery of left kidney for 60 minutes, while right kidney served as internal control. Renal blood flow was restored by releasing the microaneurysm clips to allow reperfusion. Animals were sacrificed following varying time of reperfusion. Increased lipid peroxidation and apoptotic cell number reflected the oxidative damage following renal IRI. Biochemical analysis of kidney tissues were performed to study the reduced glutathione (GSH) levels and activities of glutathione peroxidase (GPx) and glutathione reductase (GR) enzymes, the two important enzymes of the GSH redox cycle. Picroliv pretreated rats exhibited lower lipid peroxidation, better antioxidant status, and reduced apoptosis, indicating better viability of renal cells. Immunohistochemical studies revealed that picroliv pretreatment attenuated expression of ICAM-1 and CD-18 in glomerular region. Overall, picroliv pretreatment appears to protect rat kidneys from IRI, perhaps by modulation of free radical damage and adhesion molecules. Picroliv can be developed as an agent for ameliorating ischemic disorders. The implications of these developments may be important in biology and therapeutics.

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CERTIFICATE

This is to certify that the thesis entitled **"Molecular Mechanism(s) of Hypoxia/Ischemia - Reperfusion induced injury: Beneficial effects of Picroliv"**, and submitted by Subhashree Madhavan, ID No. 92PD29497 for the award of Ph.D Degree of the institute, embodies original work done by her under my supervision.

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DEDICATION

To my loving parents and my sister.

ACKNOWLEDGEMENTS

I owe a deep a deep sense of gratitude to my mentor and advisor Prof. Radha K. Maheshwari for providing a rich environment to learn and grow as a scientist. He has been a great source of inspiration. His patience and intellectual curiosity have helped steer my experiments towards a rationale goal.

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INTRODUCTION

Ever since Joseph Preistley demonstrated in 1774, the deleterious effect of a burning candle on a mouse in a bell jar, we have known that oxygen is essential for animal life. Low oxygen supply, often referred to as hypoxia, is a physiological condition that occurs during injury, ischemia/reperfusion and stroke. Traumatic injury is associated with cellular hypoxia, either localized or in hemorrhage. The mortality and morbidity associated with cardiovascular and cerebrovascular disease, respiratory disease and neoplastic disorders can be largely attributed to the effects of hypoxia. Despite the wealth of knowledge available on the mechanisms of hypoxic injury in different organs, relatively little is known about how to protect such tissues from this hypoxic insult. A better understanding of how cells adapt to hypoxia will offer new therapeutic targets for the treatment of injury. This research is directed at the development of strategies to save the lives of casualties that result from hypoxia/ischemia-reoxygenation induced traumatic injury. The ultimate goal of this study is to demonstrate advanced pharmacologic intervention strategies and to deliver far forward echelon medical support for casualties, permitting life-saving measures which will extend the "golden hour" by suspended animation and delayed resuscitation procedures.

1. Cellular responses to hypoxia:

The term "hypoxia" is derived from Greek, *hypo* meaning "sub" or "under" + oxygen and implies less than a normal amount of oxygen (Van Liere and Stickney, 1963). Hypoxia affects many cellular processes and these include changes in gene

expression, transcription regulation, mitochondrial function, glycolytic activity, lipid peroxidation, membrane fluidity, ionic imbalance and sustained hypoxia finally results in cell death. A schematic represention of various processes affected by hypoxia is presented in Fig.1. The first line of defense against hypoxia includes a balanced suppression of ATP-demand and ATP-supply pathways (Hand et al., 1996). This regulation stabilizes at new steady-state levels even while ATP turnover rates greatly decline.

1.1 *Cells become aware of limited oxygen supply:*

The cells detect when oxygen becomes limiting and they immediately switch from oxidative phosphorylation to anaerobic glycolysis. As the ATP generation by oxidative phosphorylation begins to fall off due to oxygen lack, the energy deficit is made up by activation of anaerobic ATP supply pathways (Buck et al., 1993). Classically, the concept of cell level responses to O_2 limitation was based on Pasteur effect: as ATP generation by oxidative phosphorylation begins to fall off due to oxygen lack, the energy deficit is made up by activation of anaerobic ATP supply pathways. The following argues persuasively against this classical view. Cells subjected to hypoxia, use anaerobic metabolism not to make up for the energy deficits but to sustain a reduced energy turnover state instead (Krumschnabel et al., 1994).

1.2 *Membrane alterations during hypoxia:*

Interestingly, direct estimates indicate that the electrochemical potential in hypoxic cells is essentially the same as in normoxia. The only mechanism by which we can account for the large scale drop in absolute Na^+/K^+ ATPase activity and the

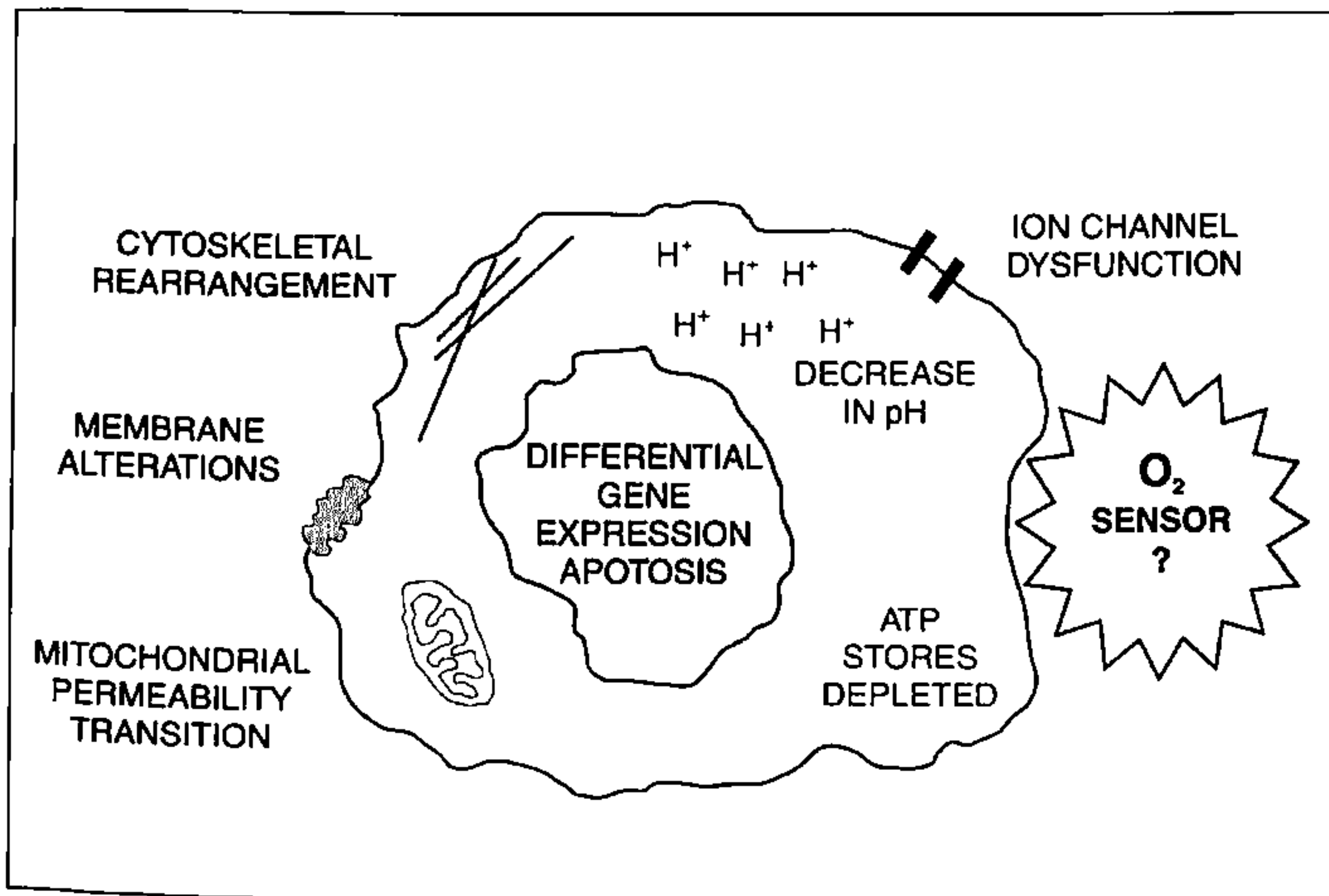


Fig.1: Hypoxia affects many cellular processes

(Adopted from the presentation of Dr. Constance Oliver, Office of Naval Research: In 'Cell Biology of Hypoxia', Edts: Nielsen TB, Kidwell JL)

simultaneous maintenance of normal electrochemical gradients is by means of a similar magnitude decrease in cell membrane permeability. Hypoxia generates free radicals via peroxynitrite production and causes lipid peroxidation and membrane dysfunction (Numagami et al., 1997). This may affect the permeability and structural integrity of lipid bilayers that comprise cellular membranes and also may alter the proteins associated with these bilayers.

1.3 *Hypoxic suppression of protein synthesis:*

One of the first effects of hypoxia on cell metabolic functions is a rapid inhibition of protein synthesis. The decline can be so rapid that its time course is difficult to ascertain accurately with currently used techniques for measuring protein biosynthesis. In principle, a hypoxia-induced block could occur at the level of gene transcription or at translation. In animals (Hoffman et al., 1994) and in plant systems (Vadya et al., 1995), hypoxic suppression of protein synthesis may be mediated by a translational block affecting both initiation and elongation.

1.4 *Preferential gene expression during hypoxia:*

Several genes have been found to be induced by hypoxia in a variety of different tissues (Helfman and Falanga, 1993). These genes can be divided into three general categories based on the direct or indirect actions of their encoded proteins. The first group includes genes which are favorable for the adaptation of the whole organism to general hypoxia such as erythropoietin (EPO) and tyrosine hydroxylase (TH). A second class comprises of local acting factors that ensure the survival of tissues exposed to systemic hypoxia due to high oxygen consumption, reduced blood supply, or injury. Examples for this category are VEGF, a potent

angiogenic factor that promotes neovascularization in affected tissues (Plate and Risau, 1995), interleukin 1α (Shreeniwas et al., 1992) and Platelet –derived growth factor beta chain (Kourembanas et al., 1990). The third group consists of intracellular factors involved in the adaptation of the cell to hypoxia such as glycolytic enzymes Aldolase A, Phosphoglycerate kinase 1, Lactate dehydrogenase A, pyruvate kinase M, which provide ATP through anaerobic glycolysis (Semenza et al., 1994); or transcription factors of the Jun and Fos family which are induced by hypoxia in cardiac myocytes and hepatoma (Goldberg and Schneider, 1994). Due to the limited scope of this introduction, only hypoxic induction of genes that are relevant to our study are examined.

2. Vascular Endothelial Growth Factor (VEGF):

Hypoxia has been shown to be an important stimulus for new blood vessel formation seen in coronary artery disease (Sabri et al., 1991), tumor angiogenesis (Shweiki et al., 1992) and neovascularization (Aiello et al., 1994). Hypoxia is an important regulator of blood vessel tone and structure. All tissue growth requires the establishment of an adequate vascular structure. In its absence the tissue becomes deprived of oxygen and nutrients and in response the cells produce angiogenic factors, which function to recruit new blood vessels into the under-vascularized tissue (Folkman and Shing, 1992). Of the angiogenic factors identified to date, VEGF is likely to be a key regulator of angiogenesis (Millauer et al., 1994). VEGF is a secreted, endothelial cell-specific mitogen that is highly expressed in solid tumors and in areas of active vascularization (Shweiki et al., 1992). Upregulation of VEGF expressin by hypoxia appears to be a critical step in the

process of neovascularization of solid tumors (Shweiki et al., 1995). Inhibition of VEGF activity *in vivo* can block both tumor establishment and progression, by inhibiting vascularization, and VEGF also appears to be required for the maintenance of tumor blood vessels, because withdrawal of VEGF leads to breakdown of the vascular structure and consequently tumor regression (Benjamin and Keshet, 1997).

2.1 *Biological functions of VEGF:*

2.1.1. Stimulation of Proliferation and tube formation of endothelial cells:

VEGF, also known as vascular permeability factor is a potent angiogenic and endothelial cell-specific mitogen (Senger et al., 1993). VEGF stimulates tubular formation on endothelial cells in Matrigel and collagen gel assays (Connolly et al., 1989). Although the process of angiogenesis *in vivo* is known to be a complex one, accumulating evidence strongly supports that VEGF is a direct angiogenic factor *in vivo* as well as *in vitro*. In addition, endothelial cells isolated from lymphatic vessels have been shown to respond to VEGF for *in vitro* angiogenesis and induction of protease genes (Pepper et al., 1994).

2.1.2. Increase in vascular permeability:

As Senger et al. (1983) demonstrated originally, VEGF bears permeability stimulatory activity on blood vessels. The effective concentration of VEGF on vascular permeability is about 1 nM, which is 100-1000 fold stronger than that of histamine and bradikinin (Connolly, 1991). Permeability of VEGF can be blocked by the co-injection of anti-VEGF antibody, indicating that the permeability activity within the VEGF preparation is an innate property of VEGF .

2.1.3. Other biological activities of VEGF on endothelial Cells:

One of the first steps in angiogenesis is the digestion of a portion of the intercellular matrix by proteases. VEGF has been reported to stimulate both the mRNA and the protein levels of the interstitial collagenase gene in human umbilical vein endothelial cells (HUVEC) (Unemori et al., 1992). In the presence of VEGF, hexose transporter GLUT-1 gene expression is enhanced (Pekala et al., 1990). Ku et al. (1993) reported that in response to VEGF, the coronary artery endothelial cells secrete nitric oxide (NO), resulting in dilation of the coronary artery in the heart. Since vascular smooth muscle cells and cardiac myocytes were shown to express VEGF mRNA in hypoxic conditions (Ladoux and Frelin, 1993), these communication systems between endothelial cells and muscle cells in coronary artery using VEGF and NO appear to be crucial for the maintenance of appropriate amounts of oxygen and nutrition supplies in the heart.

2.1.4. Effects of VEGF on non-endothelial cells:

Although the cell growth-and permeability-stimulatory activities of VEGF are restricted to endothelial cells, as a rare case VEGF was reported to stimulate the migration of human peripheral blood monocytes (Clauss et al., 1990). Further, VEGF has been shown to induce differentiation in cultured osteoblasts (Midy and Plouet, 1994), raising the possibility that VEGF might also be involved in bone remodelling.

2.2 *VEGF structure and isoforms:*

VEGF gene has been isolated from a variety of animals: human, mouse, rat, bovine and guinea pig (Leung et al., 1989, Keck et al., 1989, Senger et al., 1990,

Conn et al., 1990, Breier et al., 1992). Genomic and cDNA analysis of the human VEGF gene has revealed atleast four subtypes of VEGF, i.e., $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ (Houck et al., 1991). By alternative mRNA splicing of a single gene, VEGF may exist as one of four different molecular species, having 121, 165, 189, and 206 amino acids respectively. All subtypes of VEGF share the amino-terminal 141 amino acids, including a signal peptide encoded by exons 1-5, and the carboxy-terminal 6 amino acids encoded by exon 8. VEGF purified from a variety of species and sources is a basic heparin-binding, homodimeric glycoprotein of 45,000 daltons (Ferrara et al., 1992). These properties correspond to those of $VEGF_{165}$, the predominant isoform. $VEGF_{121}$ fails to bind to heparin (Houck et al., 1992). $VEGF_{189}$, and $VEGF_{206}$ are more basic and bind to heparin with greater affinity than $VEGF_{165}$. $VEGF_{121}$ is secreted as a freely soluble protein and $VEGF_{165}$ is also secreted but a significant fraction remains bound to the cell surface or extracellular matrix (ECM) (Park et al., 1993). In contrast, $VEGF_{189}$, and $VEGF_{206}$ are completely sequestered in the ECM. However, they may be released in a biologically active form by plasmin cleavage (Park et al., 1993). Plasminogen activation and generation of plasmin have been shown to play an important role in the angiogenesis cascade (Folkman and Shing, 1992).

2.3 *VEGF receptors:*

In agreement with the hypothesis that VEGF is an endothelial cell-specific factor, ligand autoradiography studies on foetal and adult rat tissue sections have demonstrated that high affinity VEGF binding sites are localized to the vascular endothelium of large or small vessels, but not to other cell types (Jakeman et al.,

1993). Two tyrosine kinases have been identified as VEGF receptors. The Flt-1 (fms-like-tyrosine kinase) and KDR (Kinase Domain Region) proteins have been shown to bind VEGF with high affinity (DeVries et al., 1992, Terman et al., 1992). Flk-1 (foetal liver kinase-1), the murine homologue of KDR also binds VEGF (Quinn et al., 1993). The specific roles of KDR, and Flt-1 in vascular development and function are not clear, though there is some information on the signal transduction pathways induced by specific receptor subtypes. (Waltenberger et al. (1994) have shown that binding to VEGF induces autophosphorylation of both receptors. Research from their laboratory showed that while KDR expressing cells showed striking changes in cell morphology, actin reorganization and membrane ruffling, chemotaxic and mitogenicity upon VEGF stimulation, Flt-1 expressing cells lacked such responses. Studies performed in rats sinusoidal endothelial cells have demonstrated that VEGF binding to Flt-1 leads to the activation of MAP kinase (Seetharam et al., 1995). Cunningham et al. (1995) have utilized yeast two-hybrid system to demonstrate that the Flt-1 tyrosine kinase domain interacts with p85 subunit of phosphatidylinositol 3-kinase. Like VEGF, these receptors Flt-1, and KDR are upregulated during hypoxic conditions (Brogi et al., 1996) and thus, are involved in hypoxia related angiogenesis. The transcription of Flt-1 but not that of KDR is enhanced by hypoxia (Gerber et al., 1997). KDR production is also upregulated under hypoxic conditions but the mechanism responsible for the induction seems to be posttrancriptional (Waltenberger et al., 1996). This hypoxia induced change in KDR and Flt-1 expression may be triggered indirectly, since VEGF potentiates the expression of both receptor types (Wilting et al., 1996,

Barleon et al., 1997).

2.4 *Regulation of VEGF gene expression by hypoxia:*

The mechanisms that regulate VEGF production by oxygen availability have received particular attention in recent years and are slowly being worked out. Oxygen tension plays a major role in the regulation of VEGF gene expression. VEGF mRNA expression is rapidly and reversibly induced by exposure to low pO_2 in a variety of cultured cells. Similarities have been noted between the mechanisms leading to hypoxic regulation of VEGF and Epo genes (Goldberg and Schneider, 1994). Hypoxia-induced transcription of VEGF mRNA is apparently mediated, atleast in part, by the binding of HIF-1 to an HIF-1 binding site located in the VEGF promoter (Liu et al., 1995). Activation of c-Src is known to participate in the hypoxic upregulation of VEGF gene expression (Mukhopadhyay et al., 1995). In addition to the induction of transcription, hypoxia promotes the stabilization of the VEGF mRNA by proteins that bind to sequences located in the 3' untranslated region (UTR) of the VEGF mRNA (Claffey et al., 1998). Recently one such protein has been identified as the HuR mRNA binding protein (Levy et al., 1998). It is clear that additional proteins stabilize the VEGF mRNA but their identity is still unknown.

2.5 *VEGF as a potential therapeutic agent:*

Growth factors that are able to promote the growth of new collateral vessels would be potentially of major therapeutic value for the treatment of disorders characterized by inadequate tissue perfusion. Intra-arterial or intra-muscular administration of Arterial gene transfer of $VEGF_{165}$ significantly augments perfusion and development of collateral vessels in a rabbit model where chronic hind limb

ischemia was created by removal of the femoral artery (Takeshita et al., 1994). In addition, the angiogenesis initiated by the administration of VEGF results in improved muscle function. Studies have shown that VEGF administration also leads to a recovery of normal endothelial reactivity in dysfunctional endothelium (Bauters et al., 1995). Extra luminal administration of as little as 2μg of VEGF resulted in a significant increase in coronary blood flow in a model of chronic myocardial ischemia created by occlusion of the proximal circumflex artery (Harada et al., 1996). With all these observations, an intriguing possibility is that the VEGF protein or gene therapy with VEGF cDNA may be used in the future to promote endothelial cell growth and collateral vessel formation. This would represent a novel therapeutic modality for conditions that frequently are refractory to conservative measures and unresponsive to pharmacological therapy.

The expression of VEGF mRNA in the vast majority of human tumors, the presence of elevated levels of VEGF in eyes of patients with proliferative retinopathies support the hypothesis that VEGF is a mediator of angiogenesis associated with various pathological conditions. The ability of anti-VEGF antibodies or soluble VEGF receptors to block tumor growth or neovascularization associated with ischemic disorders provides more direct evidence for such hypothesis. Therefore, VEGF antagonists have the potential to be of therapeutic value for a variety of highly vascularized and aggressive malignancies as well as for other angiogenic disorders.

3. HIF-1 as a global regulator of hypoxic gene expression:

The single most important advancement in the quest to delineate

mechanisms involved in hypoxia regulated gene expression was the discovery and subsequent characterization and purification of HIF-1 (Wang and Semenza, 1995). Since the identification of HIF-1, many investigators have sought to link its activity to general hypoxic gene expression, for reasons important to basic and clinical sciences. There has been much progress on this front, as demonstrated by identification of homologous HIF-1 binding sites in various hypoxia inducible genes (Table 1). HIF-1 is now recognized as a central regulator in the mediation of hypoxic adaptation at the systemic and cellular levels. HIF-1 binds the sequence 5'-TACGTGCT-3' in the 3' flanking region of the Epo enhancer, and this site specific binding activity facilitated its purification (by affinity purification) and subsequent cloning of its genes (Wang and Semenza, 1995). It is an α/β heterodimer, with each subunit containing a basic-helix-loop-helix (bHLH) motif and a PAS protein-protein interaction domain (derived from Per, Aryl hydrocarbon receptor and Sim, the first three transcription factors found with this domain) found in a rapidly expanding family of known, or suspected transcription factors including the mammalian proteins Aryl hydrocarbon receptor (AHR), and Aryl hydrocarbon receptor nuclear translocator (ARNT). The HIF-1β subunit is identical to ARNT, which can also serve to form a heterodimer with AHR in the cellular response to environmental toxins such as dioxin. Since HIF-1β (ARNT) is involved in the induction of at least two distinct pathways, HIF-1α is the critical hypoxia-specific component of the heterodimer transcription factor (Salceda et al., 1996).

Given the ubiquitous nature of HIF-1 in modulating the cellular response to

Table 1: HIF-1 binding sites in various genes.

Hypoxia-inducible gene	**HIF-1 binding site**	**Reference**
Erythropoietin	TAC**GTG**CTGT	Wang et al., 1993
Phosphofructokinase L	TAC**GTG**CTGC	Semenza et al., 1994
Aldolase A	GAC**GTG**ACTC	Semenza et al., 1996
Phosphoglycerate Kinase 1	GAC**GTG**CGGC	Semenza et al., 1994
Enolase 1	CAC**GTG**CGCC	Semenza et al., 1994
Lactate Dehydrogenase A	CAC**GTG**ACGG	Firth et al., 1995
Glucose Transporter 1	GGC**GTG**CCGT	Ebert et al., 1995
VEGF	TAC**GTG**GGCT	Liu et al., 1995
Nitric Oxide Synthase	TAC**GTG**CTGC	Melillo et al., 1995
Retrotransposon VL30	TAC**GTG**CT	Estes et al., 1995
Heme Oxygenase 1	GAC**GTG**CTGG	Lee et al., 1997
Transferrin	TAC**GTG**CGCT	Rolfs et al., 1997

Homologous binding sites have been identified in various genes that are induced as a result of a hypoxic response. These binding sites are known as HIE or hypoxia inducible element.

hypoxia, the past eight years of research has produced a steadily increasing number of publications devoted to delineating the pathways critical to its regulation. As shown in Table 1, many genes involved in various functions are regulated by HIF-1. Thus, multiple hypoxic responses including adaptation to anerobic metabolism, erythropoiesis, angiogenesis, vasodilation, and possibly breathing are all under the control of this single transcription factor. Clues about signal transduction from the mammalian oxygen sensor to the induction of HIF-1 have also begun to accumulate, suggesting that the pathway may involve protein phosphorylation. General inhibitors of tyrosine kinases block induction of the HIF-1 genes, while inhibitors of tyrosine phosphatases increase basal levels of HIF-1 proteins and HIF-1 activity (Wang et al., 1995). A candidate tyrosine kinase upstream of HIF-1 is c-Src, which has been shown to be activated by hypoxia, thus raising the possibility of c-Src functioning upstream of HIF-1 (Mukhopadhyay et al., 1995).

3.1 *The oxygen sensing pathway:*

With the convergence of several independent areas of hypoxia research on a common oxygen sensing pathway, HIF-1 has emerged as a central regulator of hypoxic gene expression and restoration of cellular oxygen homeostasis. A working model of the pathway is schematized in Fig.2. Oxygen directly interacts with the cellular oxygen sensor independent of mitochondrial respiration, keeping the sensor inactive. When oxygen levels drop, the deoxy form of the sensor activates a signal that may then be transduced via protein phosphorylation to factor X, which leads to increased expression of HIF-1α and β. The HIF-1 heterodimer in turn induces a

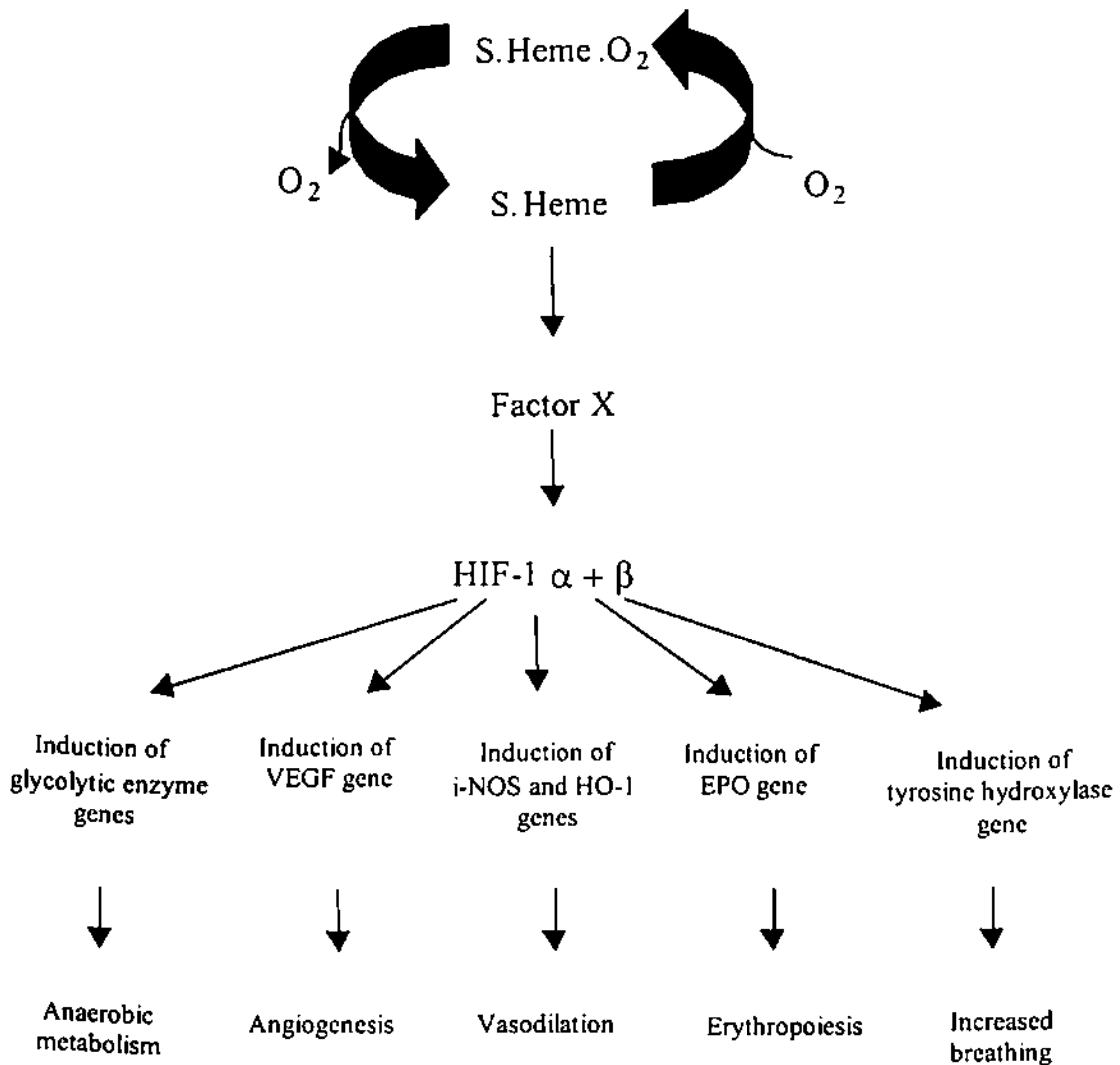


Fig.2: Oxygen sensing pathway : Involvement of HIF-1

battery of genes involved in cellular and global responses to hypoxia which allows the cells to survive at low oxygen and helps restore the normal oxygen level.While an outline of HIF-1 hypoxic response pathway is emerging, many critical questions remain. What are the identities of the cellular oxygen sensor and factor-X, and what is the mechanism of signaling between these two parts of the pathway? We also need to better understand the full range of cellular responses to low oxygen tensions and how a cell co-ordinates the HIF-1 pathway with other hypoxic responses such as the Pastuer effect (Graeber et al., 1996). Such an understanding would facilitate the development of medical treatments for rescuing ischemic/hypoxic tissues.

4. Angiogenesis:

One of the important patho-physiological responses to hypoxia is angiogenesis or blood vessel formation. Hypoxia increases vascular permeability (Kinasewitz et al., 1986) and provides a potent stimulus to neovascularization (Adair et al., 1990). Angiogenesis is a cascade process consisting of 1) Degradation of the extracellular matrix of a local venule after the release of proteases, 2) Proliferation of capillary endothelial cells, and 3) Migration of capillary tubules toward the angiogenic stimulus (Gross et al., 1983). This process is fundamental to reproduction, development, and repair. The formation of blood vessels is highly orchestrated involving both positive and negative regulators of the growth of microvessels. In view of the remarkable physiological and pathological importance of angiogenesis, much work has been directed in recent years to the elucidation of the factors capable of regulating this process.

4.1 *Mechanism of blood vessel formation:*

In established blood vessels of mature organisms, the endothelial cells remain in a quiescent, non-proliferative state until provoked by wounding, inflammation or other pathological conditions that stimulate angiogenesis. Formation of new vessels can be considered as the culmination of several steps: Dissolution of the matrix underlying the endothelium; migration, reattachment (adhesion), and proliferation of endothelial cells; and, finally, formation of a new three-dimensional tube, which then lengthens from its tip as circulation is re-established. The research conducted after the discovery of VEGF, the growth factor upregulated by hypoxia, revealed that VEGF is a central regulator of angiogenesis.

4.2 *Growth factors involved in angiogenesis:*

A number of naturally occuring growth factors and cytokine can induce and/or promote angiogenesis by stimulating endothelial cell growth and migration. Vascular growth factors are polypeptides, originally isolated in studies of tumor growth (Shing et al., 1984) and more recently demonstrated to be responsible for natural as well as pathologic angiogenesis. A large variety of growth factors belonging to several groups may play a role in angiogenesis. Basic (bFGF) and acidic (aFGF) fibroblast growth factors enhanced collateral flow in acute and chronic animal models of hindlimb ischemia (Baffour et al., 1992).

VEGF is currently the focus of major interest as a possible substance to promote therapeutic angiogenesis. Its high affinity binding sites are present on endothelial cells and VEGF has no mitogenic effect on smooth muscle cells and fibroblasts (Ferrara et al., 1989) unlike aFGF and bFGF (Klagsbrun, 1991). This

makes VEGF an excellent substance for study in the circumstances of endothelial disruption or ischemia where angiogenesis is desirable. It is likely that a number of yet to be discovered angiogenic growth factors will have advantages and effects superior to those presently under study. Another aspect of angiogenesis where molecular intervention could be considered is the regulation of cellular growth factor receptors.

VEGF receptor family members are increased in appropriate cell types during physiological circumstances requiring neovascularization. Studies on tumor angiogenesis have shown that expression of KDR and Flt-1 in endothelial cells correlates with vascularity (Takahashi, et al., 1985). The increased expression of receptor appears to be essential for the progression of tumors, since inhibition of KDR by neutralizing growth factor antibody (Kim et al., 1983) blocks tumor progression. In addition to its role in tumor angiogenesis, increased expression of VEGF receptors contributes to the progression of other physiological processes requiring neovascularization. Expression of KDR and Flt-1 is increased in the developing embryonic vasculature (Matthews et al., 1991,) and in vessels bordering healing skin wounds (Peters et al., 1993).

4.3 *Angiogenesis and matrix metalloproteinases (MMPs):*

Invasive processes of angiogenesis involve degradation of extracellular matrix, basement membranes, basal laminae and interstitial stroma required for invasive cells to migrate into adjacent tissues. For the degradation of the different components of ECM, a complex array of lytic enzymes is necessary (Schmitt et al., 1990). During angiogenesis, angiogenic factors like aFGF, bFGF, and TGF-β

(Gross et al., 1982, Pepper et al., 1990) have been shown to induce the synthesis of matrix proteinases by endothelial cells. ECM components have unique proteolytic requirements for their degradation performed by hydrolytic enzymes, MMPs being one of them. Metalloproteinases are divided into three major subclasses based on their substrate specificity. They are, 1) Interstitial collagenases that degrade I, II and II collagens; 2) 72 Kda Type IV collagenase that acts on IV, V and VII collagens, fibronectin and gelatin; 3) Stromelysin which digests proteoglycans and laminin. The expression of MMP activity is controlled at three levels, gene transcription, proenzyme activation and inhibition by specific tissue inhibitors. In most cells types the MMP genes are not constitutively expressed (Matrisian, 1990). However transcription can be induced by a number of agents like phorbol esters (Chin et al., 1895) and oncogene products (Kerr et al., 1990). Thus metalloproteinases are key components of an enzyme system and play major role in angiogenesis.

4.4 *Models used for studying angiogenesis:*

Several models have been reported in literature, to study the mechanism and regulation of angiogenesis. They are:

i. Corneal micropocket technique, which permits linear measurement of individual capillaries as they grow towards a tumor or an angiogenic substance implanted in cornea (Gimbrone et al., 1974).

ii. *Chick embryo chorioallantoic membrane* (CAM), which is used to detect angiogenic activity of partially purified fractions from tumor extracts (Ausprunk et al., 1974). In this method, the test substance is usually adsorbed onto a

polymer disc from which it is released slowly.

iii. *Subcutaneous rabbit sponge model*, in which the angiogenic stimulators or inhibitors are implanted into polyester discs in rats after creating a lesion (Andrade et al., 1987) and the sponges are gradually infiltrated by blood vessels.

iv. *Rat-aortic ring model*, wherein thoracic aortas are excised, cultured in collagen gels and treated with the compound under investigation (Diglio et al., 1989).

v. *Collagen gels*, an *in vitro* model in which the endothelial cells are cultured on the surface of a three-dimensional gel of type I collagen and angiogenic factors studied by their invasion into the underlying collagen matrix.

vi. *Matrigel assay*, a more recently developed *in vitro* differentiation model, wherein endothelial cells rapidly and completely form a capillary like network on reconstituted basement membrane (Grant et al., 1989). The basement membrane (matrigel) contains laminin, collagen IV, nidogen/entactin and proteoglycan. Matrigel affects a variety of biological activities including cell adhesion, migration, growth and morphogenetic changes during development and differentiation (Carey, 1990). When endothelial cells are layered on matrigel, the cells attach and differentiate into capillary like tubes. A representation of *in vitro* matrigel model is shown in Fig.3.

4.5 *Therapeutic implications: Pro-angiogenesis:*

Whereas, the role of new blood vessel formation (angiogenesis) in cancer (Folkman et al., 1995) and development has been well documented, the use of angiogenesis to restore blood flow to ischemic tissues, a novel form of therapy currently undergoing clinical trials, has received less attention. The concept of pro-

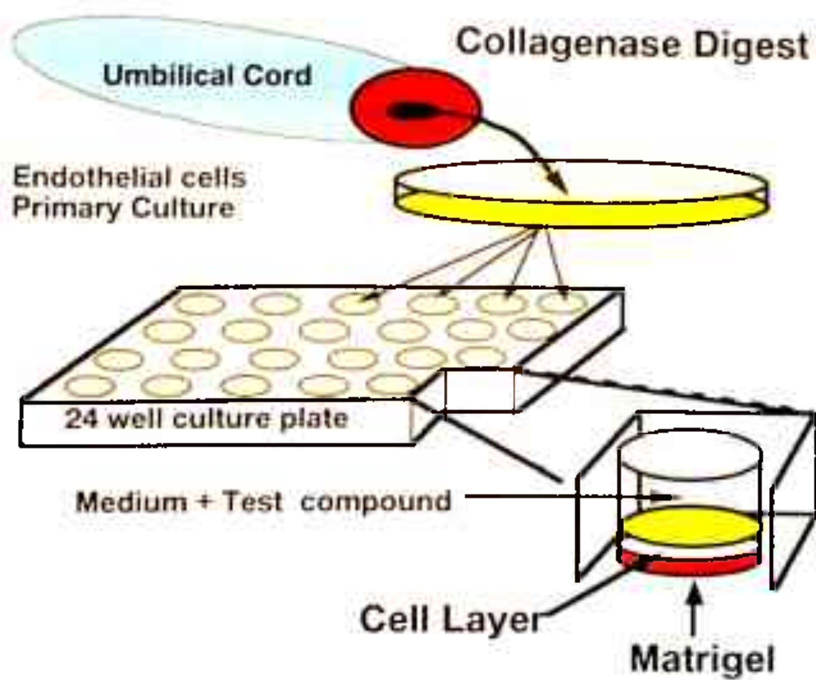


Fig.3: *In vitro* Matrigel model

(Kindly provided as part of a protocol by Dr. Dr. Hynda Klienman CDBRB/NIDR/National Institutes of Health, Bethesda, MD)

angiogenesis (therapeutic angiogenesis) is based on the hypothesis that induction of blood vessel growth represents a rational therapy for ischemic diseases. The observation that collateral vessels are often formed in chronic ischemic states together with the finding that blood vessel number is often increased around ischemic areas has led to the concept that enhancement of endogenous angiogenesis could prevent hypoxic/ischemic tissue damage. A lot of studies using animal models have encouraged several clinical trials to test whether angiogenic growth factors provide benefits to patients with ischemic heart disease. The first report of angiogenic therapy of human coronary heart disease involved patients with three-vessel coronary heart disease who received aFGF as a test material. This induced local angiogenesis and resulted in better patient conditions (Schumacher et al., 1998). Application of angiogenesis augmentation to patients with severe atherosclerosis has been reported to establish normal blood flow and vessel growth (Wall Street Journal, 1998). Thus, there is ample evidence of growth factor-induced angiogenesis in ischemic tissues that restore normal blood supply by formation of new blood vessels. Therapeutic angiogenesis appears to be potentially feasible in humans with ischemic disorders, but more studies and data are awaited.

4.6 *Angiogenesis in wound healing:*

Normal wound repair includes a vigorous angiogenic response that delivers nutrients and inflammatory cells to injured tissues. Angiogenesis can be conceived as a coordinated response at the wound site aimed at restoring favorable oxygen and energy balance (Constant et al., 1996). This angiogenic response facilitates the removal of debris and assists in the development of a granulation tissue

framework for wound closure (Battegay, 1995). The mediators of wound angiogenesis are thought to include numerous soluble factors that have been identified in various wound models (Orredson et al., 1983). Both angiogenic agonists and antagonists have been identified at various times during wound repair (Dipietro et al., 1996) suggesting that the net angiogenic stimulus may change as the balance of factors alternatively favors either vessel growth or regression (Martin, 1997). VEGF has been shown to mediate angiogenic activity within surgical wounds (Nissen et al., 1998). Angiogenic growth factors like bFGF accelerate dermal wound healing in rabbits via effects on angiogenesis, deposition of ECM (Pierce et al., 1992) and by thickening granulation tissue in guinea pig excision wounds (LeGrand et al., 1993). Taken together, the mechanisms responsible for initiating and driving angiogenic response in wounds might find broad application in areas such as augmentation of wound repair, improving collateralization of ischemic limbs and myocardium, and understanding tumor growth.

Although elucidating the mechanisms that orchestrate the various regulatory pathways within the cell are crucial for understanding hypoxia, the need for using *in vivo* models cannot be more emphasized. In a clinical frame of reference, situations involving organ transplants, tumors and combat casualties involve a period of ischemia to various organs of the body. Animal models are necessary to mimic these conditions so as to test the investigational agents that are critical to the discovery of new drug therapies.

5. Ischemia-Reperfusion injury (IRI):

Ischemia-induced pathologic processes are responsible for the greatest

causes of death – heart disease, and haemorrhagic shock involving cerebral ischemia. IRI is a complex interrelated sequence of events that classically involves the vascular endothelium and activated leucocytes. Due to limited scope of this introduction, only renal IRI pertinent to our study is elaborated. The mechanisms underlying ischemia/repurfusion damage to kidneys are multifactorial and interdependent, involving hypoxia, free radical damage and inflammatory response (Paller, 1994). The most common cause of acute renal failure is renal ischemia, which causes renal functional impairment through a combination of renal vasoconstriction, renal tubular obstruction, tubular back leakage of glomerular filtrate, and decreased glomerular permeability (Paller et al., 1984).

As a standard model to study renal IRI in rats, researchers have used silicone rubber tourniquet around the left renal pedicle to prevent blood flow to the left kidney. Various time courses of ischemia and reperfusion are performed and during the procedure the right kidney serves as the non-ischemic control. A schematic is shown in Fig.4. This is a good model to mimic renal IRI and is useful in studying the following parameters involved in various deleterious effects on tissues during such an insult.

5.1 *Free radicals in IRI:*

A free radical is an atom or molecule with one or more unpaired electrons in the outer orbit (Halliwell et al., 1990). These highly reactive molecules cause damaging effects on cells and tissues. O^{2-}, H_2O_2, and OH^- are extremely reactive free radicals amongst which OH^- is the most reactive in biologic systems (Halliwell et al., 1986). During ischemia, because of limited oxygen availability, ATP is

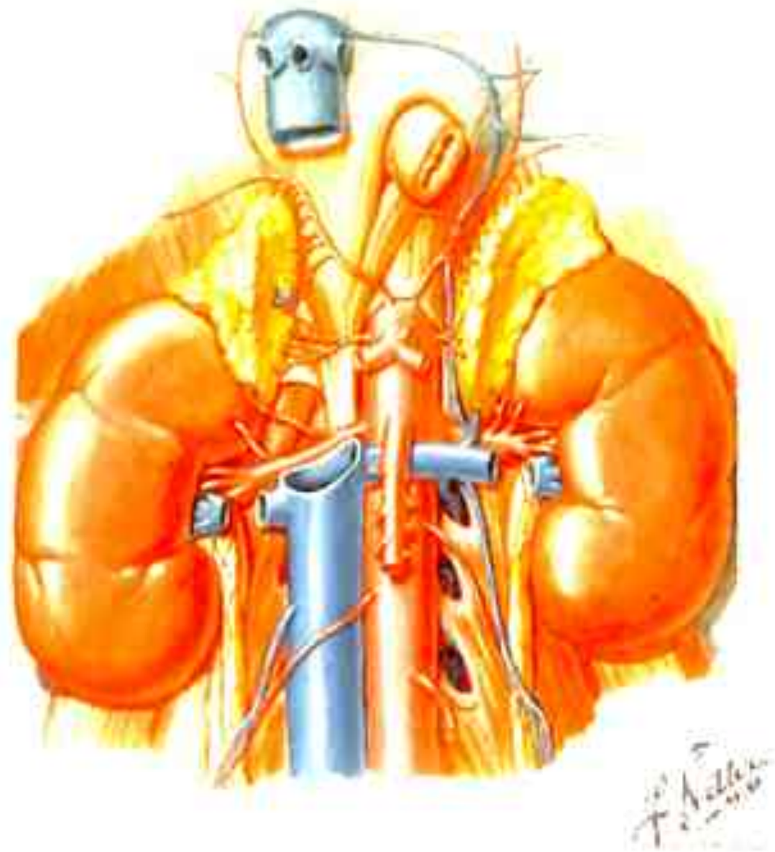
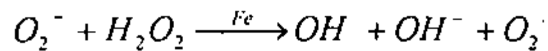


Fig.4: Renal IRI model

(Adopted from 'Netter Atlas': Ciba-Geigy corporation)

dimished (Takano et al., 1985). Calcium ion influx occurs because of deranged membrane ion gradience. This leads to an elevated cytosolic calcium concentration that activates proteases capable of transforming xanthine dehydrogenase into xanthine oxidase (Malis et al., 1986). Simultaneously, during the ischemic period, while ATP production is abated, the concentration of AMP increases. AMP is further metabolized into hypoxanthine. This accumulation of hypoxanthine during renal ischemia might lead to the generation of highly reactive oxygen free radicals, since the enzymatic conversion of hypoxanthine to xanthine by xanthine oxidase generates superoxide radical (O_2^-) as a reduction product of molecular oxygen (Fridovich et al., 1970). This superoxide radical reacts with H_2O_2 to form OH^- and $OH^\cdot$ by the iron-catalyzed Haber-Weiss reaction (Halliwell et al., 1986).



$$O_2^- + H_2O_2 \xrightarrow{Fe} OH^\cdot + OH^- + O_2$$

5.1.1. Cellular injury due to free radicals:

Superoxide radical and its reaction products can produce cellular injury through lipid peroxidation of mitochondrial, lysosomal, and plasma membranes, which can alter both membrane structure and function (Kellogg et al., 1975). A role for participation of oxygen free radicals in injury after hypoxia/ischemic insult has been found in the brain, intestine, and kidneys of experimental animals (Flamm et al., 1978, Guarnieri et al., 1980, Granger et al., 1981, and Paller et al., 1984). Lipid peroxidation is caused by the abstraction of allylic hydrogen from a poly unsaturated fatty acid by O^{2-}, H_2O_2, or $OH^\cdot$. Lipid containing cell and organelle membranes that are critical components for cell structure and function are exceedingly susceptible

to injury by lipid peroxidation. Lipid peroxidation disrupts the structural integrity of the lipid bilayer and leads to increased membrane permeability, impaired electron transport for oxidative phosphorylation in mitochondria, and increased lysosomal permeability. Increased lysosomal permeability would cause the release of hydrolytic enzymes further enhancing cell injury. Hydroxyl radical is capable of oxidizing sulfhydryl compounds, reacting with DNA leading to subsequent degradation. Sodium-pottasium ATPase is inactivated by sulfhydryl oxidation (Thomas et al., 1990). Given the central role of sodium-potassium ATPase in the maintenance of cell ion gradients and cell transport, oxygen free radicals can markedly disrupt cell function.

5.1.2. Antioxidants and oxygen free radicals scavenger:

An antioxidant may be defined as any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell, 1990). Antioxidants thus play a substantial role in attenuating injury caused by reactive oxygen intermediates. Well-known biological antioxidants include superoxide dismutase (SOD), glutathione peroxidase, catalase, vitamin E, glutathione, ascorbic acid, ceruloplasmin, and amino acids such as tryptophan (Halliwell, 1990). Many antioxidants have several mechanisms of action. They perform as free radical scavengers by binding metal ions, degrading peroxides to nonradical compounds, preventing chain initiation by scavenging initiating radicals and breaking chain reactions. SOD is an enzyme that is ubiquitously and evenly distributed among aerobic organisms and tissues. This enzyme catalyzes the dismutation of superoxide radical to H_2O_2 and O_2 (Fridovich,

1986). Catalase, a heme containing enzyme subsequently catalyzes the degradation of H_2O_2 to yield O_2 and H_2O. Catalase is specific for hydrogen peroxide, where as glutathione peroxidase can catalyze the degradation of H_2O_2 or lipid hydroperoxides. H_2O_2 reacts with glutathione (GSH) in the presence of glutathione peroxidase to form water and glutathione disulfide (GSSG). Vitamin E interferes with the propagation of lipid peroxidation by reacting with lipid peroxy radicals is the most important hydrophobic scavenger (Grisham et al., 1986).

Oxidative DNA damage induced by ROS occurs at the rate of about 10,000 events per cell per day in humans (Ames and Gold, 1991) and increase in extent and rate of formation with age (Holms et al., 1992). There is accumulating evidence that oxygen free radicals are involved in acute renal failure induced by ischemia. The bulk of evidence to support this role of oxygen radicals has consisted of tests of the efficiency of antioxidants in renal ischemia and measurements of by-products of free-radical reactions such as lipid peroxidation products. It has been demonstrated that SOD infusion before ischemia and again at the time of reperfusion provides functional and histologic protection against injury (Shanley et al., 1990). Thus although minor differences are likely to exist, over all there is a consistancy in findings implicating reactive oxygen in postischemic injury of the kidneys in all species and man is not likely to be an exception.

Nitric oxide is a free radical derived from the metabolism of L-arginine (Palmer et al., 1988) under the control of nitric oxide synthase (NOS). NO has a clearly defined place in normal renal homeostaesis while there is a continuing debate as to its role under pathophysiological conditions. The complex, interrelated

sequence of events that underlies IRI involves priming the endothelium during ischemia to produce both free radicals and chemoattractants which, upon reperfusion, sequester and activate neutrophils, thus amplifying the injury (Weight et al., 1996). On the other hand, NO has been defined as an important factor in the maintenance of normal diuresis and glomerular filteration rate (GFR) (Salazar et al., 1992). There is also evidence of protective effect of exogenous NO on renal function in a model of ischemia-reperfusion (Garcia-Criado et al., 1998). There remains uncertainty about the role of NO in the postischemic kidney and also as to whether NO production is increased or depressed (Yaqoob et al., 1996).

5.2 *Leucocyte adhesion molecules in ischemic renal injury:*

Infiltrating leucocytes serve to clear tissue of pathogens and probably clean up the cellular debris during healing. However, misdirected inflammation, like IRI, can lead to leucocyte-mediated diseases. In renal IRI, leucocyte infiltration is a classic feature of pathological finding of acute tubular necrosis and glomerular damage in the kidney. Three main family of leucocyte adhesion molecules act in concert to facilitate migration of leucocytes from blood to extravascular sites of inflammation (Hynes, 1992). Circulating leucocytes (PMN) are initially tethered loosely to endothelium through interaction of selectins on leucocytes or endothelial cells (EC) with their ligands. These selectin-mediated cell-cell interactions cause leucocytes to role on endothelium. Upon activation by cell-associated mediators of inflammation, loosely adherent rolling leucocytes are immobilized on endothelium through interaction of leucocyte integrins with endothelial counter receptors, many of which are members of immunoglobulin-superfamily (Ig superfamily), such as

intercellular adhesion molecules – 1 (ICAM-1). This PMN-EC interaction leads to infilteration of leucocytes into tissues.

Integrins are heterodimeric transmembrane receptors whose sub-families are defined by a common β-subunit non-covalently linked with unique α-subunits. With respect to kidney diseases, the VLA-4 (very late antigen – 4) β_1 integrin and CD11/CD18 β_2 integrins have received most attention (Rabb, 1994). CD11/CD18 is a major ligand for ICAM-1. Studies involving anti-CD11 mAb resulted in reduced tubular necrosis in renal ischemic injury (Rabb et al., 1994).

ICAM-1 is a single chain, transmembrane glycoprotein member of Ig superfamily (Springer, 1990). ICAM-1 is found on leucocytes, epithelial cells, endothelial cells, and many other cell types. In order to evaluate the role of ICAM-1 in renal IRI, Kelly et al. (1994) administered mAb to ICAM-1 in a rat model of 30 min renal ischemia and found a profound protection of renal function, even when mAb was given 2 h postischemia. Further evidence demonstrating the role of ICAM-1 in renal IRI emerged from the work of Linas et al. (1995) with their *ex vivo* system of ischemic rat kidney.

Selectins are single chain transmembrane glycoproteins characterized by complement regulatory repeats, an epidermal growth factor domain and an N-terminal lectin domain (Lasky, 1992). P-selectin (expressed on platelets and endothelium) blockade significantly attenuates IRI (Seekamp et al., 1994).

The complex vascular circuitry in the kidney, further modified functionally by ischemia and resultant sluggish capillary flow, could obviate the role of adhesion

molecules in IRI.

5.3 *Apoptosis in IRI:*

Ischemia of tissues during arterial occlusion, shock and organ transplantation is a common and important cause of cell death. Cells may die during ischemia by early membrane damage that leads to activation of multiple degradative systems in an uncontrolled fashion. This form of death is "necrotic". Dead cells are found in the course of ischemic regions but may also be diffusely distributed if the ischemic period is relieved by reperfusion of blood. Sublethal ischemic damage may cause some cells to undergo reperfusion injury when reoxygenated by blood reflow. Availability of oxygen and nutrients during reperfusion allows ischemic cells to restore ATP pools. So, additional losses of cells during reperfusion could be "apoptotic", since this form of cell death requires energy (Eguchi et al., 1997). However, these two types of cellular demise can occur simultaneously in tissues exposed to ischemia-hypoxia and reoxygenation (Noda et al., 1998, Wiegele et al., 1998). Cell death during IRI could follow various pathways. First, it has been reported that expression of Fas ligand during hypoxia/reoxygenation may trigger fast dependent death pathways (Nogae et al., 1998, Vogt et al., 1998). Second, reoxygenation injury has been attributed to the toxic effect of oxygen free radicals generated during reperfusion of organs with oxygenated blood. Ample evidence exists for the over production of radicals in reperfused organs (Chan, 1994), but following an initial increase, cellular concentrations are buffered down sharply by endogenous antioxidants. A third possibility, for which substantial support exists, is that irreparable mitochondrial damage underlies cell death by IRI. Since

mammalial cells are obligately dependent on mitochondria for long term viability, it follows that irreversible mitochondrial damage (electron conduction defects) (Piper et al., 1994) should have lethal consequences and lead to apoptosis. It has also been reported that Bax and Caspases are involved in hypoxic death (Koushal et al., 1998). An important clinical consideration that follows from above observations is that apoptosis inhibitors can offer long term protection to ischemic tissues.

6. Potential therapeutic approaches for IRI:

A. *Calcium channel blockers* : Several studies have demonstrated the role of calcium channel blockers in improving ischemic disorders following renal transplantation (Frei et al., 1987). The fact that dehydrogenase to oxidase conversion is calcium dependent may be one reason that calcium channel blockers like nephidipine prevent hypoxic injury.

B. *ATP administration* : Although ATP levels fall quickly during ischemia, tissue viability is not affected in a drastic manner. It appears that the ability to resynthesize ATP after ischemia is more important than the temporary reduction in ATP levels (Neumayer et al., 1989). *In vitro* studies using rabbit renal proximal tubules have demonstrated that the administration of extracellular ATP – magnesium chloride significantly improves cellular respiratory function during recovery from ischemia (Takano et al., 1985). It also restores membrane permeability to normal in kidneys (Mandel et al., 1988).

C. *Free radical scavengers*: Both SOD, a scavenger of reactive oxygen species, and allopurinol, a xanthine oxidase inhibitor (Green et al., 1989), have a protective effect in most models of renal ischemia (Baker et al., 1985).

Biological antioxidants like Curcumin have tested positively against ischemic injury in kidney and heart (Shoskes, 1998). The use of iron clelators desferrioxamine and deferoxamine to minimize the production of hydroxy radical has shown some success both *in vitro* and *in vivo* (Radi et al., 1991). It has been demonstrated that deferoxamine limits the production of reactive oxygen species and lipid peroxidation. NO for various purposes is considered a free radical and agents that specifically inhibit NO production, like nitroindazole, but do not block vasodilation due to endothelial NO production, appear promising in experimental ischemia (Yoshida et al., 1994).

D. *Membrane stabilizers*: Pavlock et al. (1981) have demonstrated that chlorpromazine pretreatment offers protection in rabbit kidney 2 h ischemia model. Membrane stabilizers like 21-amino steroids or lazaroids have been shown to inhibit iron-catalyzed lipid peroxydation.

E. *Manipulation of arachidonic acid metabolism*: Inhibition early in the pathway using the cyclo-oxygenase inhibitor ibuprofen prevents the rise in thromboxane levels normally seen in post-ischemic kidneys (Klausner et al., 1989). The use of specific thromboxane synthetase inhibitor has been shown to decrease histological damage in renal IRI (Lelcuk et al., 1985).

F. *Anti-adhesion Therapy*: Several complementary strategies have been employed to probe the contributions of leucocyte adhesion molecules to the pathophysiology of IRI, and to access the potential of anti-adhesion therapy as a therapeutic modality. Monoclonal antibodies to ICAM-1 and CD11/CD18 integrins have been successfully used to improve the damaged ischemic areas

in many IRI models. An alternative approach is to administer soluble forms of adhesion molecules or of synthetic oligosaccharides that engage leucocytes or endothelial binding sites and render them unavailable for leucocyte-endothelial cell adhesion.

6.1 *Future goals:*

Acute ischemic intervention is only at its infancy. Objectives for the future include efforts to: (1) refine animal models of IRI to better reflect real life human situations; (2) discover agents that protect against specific pathological events involved in IRI; (3) combine agents with protective properties that are synergistic; and (4) determine the time frame during which an intervention is likely to be effective. With the speed at which this field is progressing, we do not have to hold our breath for long for specific answers to all the IRI questions.

Given the multifarious properties of picroliv, a novel pharmacological agent, like antioxidant, hypolipidemic, membrane stabilizing and iron chelator functions, we decided to investigate the protective efficacy of this drug against hypoxic and reoxygenation injuries. We have delineated various regulatory check points where picroliv intervenes and protects the cells and tissues from ischemia-reperfusion mediated injuries. These studies directed towards modulation of hypoxic molecules have greater implication in developing a potent drug against Hypoxia/Ischemia - reoxygenation injuries.

PICROLIV

Picroliv, a natural product, is obtained from the ethanolic extract of the roots of the rhizome Picrorhiza kurroa (Scrophulariaceae). It is a standardized preparation containing mainly a mixture of two iridoid glycosides, picroside-I and kutkoside (1:1.5 w/w) (Chander et al.,1992). The structures of the two major constituents are represented in Fig.5. Picrorhiza kurroa grows abundantly in Himalayan and sub-Himalayan regions at 3300-3400 m. Preparation of picroliv involves an elaborate extraction and purification process. Roots and rhizomes of this plant are collected, dried, powdered and extracted with alcohol by cold percolation. The extract is evaporated *in vacuo* below 50°C. It is then dissolved in methanol:water (1:1 v/v) and extracted with chloroform. The aqueous methanolic phase is extracted with ethyl acetate followed by another extraction with butanol. The ethyl acetate and butanol-soluble fractions are combined and vaporized *in vacuo* to yield picroliv (Dwivedi et al., 1991).

Orginally developed as a hepatoprotective agent against a variety of toxins (Dwivedi et al.,1993), it has potent anti-inflammatory and antioxidant properties (Dhawan, 1995). It has been recently documented to possess protective activity against myocardial ischemia (Tandon et al.,1995). It is a known anti-oxidant with activities like that of xanthine oxidase and helps in the recovery of glutathione metabolism and has also been suggested to act as an iron chelator (Chander et al.,1992). Picroliv prevented thiocacetamide induced damage of liver in rats by reversing the biochemical changes Thioacetamide causes respiratory metabolic disturbances in liver by higher influx of Ca^{++} into hepatocytes and inhibition of

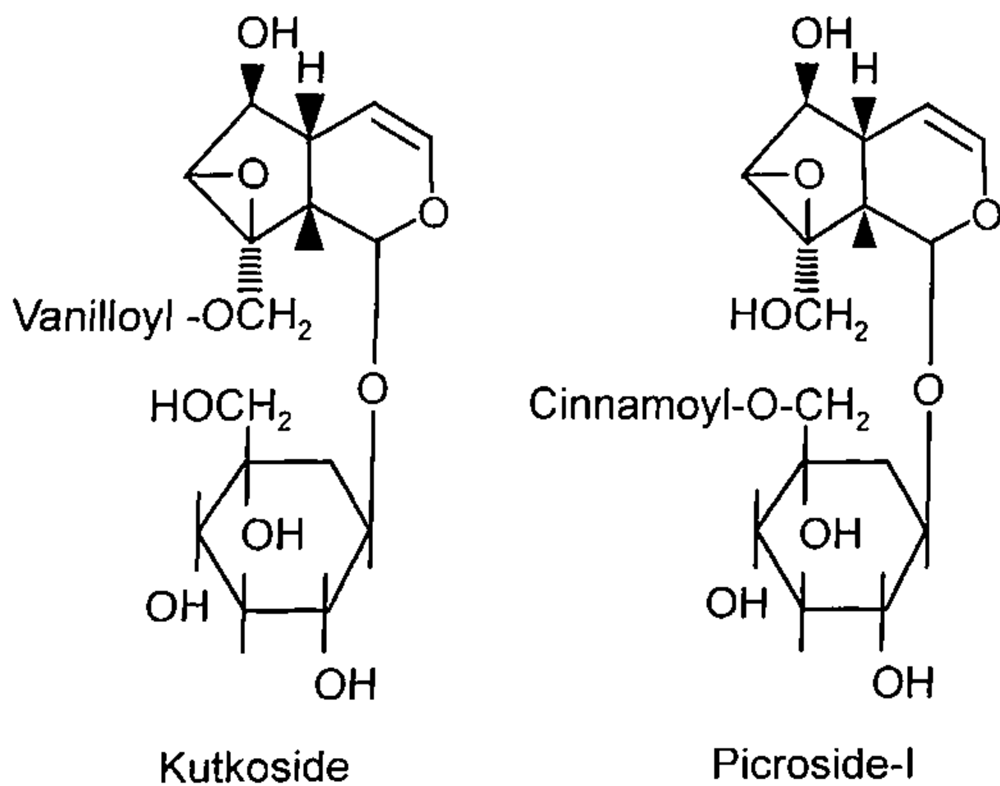


Fig.5: Structure of constituents of Picroliv

oxidative phosphorylation (Jeong et al., 1956). The elevated levels of 5'-nuceotidase, γ-glutamyl transpeptidase, succinate dehydrogenase, acid ribonuclease, glucose 6-phosphatase in liver and serum parameters like, glutamate pyruvate transaminase, glutamate oxaloactate transaminase and alkaline phosphatase levels caused by thioacetamide were significantly restored towards their normal values by picroliv. The decreased levels of the enzymes attribute liver protective properties to picroliv, by effecting the cellular membrane transport and mitochondrial transport chain.

In a similar study, hepatoprotective activity of picroliv was demonstrated in rat livers damaged by paracetamol (Shukla et al., 1991). Paracetamol causes severe cellular oxidative stress and lipid peroxidation (Albano et al., 1983). The increased levels of lipids, lipid peroxides and γ-glutamyl transpeptidase caused by paracetamol in rat livers were reduced by picroliv. Decreased contents of other biochemical parameters like 5'-nucleotidase and succinate dehydrogenase, total protein and glycogen caused by paracetamol were shown to be increased to normal levels by picroliv. The results suggest picroliv reverses the paracetamol induced alterations in the enzymes of plasma membrane, mitochondria, microsomes and lipid peroxides. These protective properties of picroliv that prevent hepatocyte damage from various hepatotoxins including carbon tetrachloride, monocrotaline and hepatitis B virus, have been ascribed to its membrane stabilizing action (Dhawan et al., 1995). Picroliv significantly prevented the changes in activities of aldehyde dehydrogenase (ALDH), SOD, catalase, peroxidase, glutathione-S-

transferase and lipid peroxides that occur by alcohol induced chronic hepatotoxicity in rats (Rastogi, et al., 1996). Picroliv was also shown to possess antioxidant properties and inhibited the generation of superoxide (O_2^-) anions in both xanthine-xanthine oxidase and non-enzymic phenazine methosulphate NADH systems. Picroliv inhibited generation of malonaldehyde (MDA) in rat liver microsomes by ascorbate-Fe^{2+} and NADPH-ADP-Fe^{2+} systems. The mechanism of antioxidant action was like that of SOD, xanthine oxidase inhibitors and metal ion chelators (Chander et al. 1992). It was suggested that picroliv has a dual role by chelating Fe^{2+} ions and scavenging the free radicals from fatty hydroperoxides to result in lipid peroxide inhibition and membrane stabilization.

We have been collaborating with Central Drug Research Institute (CDRI), Lucknow, India to identify pharmacological activities of natural compounds. In that endeavor, Picroliv, description of which dates back to the time tested form of Indian traditional medicine, Ayurveda, appeared to be a good candidate for testing protective efficacy in ischemia-reperfusion mediated injury. In this report we present evidence at various cellullar and molecular levels to demonstrate that Picroliv may be developed as a pharmacological agent against hypoxia/ischemia.

MATERIALS AND METHODS

Reagents

Picroliv was obtained from Central Drug Research Institute, India. The mouse monoclonal anti-phosphotyrosine antibody was purchased from Upstate

Biotechnology Inc. (Lake Placid, NY) and rabbit polyclonal anti-VEGF antibody was from Santa cruz Biotechnology,CA . Rat monclonal anti-ICAM-1 and anti-CD-18 were from Pharmingen, San Diego, CA and human polyclonal anti-Cu/Zn SOD was obtained from Calbiochem-Novabiochem Corporation, San Diego, CA. Fluorescein isothiocynate (FITC) conjugated anti-rabbit IgG and HRP-linked anti mouse IgG were acquired from Cappel and Santa Cruz respectively.). Monoclonal PKC (ab-2) antibody was obtained from Calbiochem (Cambridge, MA). [α-^{32}P]dCTP and [α-^{32}P]ATP were purchased from NEN Life Science Products (Boston, MA). Phototype-HRP Western blot detection kit was obtained from New England Biolabs (Boston, MA). Human hepatocellular carcinoma cells (Hep 3B) and human glioma cells (Hs 683) were obtained from ATCC (Rockville, MD) and human umbilical vein endothelial cells (HUVEC) from Clonetics (San Diego, CA). Rats (250-350 gm) were purchased from Charles River Laboratories, Kingston, NY. The animals were used in compliance with the U.S. Public Health Service Policy on human care and use of animals.

Maintenance of cell culture

Glioma cells were grown in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum and Hep 3B liver cells in Eagle's MEM with non-essential amino acids, sodium pyruvate, Earle's BSS and fetal bovine serum. HUVEC were cultured in Media-199 with ECGS (50 mg), fetal bovine serum(10%) and antibiotics. Cells were routinely cultured in an atmosphere of 21% O_2, 5% CO_2 and 74% N_2 at 37 °C and were made hypoxic by placing them in a

modular incubator chamber (Billups-Rothenberg inc., Del mar, CA) maintained at 1% O_2, 94% N_2 and 5% CO_2. For reoxygenation the cells were grown in the same condition as in normoxia.

Cell viability assay

To determine the non-toxic doses of picroliv, MTT assays were performed. Cells were cultured in 96 well tissue culture plates (Costar, Amherst, MA) in a range of 1.5 X 10^4 cells per well in a final volume of 100 μl. Cell viability was measured using the cell proliferation kit (Boehringer Mannheim, Indianapolis, IN) that assays cell growth by cellular conversion of tetrazolium salt into blue formazan crystals. The cells were treated with varying doses of picroliv (0.5-100μg/ml) for 24 h. The chromogenic methyl thiazol tetrazolium bromide (MTT) dye, an indicator of metabolically active mass, was added to the cells and incubated for 4 h at 37°C. Cells were lysed and the reduced intracellular formazan product was dissolved in a solution containing 10% SDS and 0.01 N HCl. The absorbance was recorded at 570 nm and percentage viabilities were tabulated against untreated control.

Lactate dehydrogenase (LDH) activity

Calorimetric assay for release of LDH, an indication of cytolysis or membrane damage was performed as follows. Cells (3 x 10^4/well) were plated in 96 well microtiter plates and then treated with picroliv (0-100 μg/ml media) under hypoxic condition for 24-48 h. At the end of incubation period, cells were simultaneously

sedimented by centrifugation at 400 X g for 10 min and the supernatant was collected. For determination of the total LDH released, the cells were completely lysed with Triton X 100 and this was used as a positive control. LDH contents in the supernates were measured spectrophotometrically using cytotoxicity detection kit (Boehringer Mannheim, IN), following the manufacturers instructions. The absorbance was measured at 490 nM using microplate reader (Dynatech Laboratories, VA) and percentage LDH release was plotted against the untreated control. Significant differences between groups were determined by student's *t*-test. $p < 0.05$ were considered significant.

Study design for *in vitro* system

A typical set experiments in the study included the following nine treatments: 1. normoxia; 2. 1% hypoxia for 48 h (hypoxic control); 3. hypoxia for 48 h followed by reoxygenation for 24 h; 4. treatment with picroliv for 24 h at normoxia (drug control); 5. treatment with picroliv for 24 h at normoxia followed by hypoxia for 48 h; 6. treatment with picroliv for 24 h at normoxia followed by hypoxia for 48 h and reoxygenation for 24 h; 7. Simultaneous treatment of cells with picroliv and hypoxia; 8. Simultaneous treatment of cells with picroliv and hypoxia followed by reoxygenation for 24 h ; 9. Hypoxia for 48 h followed by reoxygenation along with picroliv for 24 h. Treatments 1-4 were controls and 5-9 were experimental groups. Taking lead from viability studies, a concentration of 1 μg/ml of picroliv was chosen for the above treatments.

RT-PCR analysis

Following the treatments, the cells were harvested and total cellular RNA was prepared using Trizol method (Life Technologies, Gaithersburg, MD). 1-2 μg of total RNA was reverse transcribed to cDNA using Superscript II RNase H-reverse transcriptase (Life Technologies, Gaithersburg, MD) following the manufacturer's instructions. Amplification reactions were carried out through 25-30 cycles (95°C-30 sec; 60°C-60 sec), using 2 μl of cDNA. The reaction mixture contained 10 pmol of each primer pair and 0.5 U of Taq DNA polymerase (Life Technologies, Gaithersburg, MD). PCR products were analyzed by electrophoresis on 2% agarose gels. The primer sequences of the oligonucleotides used for PCR were as follows: VEGF, sense 5'-TCT TCA AGC CAT CCT GTG TGC-3', antisense 5'-CAC ATT TGT TGT GCT GTA GGA AGC-3'; HIF-1α sense 5'-TGT AAT GCT CCC CTC ACC CAA CGA A-3', antisense 5'-CAG GGC TTG CGG AAC TGC TTT CTA A-3'; HIF-1β sense 5'-TTT GCC AGG TCG GAT GAT GAG C -3', antisense 5'-TCA TGT GAG AAA CTG CCA TGC G -3'; Flt-1 sense 5'-GTC ACA GAA GAG GAT GAA GGT GTC TA-3', antisense 5'-CAC AGT CCG GCA CGT AGG TGA TT-3'; KDR sense 5'-GTG AAC GAC TGC CTT ATG ATG C-3', antisense 5'-TCT TGA GTT CAG ACA TGA GAG GA-3'; Flt-4 sense 5'-CAG GAT GAA GAC ATT TGA-3', antisense 5'-AAG AAA ATG CTG ACG TAT GC-3' and L28 5'-CGC AAT TCC TTC CGC TAC AAC G-3', antisense 5'-ATT CTT GTT GAT GGT GGT CCG C-3', MMP-1; sense 5'-CTG GAA TTG GCC ACA AAG TT-3', antisense 5'-TGT ACC CAC CAT TTG TGG AA-3', MMP-3;sense 5'-TTC ACA GTT GGA GTT TGA CCC-

3',antisense, 5'-GCA AGC TAA GCA GCA GCC-3' and MMP-9; sense 5'-TCT TCC AGT ACC GAG AGA AAG C-3', anti sense 5'-GAG AAG AGA GGG CCC AGC-3'.

Amplification was performed on samples in which reverse transcriptase was omitted or no RNA was added to the reaction mixture to act as controls to check for any contamination. Densitometric analysis of the PCR products (10 μl each) was performed with Instant Imager (Packard, Meriden, CT). NIH image analysis was used for the quantitative estimation of the intensity of the experimental bands as compared with that of the controls. The gel plotting macros of the NIH image program (version 1.59) was used and the area under the curve for each band was tabulated.

Northern blot analysis

Total RNA (20 μg) isolated from cells following the treatments, were electrophoresed on 1% agarose gel containing fomaldehyde and transferred to nylon membrane (Micron Separations Inc.) by electroblotting. Amplified DNAs generated by RT-PCR, using human placental DNA and primers described in the RT-PCR analysis section were used for probes. The PCR products were verified by restriction endonuclease mapping and electrophoretic analysis. The probes were labeled by random labeling using Klenow fragment (Life Technologies, Gaithersburg, MD) and [α-^{32}P]dCTP. Blots were sequentially hybridized with [α-^{32}P]dCTP random labeled VEGF, HIF-1α, HIF-1β and L28 probes in 50% formamide, 5 X Denhardt's solution, 5 X SSPE (1 X SSPE = 0.18 M NaCl, 10 mM

sodium phosphate, pH 7.7, 1mM EDTA), 0.2% SDS, 100 μg/ml denatured DNA and 10% dextran sulfate at 42°C. After hybridization, the membranes were washed to a final stringency of 65°C in 0.1 X SSC (1 X SSC = 150 mM NaCl and 15 mM trisodium citrate) and 0.1% SDS. Then the blots were exposed to x-ray film with intensifying screens. After autoradiographic imaging, membranes were stripped and rehybridized with another radiolabeled probe. Densitometric analysis of gene expression was performed with Instant Imager (Packard, Meriden, CT).

Immunofluorescence staining

Cells grown on cover slips under different culture conditions were fixed in chilled acetone. Expression of VEGF was determined by immunofluorescence staining method. Fixed cells were rehydrated in PBS and incubated at 37°C with 50 μl of anti-VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology.,CA) at a dilution of 1:200, for 30 min. After a series of washes with PBS containing 0.1% BSA, the cells were incubated with FITC (fluorescein conjugated iso thiocyanate) conjugated goat anti-rabbit IgG (1:100dilution) for 30 min. Cells were then washed and mounted on glass slides using fluoramount. Untreated cells grown in normoxia were employed to demonstrate the specificity of staining. Cells were viewed and photographed using Olympus 1 x 70 fluorescence inverted microscope.

Phosphorylation studies

To enhance the detection of the tyrosine phosphoproteins, immunoprecipitation followed by western blot analysis was performed using

monoclonal anti-phosphotyrosine antibodies according to Rui et.al.,1994. Cells cultured under the treatment conditions mentioned in the study design, were rinsed with ice-cold phosphate buffered-saline and solubilized in 1 ml of lysis buffer (modified RIPA buffer) containing 10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovandate, 1% Triton X-100, 1mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A and 2 μg/ml leupeptin for 30 min on ice. The cells were disrupted by repeated aspirations through a 21 gauge needle. Cellular debris was pelleted by centrifugation at 3000 rpm at 4°C for 15 min. After the quantitation of protein, equal amount of protein for each sample was incubated rotating end over end for 4 h at 4°C with either mouse monoclonal anti-phosphotyrosine antibodies (Upstate Biotechnology Inc., Lake Placid, NY) or monoclonal PKC (Ab-2) depending on the experiment. The antibodies were precipitated with protein A-agarose beads (Santacruz biotechnology.,CA) overnight at 4°C. Precipitated material was eluted off the beads by boiling in SDS sample buffer for 5 min, subjected to 7.5% SDS-PAGE under reducing conditions, and transferred to nitrocellulose membrane (Micron Separations Inc.) by electroblotting. Subsequently, the filter was blocked in TBS (10 mM Tris-HCl, pH 8.0; 150 mM NaCl) with 5% blocker (Bio-Rad Laboratories, Hercules, CA) for at least 1 h on a rocking platform. All steps were carried out at room temperature. For detection of the phosphotyrosine proteins, blots were incubated for 90 min with either mouse monoclonal anti-phosphotyrosine antibody (clone 4G10., Upstate biotechnology, NY) or monoclonal PKC (Ab-2) as

per the experiment at a concentration of 0.5 μg/ml in blocking buffer. The blots were washed thrice for 5 min each and incubated for 45 min with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santacruz Biotechnology,CA) diluted to 1:1000 in blocking buffer. The blots were washed three times. The proteins were detected by enhanced chemiluminescence (ECL) using Phototype-HRP Western blot detection kit. Blots were incubated in Luminol ECL reagent (SantaCruz Biotechnology, CA) according to the manufacturer's instructions for 1 min and exposed to autoradiograph for 1-20 min.

Kinase activity of tyrosine phosphorylated proteins

For *in vitro* kinase assay of tyrosine phosphorylated proteins, the lysates were prepared with lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 1% NP-40, 10% glycerol). The cell extracts were incubated with monoclonal agarose conjugated anti-phosphotyrosine antibody (4G10 clone) for 4 h at 4°C. The extracts were centrifuged, and agarose beads were washed with PBS. The beads were again washed with Tris buffer (20 mM Tris, pH 8.0, 0.5M LiCl) twice and once with kinase buffer (10mM HEPES, pH 7.6, 50 mM Nacl, 100μM Na_3VO_4, 5 mM $MnCl_2$, 5 mM $MgCl_2$). Agarose beads were resuspended in 40 μl of fresh kinase buffer containing 10 μCi of γ-^{32}P-ATP(3,000 Ci/mmol) and incubated at room temperature for 20 min. The kinase reaction was stopped by adding 30 μl of SDS-PAGE sample buffer, and boiled for 5 min. Peptides were separated from unincorporated label on 10% SDS-PAGE and visualized by autoradiography.

Zymogram Analysis

The culture supernatant from the treated and control cells were estimated for ther protein content using the BCA* Protein Assay Reagent (Pierce, Rockford, IL). Equal protein from each sample was mixed with sample buffer (10% SDS, 4% glycerol, 0.25M Tris-HCl pH 6.8 and 0.1% bromophemol blue) in a ratio of 3:1. The samples were loaded on a 10% Zymogram (Gelatin) gel (Novex, San Diego, CA). Gels were run at 15mA/h while stacking and 20mA/h during resolvingphase at 4^0C. After electrophoresis, the gels were soaked in 2.5% Triton x-100 with gentle shaking for 30 min at ambient temperature with one change of detergent solution. The gels were rinsed with water for 20 min and incubatede overnight at 37^0C, in substrate buffer (50mM Tris-HCl pH 8, 5mM $CaCl_2$ and 0.02% Tween 20). After incubation the gels were stained for 1h in 0.5% Coomassie blue R-250 in acetic acid, methanol and water (1:3:6), destained in acetic acid, methanol and water (1:4:6). After intensive destaining, proteolyic areas appeared as clear bands against a blue background. The molecular weights of the proteolytic bands were determined in relation to the reference protein marker which was simultaneously loaded in the gel. The molecular weight markers were: Myosin 205,000; β–galactosidase 121,000; Bovine serum albumin 86,000; Ovalbumin 50,500; Carbonic anhydrase 33,600; Soybean trypsin inhibitor 27,800; Lysozyme 19,400 and Aprotinin 7,400 (Bio-Rad, Hercules, CA).

***In vitro* angiogenesis assay**

HUVEC cells (Passage 3-5) were treated with picroliv at 0, 0.5, 1 and 5 μg/ml media concentrations under normoxic condition for 24 h. Twenty-four-well culture plates were coated with 0.3 ml of matrigel (14-20 mg protein/ml), which was then allowed to solidify at 37 ° C for 1 h. Subsequently, the treated cells were trypsinized using trypsin-EDTA (Gibco BRL, Gaithersburg, MD) and plated (3×10^4/well) in triplicates in 500 μl of serum free RPMI 1640 media (Life Technologies, Gaithersburg, MD). The plates were incubated for 6 h at 37°C in a 5% CO_2 humidified incubator, the culture supernatant was aspirated and the cells were fixed in 10% neutral buffered formalin solution (Sigma Chemical Co.) and stained with Giemsa blue stain. The total area covered by the tube network was measured with an optical imaging technique by scanning the pictures of the tubes using Adobe Photoshop and quantitated in NIH image program. Cells along with the tube network were photographed using Olympus microscope at a magnification of 20 x. All experiments were performed at least three times.

Ex vivo angiogenesis assay using rat aorta ring model

Aorta isolated from male Sprague Dawley rats (Charles River Laboratories, Kingston,NY) weighing approximately 150 g, was cleaned with DMEM medium. The cleaned aorta was cut into rings of 1-2 mm thickness and placed in 48-well plate precoated with matrigel. The ring was covered with 50 μl of matrigel and allowed to solidify for 15 min at 37°C. Serum free human endothelial-SFM basal growth medium (Life Technologies, Gaithersburg, MD) containing 0.5, 1 and 10

μg/ml picroliv, in a final volume of 1 ml with or without endothelial cell growth supplement (ECGS) was added and the plate was incubated at 37° C for 90-96 h. After the incubation, the rings were fixed in 1% buffered formalin and stained with Wright-Giemsa. The rings were photographed using Olympus 1 X 70 inverted microscope.

Creation of Wounds

The animals were anesthetized using pentobarbital. Hair on the dorsal side was shaved and the skin was cleaned with 70% ethanol. An 8-mm skin biopsy punch (Acuderm Inc., Ft. Lauderdale, FL) was used to create full thickness cutaneous wounds under aseptic conditions. Six wounds were created in the rats, three each on the left and right sides of the midline, while two wounds were made in mice, one on each side of the midline. Thereafter, the animals were individually caged.

Picroliv treatment to test wound healing in rats

Oral dosage was based on previous studies that have shown that picroliv was most effective when given in a dose of 12 mg/kg body weight/ day. Picroliv was dissolved in water and given once daily by oral gavage. Control animals were similarly handled, but fed only water instead of picroliv. No dressing was placed on any of the wounds.

Histopathological studies

Six animals from each group were sacrificed on the 7th day after wounding, using pentobarbital. Wound tissues were excised in full depth to include subcutaneous fat, including a margin of at least 5-mm of healthy uninjured skin around each wound. Tissues were fixed in 10% buffered neutral formalin, routinely processed, and embedded in paraffin. At least six serial sections (5-6μ) were stained with hematoxylin and eosin (HE) for histologic evaluation and morphometry.

Induction of ischemia-reperfusion injury (IRI)

Ischemia-reperfusion injury was induced in the left kidney of adult male Spargue Dawley rats (250-325g; Charles River Labs, Kingston, NY). Twenty four animals were randomly divided in two main groups. One of the group (picroliv treated; n = 12) was pre-treated with 12 mg/kg dose of picroliv once daily for 7 days by oral route, while the other (Vehicle; n = 12) was administered same volume of water. Rats were anesthetized by interaperitoneal injection of Pentothal (50 mg/kg; Abbot Laboratories, North Chicago, IL, USA). Autoclave sterilized surgical instruments were used for the procedure. A laparotomy was performed using a vertical midline incision and the left renal artery was exposed by a blunt dissection. A hemostatic micro clamp was applied on the renal artery left kidney for 60 minutes to create complete renal ischemia. Right kidney was left as such to serve as control kidney. The clamp was later removed to allow restoration of blood flow to the kidney, for varying time of reperfusion. Animals were sacrificed later following 5, 60 120, 240 min initiation of reperfusion. After sacrifice both right and left kidneys were

dissected out and split into two equal halves vertically. At random, one half was fixed in 10% buffered formalin for paraffin embedded sections and the other was stored at -70° C for biochemical estimations.

Biochemical parameters

a) Measurement of free radical mediated lipid peroxidation : Frozen kidneys were homogenized in ice cold 20 mM Tris-HCl buffer (pH=7.4) to prepare 10 % w/v homogenates. Homogenates were centrifuged at 3000 X g for 10 minutes at 4° C, to remove cell debris. Free radical mediated lipid peroxidation was assessed in tissue homogenates in terms of μM malondialdehyde (MDA)/mg tissues protein using a colorimetric biochemical kit (Calbiochem-Novabiochem Corporation, San Diego, CA, USA), following manufacture's protocol. MDA contents were calculated using standard MDA curve plotted using the standard provided with the kit.

b) Measurement of Reduced Glutathione: Reduced glutathione was estimated colorimetrically using a Glutathione assay kit procured from Calbiochem-Novabiochem Corporation, San Diego, CA, USA. Briefly, 5% tissue homogenates were prepared in ice cold 5 % metaphosphoric acid, which were centrifuged at 3000 X g for 10 minutes at 4° C, to remove cell debris. 100 μl supernatant was used for GSH estimations, to which 900 μl 200mM potassium phosphate buffer, pH 7.8 (containing 0.2mM diethylene triamine pentaacetic acid and 0.025% LUBROL) was added. 50 μl of chromogenic agent in 0.2N HCl was added to this mixture to form thioesters. Further, 50 μl of 30 % NaOH was added for beta elimination in alkaline

conditions which transforms substitution product obtained with GSH into a chromophoric thione which was read at 400 nm.

Glutathione peroxidase (GPx) and Glutathione reductase (GR) activities were assayed using biochemical kits obtained from Oxis International Inc., Portland, OR, USA. Briefly, 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. 400 µl of GSSG was added to 200 µl of diluted samples which was incubated at 25° C. 400 µl of NADPH was added to this reaction mixture and reaction kinetics was followed at 25° C for 6 minutes at 340 nm, spectrophotometrically. Each sample was assayed against its own blank containing potassium phosphate buffer pH 7.5 to replace GSSG. GR activity is based on the oxidation of NADPH to NADP catalyzed by GR. For Gpx activity, 5 mM EDTA and 1 mM mercaptoethanol were added to homogenizing buffer. Reaction mixture containing 350 µl assay buffer, 350 µl NADPH reagent and 70 µl of diluted sample was incubated at 25° C. 350 µl of enzyme substrate (0.007% tert-butyl hydroperoxide) was added to initiate the reaction which was estimated by following the oxidation of NADPH to NADP+ for 3 minutes at 340 nm to monitor GPx activity, spectrophotometrically.

c) Estimation of total nitric oxide levels : Tissue homogenates prepared for MDA levels were filtered through 10,000 molecular weight cut off microcentrifuge filters (NanoSpin plus, USA) Filtrates were used for the measurement of total nitric oxide 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, USA. Briefly, samples were incubated with reaction buffer, reduced-βNADH and diluted nitrate reductase, provided in the kit, for 30 minutes at 37° C, to allow conversion of sample total nitrate into nitrite. Reaction mixtures were then added with Griess reagent, also provided in the kit, and allowed to stand at room temperature for 10 minutes. Finally, samples were read at 570 nm for their total nitrite contents, which were calculated using a sodium nitrate standard. Protein estimation in all samples was carried out using BCA protein assay kit obtained from Pierce, Rockford, IL, USA.

Immunohistochemical staining

Immunostaining for adhesion molecules CD-54 (ICAM-1), CD-18 (Integrin 2 chain) and free radical scavenging enzyme Copper-Zinc superoxide dismutase (Cu/Zn SOD) was performed using rat monclonal anti-ICAM-1, anti-CD-18 (Pharmingen, San Diego, CA, USA) and human polyclonal anti-Cu/Zn SOD (Calbiochem-Novabiochem Corporation, San Diego, CA, USA), respectively, by an indirect avidin-biotin-immunoperoxidase technique (Quick Universal Kit, Vector laboratories, CA, USA). Tissue sections placed on poly-L-lysine coated slides were deparaffinized and hydrated using graded ethanol. They were then treated with 3 % hydrogen peroxide in methanol for 10 minutes at room temperature to inactivate endogenous peroxidase. Blocking serum, provided in the kit, was used to block non-specific staining. Sections were then incubated with respective antibody for 1 hr at room temperature in humidified chamber. Slides were washed with phosphate

buffered saline (PBS) after each incubation with antibody/kit reagents. Respective biotinylated secondary antibody IgG (H+L) was added on to sections for 15 minutes at room temperature, followed by avidin-biotin-peroxidase complex for 15 minutes. Diaminobenzidine (DAB) was used as a substrate for peroxidase and slides were incubated in dark for 5-8 minutes and sections were counter stained with Gill's hematoxylin (Vector laboratories, CA, USA). Specificity of test antibody was ascertained by incubating sections from each set with normal serum IgG separately. The slides were dehydrated and mounted on Permount (Southern Biotechnology Associates, Inc., Birmingham, AL) and were examined under the Olympus light microscope equipped with a camera, and photomicrography was performed.

Apoptotic Studies

The extent of cell death, apoptosis, was determined using TUNEL (terminal deoxynucleotidyl transferase (TdT) mediated d-UTP nick end labeling) technique using Apoptag kit (Oncor lab, Gaithersburg, MD) by following manufacturer's instructions. The TUNEL method is based on the specific binding of TdT to 3'-OH end of DNA and ensuring synthesis of a polydeoxynucleotide polymer. Tissue sections were digested using proteinase K and the endogenous peroxidase activity was blocked using 2% hydrogen peroxide in PBS. These slides were then placed in equilibration buffer and incubated with working concentration of TdT enzyme. The reaction was terminated after 30 min using stop/wash buffer that was provided with the kit. The apoptotic nuclei were visualized by direct immunoperoxidase detection of digoxigenin-labeled DNA in test sections.

Statistical analysis

Determinations, where applicable, were performed in triplicate, and experiments were repeated at least three times. Statistical calculations were done using statview progam for the Macintosh. Results are expressed as means of percentages $\pm$ SD and were analyzed using a non paired *t* test. $P<0.05$ was considered statistically significant.

RESULTS

A. Effect of picroliv on in vitro system of hypoxia-reoxygenation:

Effect of picroliv on the viability of Hep 3B, Glioma and HUVEC cells

To establish the non-toxic doses of picroliv on Hep3B, Glioma and HUVEC cells, MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) assays were performed. Cell viability data (Table 2) show that picroliv (0.5 μg-10 μg/ml) did not significantly affect the viability of all the three cell lines used in the studies. A dose of 1 μg/ml of picroliv was chosen for the hypoxia *in vitro* experiments.

Picroliv reduced the LDH release from hypoxic cells

Cellular LDH release was determined as a usual marker of cell membrane permeability to characterize hypoxia-induced membrane dysfunction. Picroliv reduced the LDH release from Hep 3B and Glioma cells, thus protecting them from hypoxia induced membrane damage. Post-hypoxic LDH release in untreated

control cells was taken as 100% and the percent reduction in LDH released from picroliv treated cells was calculated. As indicated in Fig.6, treatment with 1-50 µg/ml of picroliv showed a statistically significant ($P<0.05$, by student' 't' test) decrease in percentage LDH release as compared to untreated control. Triton X-100, a cytotoxic agent was included as a positive control and shows maximum LDH leakage.

TABLE 2: Effect of picroliv on HUVEC, Hep 3B and Glioma cell growth *in vitro.*

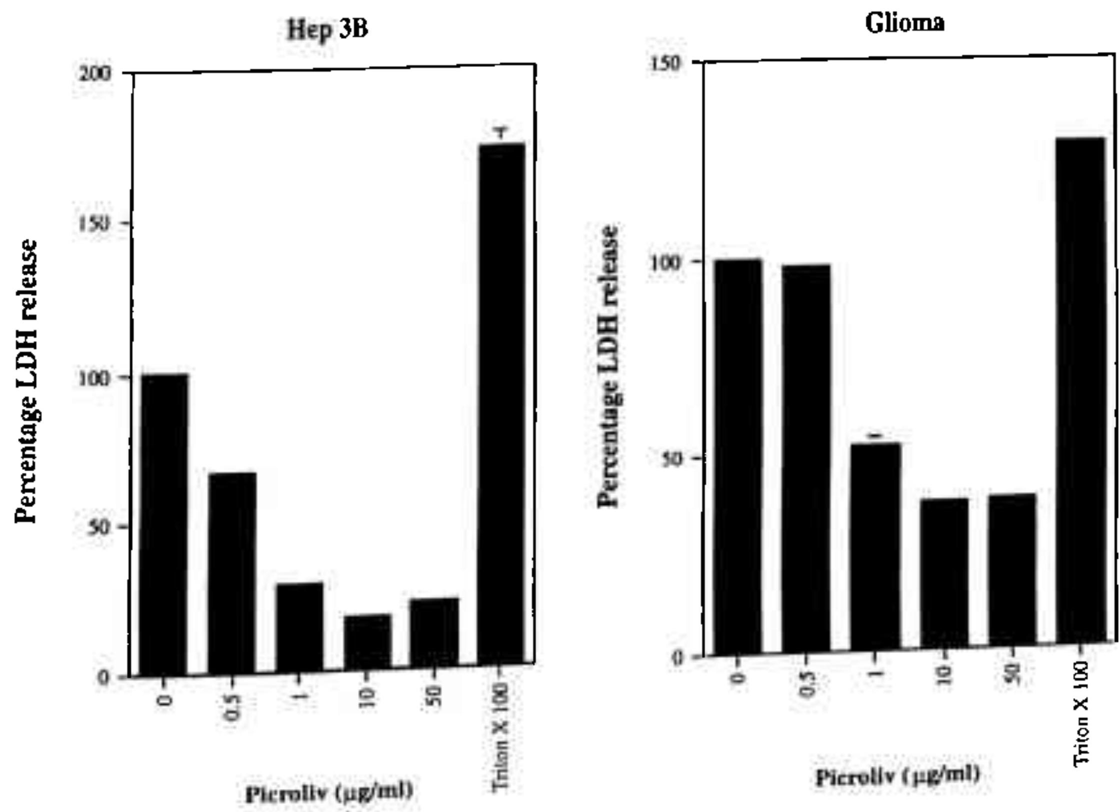
Cell type	Cell Growth[1]				
	Concentration of picroliv (μg/ml media)[2]				
	0	0.5	1	10	50
HUVEC	100	99±0.3	94±1.2	92±2.4	91±2.7
Hep 3B	100	97±0.9	97±2.4	104±0.3	127±1.0
Glioma	100	103±1.5	102±2.7	110±0.5	110±0.7

[1] Cell growth expressed in percentage compared to the untreated control (100%).

Values are mean ± SD from three experiments.

[2] Cell growth was determined at 24 h of treatment.

Fig.6: Effect of picroliv on LDH release: Hep3B and Glioma cells were plated at concentrations of $3X10^4$ cells/well in 96 well plates. Cells were treated with picroliv (0-100 μg/ml) and subjected to hypoxia for 24 h. LDH rcontents in the suparnatant were measured spectrophotometrically using cytotoxicity detection kit (Boehringer Mannheim, IN). Amount of LDH released by untreated cells was taken as 100% and the percentage LDH released by the treated cells was calculated in comparison to the control. Statistical significance was calculated by student's *t*-test. *Values expressed in percentage compared to control and are mean ± SE from six observations.


Hep 3B
Percentage LDH release
200
150
100
50
0
0
0.5
1
10
50
Triton X 100
Picroliv (µg/ml)
Glioma
Percentage LDH release
150
100
50
0
0
0.5
1
10
50
Triton X 100
Picroliv (µg/ml)

Picroliv affects the expression of hypoxia inducible genes

We assessed the ability of picroliv to regulate the expression of hypoxia inducible genes, VEGF, HIF-1α and HIF-1β in HUVEC, Hep3B and Glioma cells by RT-PCR and Northern blot analysis. Figs. 7, 8, 9 represent amplification products of the genes in all the three cell lines used, following the nine treatments mentioned in the study design. As expected, hypoxia induced the expression of these genes in all the three cell lines (lane 2) compared to untreated cells (lane 1). Reoxygenation of HUVEC and Hep3B cells with picroliv resulted in a striking reduction in the VEGF, HIF-1α and HIF-1β transcripts, as suggested by the PCR products in Figs. 7 and 8 (lanes 6, 8 & 9) in comparison with control reoxygenated cells (Figs. 7 and 8, lane 3). The expression pattern for these genes was different in Glioma cells where the expression of these genes plummeted to undetectable levels by simultaneous picroliv treatment during hypoxia and hypoxia followed by reoxygenation (Fig. 9, lanes 7 and 8). Post-hypoxic treatment of Glioma cells with picroliv did not result in the downregulation of these genes during reoxygenation (Fig. 9, lane 9). The *per se* effect of picroliv on the expression of these genes (Fig. 7, 8 and 9, lane 4) was similar to the hypoxic control (lane 2), indicating that picroliv induced the expression of these genes under study in normoxic conditions. We sought to determine the expression of VEGF receptors KDR, Flt-1 and Flt-4 in the presence of picroliv under normoxic and hypoxic conditions. VEGF receptors are known to be mostly expressed in endothelial cells. We could detect the expression of these genes only in HUVEC. Picroliv treatment of HUVEC during various hypoxic conditions modulated the expression of both the receptors, Flt-1 and KDR similar

to VEGF expression (Fig. 10). However, the quantitative differences in the expression between the treatments may not be statistically significant. Picroliv by itself moderately enhanced the levels of KDR and Flt-1 under normoxic conditions. In Hep 3B and Glioma cells, all the three receptors were not expressed. L28 expression indicates equal amount of mRNA in all the lanes (Fig. 7, 8 and 9). Northern blot analysis using total RNA from glioma cells (Fig.11) confirmed the results obtained by RT-PCR. Equal loading of RNA in Northern hybridization was ensured by reprobing the stripped blot with a house keeping gene, L28 (Fig.11).

Fig.7: Expression of VEGF, HIF-1α, HIF-1β and L28 in HUVEC: RT-PCR analysis was performed in picroliv treated HUVEC under conditions of normoxia, hypoxia and reoxygenation as explained in methods section. Lanes: M. marker, 1. normoxia, 2. Hypoxia for 48 h, 3. reoxygenation for 24 h after 48 h hypoxia, 4. picroliv treatment during normoxia for 24 h, 5. picroliv treatment during normoxia followed by hypoxia, 6. picroliv treatment during normoxia followed by hypoxia/reoxygenation, 7. treatment with picroliv during hypoxia, 8. treatment with picroliv during hypoxia followed by reoxygenation and 9. picroliv treatment during reoxygenation. Equal amounts of RNA in the RT-PCR reactions, was checked by analyzing L28 expression.

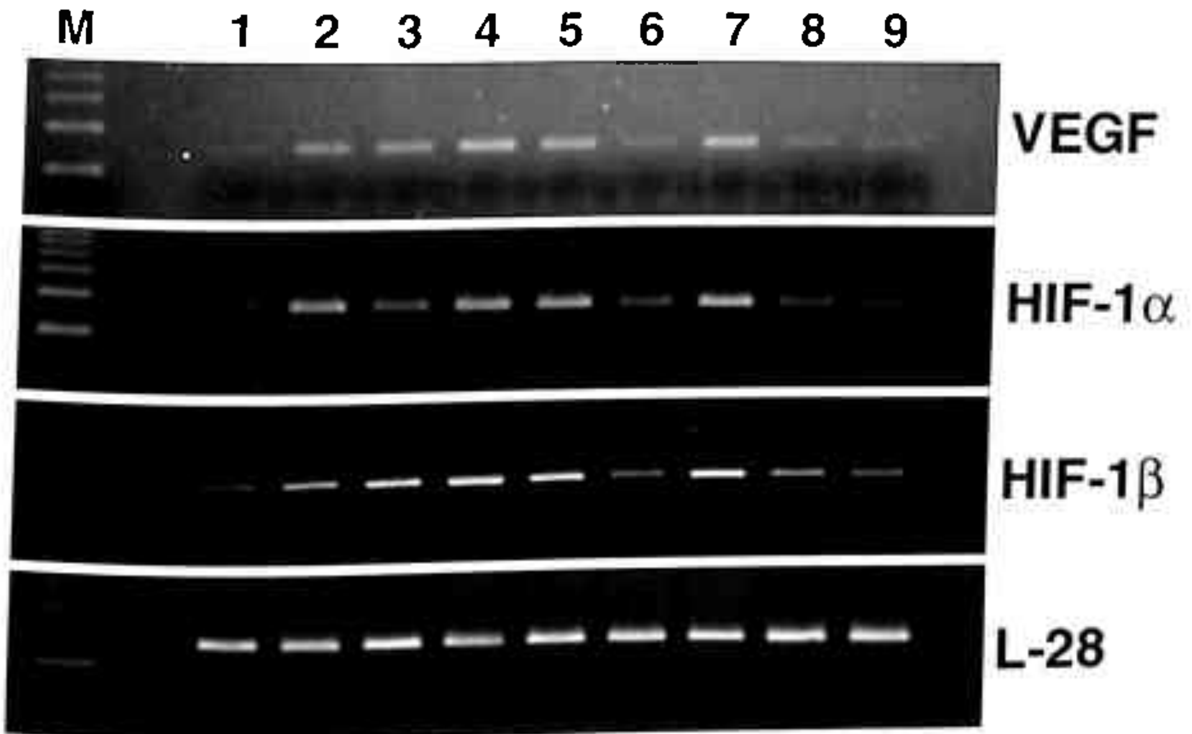

M
1
2
3
4
5
6
7
8
9
VEGF
HIF-1α
HIF-1β
L-28

Fig.8: Expression of VEGF, HIF-1α, HIF-1β and L28 in Hep 3B cells: RT-PCR analysis was performed in picroliv treated Hep 3B cells under conditions of normoxia, hypoxia and reoxygenation as explained in methods section. Lanes: M. marker, 1. normoxia, 2. Hypoxia for 48 h, 3. reoxygenation for 24 h after 48 h hypoxia, 4. picroliv treatment during normoxia for 24 h, 5. picroliv treatment during normoxia followed by hypoxia, 6. picroliv treatment during normoxia followed by hypoxia/reoxygenation, 7. treatment with picroliv during hypoxia, 8. treatment with picroliv during hypoxia followed by reoxygenation and 9. picroliv treatment during reoxygenation. Equal amounts of RNA in the RT-PCR reactions, was checked by analyzing L28 expression.

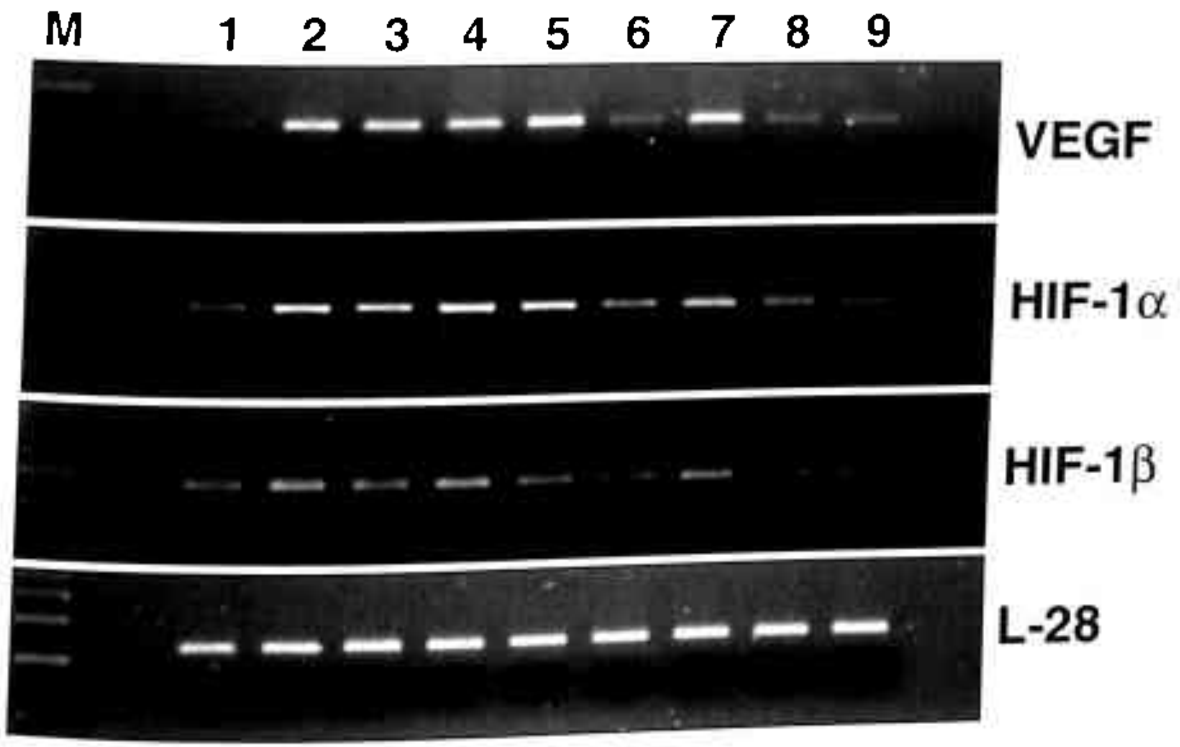

M
1
2
3
4
5
6
7
8
9
VEGF
HIF-1α
HIF-1β
L-28

Fig.9: <u>Expression of VEGF, HIF-1α, HIF-1β and L28 in Glioma cells:</u> RT-PCR analysis was performed in picroliv treated Glioma cells under conditions of normoxia, hypoxia and reoxygenation as explained in methods section. Lanes: M. marker, 1. normoxia, 2. Hypoxia for 48 h, 3. reoxygenation for 24 h after 48 h hypoxia, 4. picroliv treatment during normoxia for 24 h, 5. picroliv treatment during normoxia followed by hypoxia, 6. picroliv treatment during hypoxia/reoxygenation, 7. treatment with picroliv during hypoxia, 8. treatment with picroliv during hypoxia followed by reoxygenation and 9. picroliv treatment during reoxygenation. Equal amounts of RNA in the RT-PCR reactions, was checked by analyzing L28 expression.

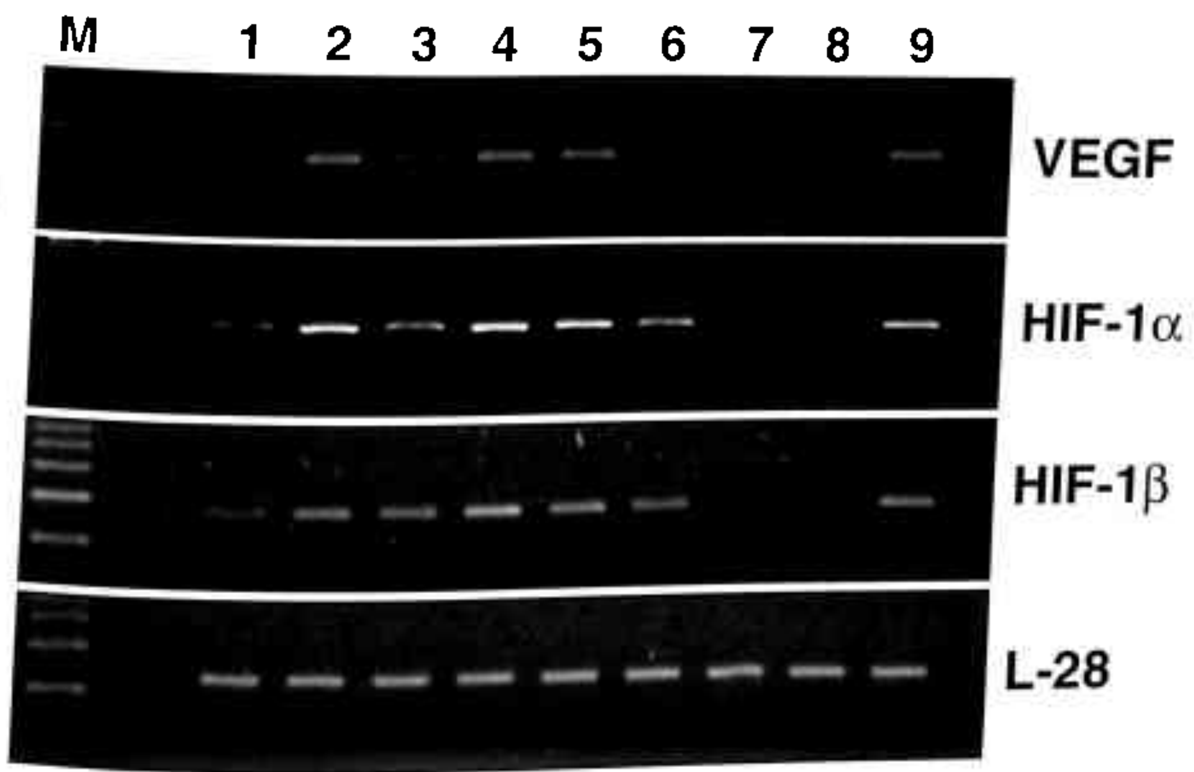

M
1
2
3
4
5
6
7
8
9
VEGF
HIF-1α
HIF-1β
L-28

Fig.10: Expression of VEGF receptors in HUVEC: VEGF receptor expression was examined by RT-PCR using primers for KDR, FLT-1 and FLT-4 primers upon treatment with picroliv under conditions of normoxia, hypoxia and reoxygenation as explained in methods section. Lanes: M. marker, 1. normoxia, 2. Hypoxia for 48 h, 3. reoxygenation for 24 h after 48 h hypoxia, 4. picroliv treatment during normoxia for 24 h, 5. picroliv treatment during normoxia followed by hypoxia, 6. picroliv treatment during normoxia followed by hypoxia/reoxygenation, 7. treatment with picroliv during hypoxia, 8. treatment with picroliv during hypoxia followed by reoxygenation and 9. picroliv treatment during reoxygenation.

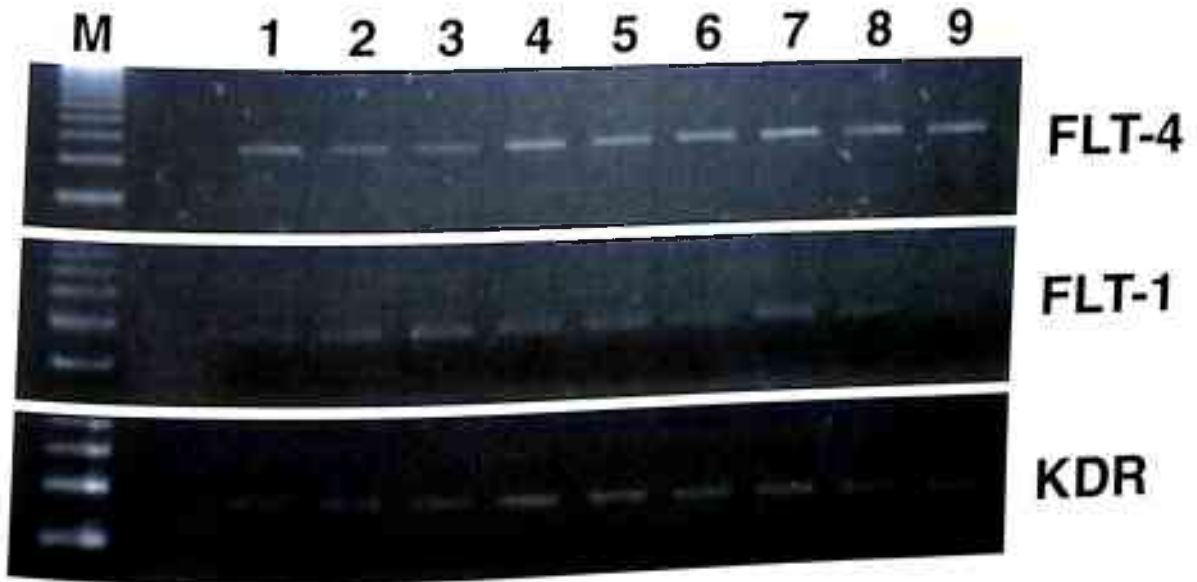
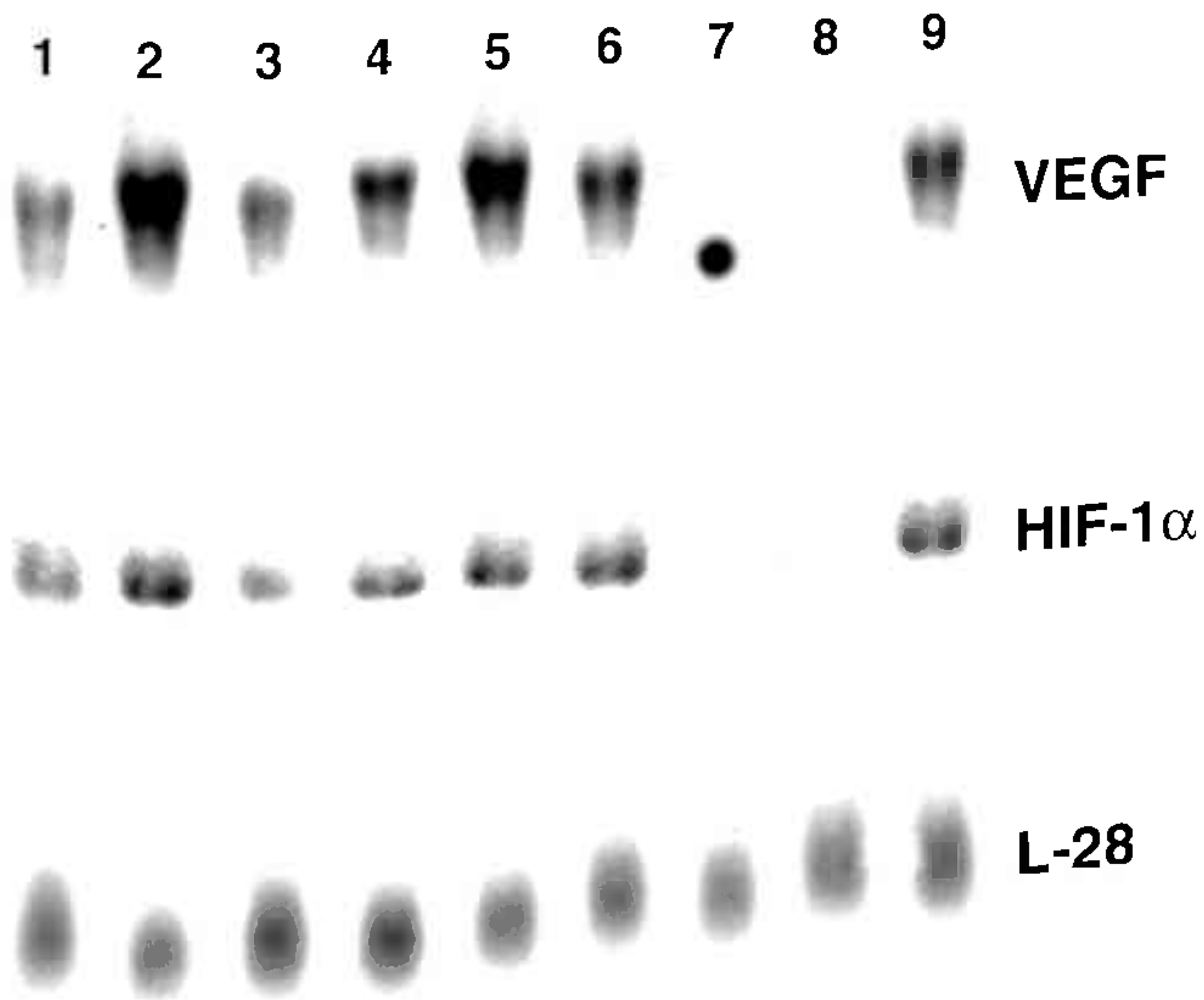

M
1
2
3
4
5
6
7
8
9
FLT-4
FLT-1
KDR

Fig.11: Northern blot analysis: mRNA analysis of VEGF and HIF-1α in glioma cells during normoxia, hypoxia and hypoxia/reoxygenation conditions were performrd by northern blotting.. Lanes: 1. normoxia, 2. Hypoxia for 48 h, 3. reoxygenation for 24 h after 48 h hypoxia, 4. picroliv treatment during normoxia for 24 h, 5. picroliv treatment during normoxia followed by hypoxia, 6. picroliv treatment during normoxia followed by hypoxia/reoxygenation, 7. treatment with picroliv during hypoxia, 8. treatment with picroliv during hypoxia followed by reoxygenation and 9. picroliv treatment during reoxygenation. The blot was stripped and reprobed with L-28 to account for equal loading of RNA.


1
2
3
4
5
6
7
8
9
VEGF
HIF-1α
L-28

Modulation of VEGF expression at the Post-transcriptional level by picroliv

Direct immunofluorescence studies were performed on HUVEC and glioma cells that were acetone fixed following all the nine treatments, to localize VEGF protein expression. These studies revealed that the alterations at the transcriptional stage by picroliv were reflected in the protein levels also. Hypoxia enhanced VEGF protein expression in both HUVEC (Fig.12C) and Glioma (Fig.13C) cells. VEGF expression was very minimal, comparable to the normoxic expression (Fig.12B), in the reoxygenated HUVEC cells treated with picroliv (Fig. 12.G, I & J). This expression was found to be lower than the untreated reoxygenated control (Fig.12C). Glioma cells responded differently to the picroliv treatments. Here, we observed significant reduction in VEGF protein expression in the simultaneous treatment of glioma cells with picroliv during hypoxia and when reoxygenation followed the same treatment (Fig.13H & I). Very low fluorescence, lesser than the normoxic control was witnessed in these two cases. However, there was an increased expression of VEGF tantamount to the hypoxic induction in both HUVEC and glioma cells (Fig.12C & 13C) that were subjected to picroliv treatment alone for 24 h (Fig.12E & 13E) under normoxic conditions. Cells were incubated with FITC conjugated goat ant rabbit IgG alone as a test for non-specific binding (Fig.12A & 13A). The pattern of VEGF protein expression was similar to mRNA expression in both HUVEC and Glioma cells.

VEGF up-regulation by picroliv is dose-dependent in Glioma cells

Enhancement of VEGF expression by picroliv in normoxic control (Fig.14, lane 4) prompted us to perform a dose response for VEGF in the presence of picroliv. Evidence is provided that picroliv at the doses of 1 & 10 µg/ml induced VEGF expression at both 12 & 24 h time points (Fig.14, lanes 3, 4, 3' & 4'). VEGF message increased by almost 3-4 folds as compared to the untreated control. Equal mRNA levels are demonstrated by L28 expression (Fig.14).

Fig.12: VEGF immunofluorescence staining of HUVEC: VEGF protein was localized in picroliv treated and untreated cells using immunofluorescence technique. A. negative control shows the specificity of the primary antibody, B. normoxia, C. 48 h hypoxia, D. 24 h reoxygenation after hypoxia, E. 24 h picroliv treatment during normoxia, F. hypoxia after picroliv treatment during normoxia, G. picroliv treatment during normoxia followed by hypoxia/reoxygenation, H. simultaneous treatment of picroliv and hypoxia, I. Picroliv treatment during hypoxia followed by reoxygenation, and J. picroliv treatment during reoxygenation.

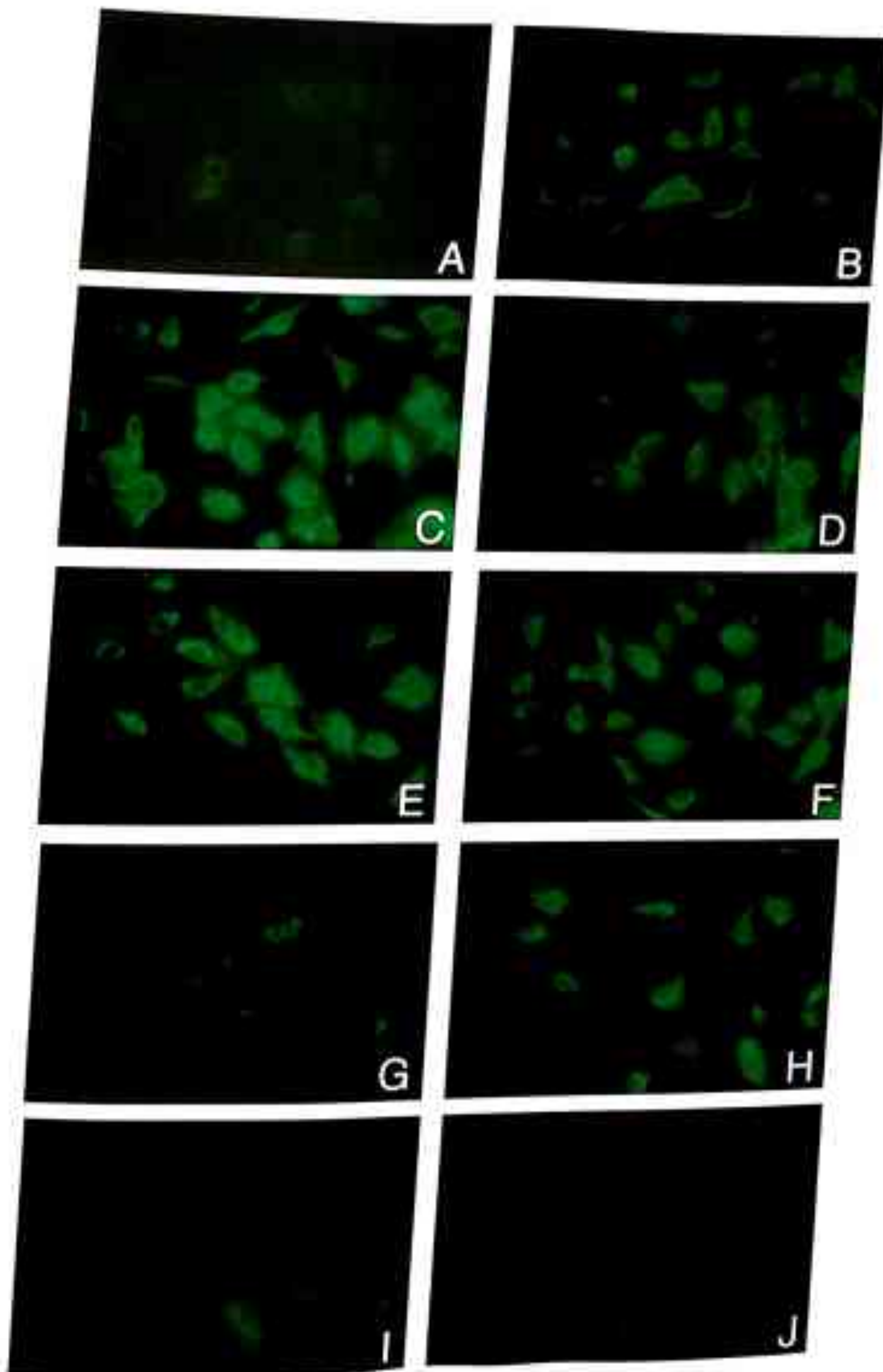

A
B
C
D
E
F
G
H
I
J

Fig.13: VEGF immunofluorescence staining of Glioma cells: VEGF protein was localized in picroliv treated and untreated cells using immunofluorescence technique. A. negative control shows the specificity of the primary antibody. B. normoxia, C. 48 h hypoxia, D. 24 h reoxygenation after hypoxia, E. 24 h picroliv treatment during normoxia, F. hypoxia after picroliv treatment during normoxia, G. picroliv treatment during normoxia followed by hypoxia/reoxygenation, H. simultaneous treatment of picroliv and hypoxia, I. Picroliv treatment during hypoxia followed by reoxygenation, and J. picroliv treatment during reoxygenation.

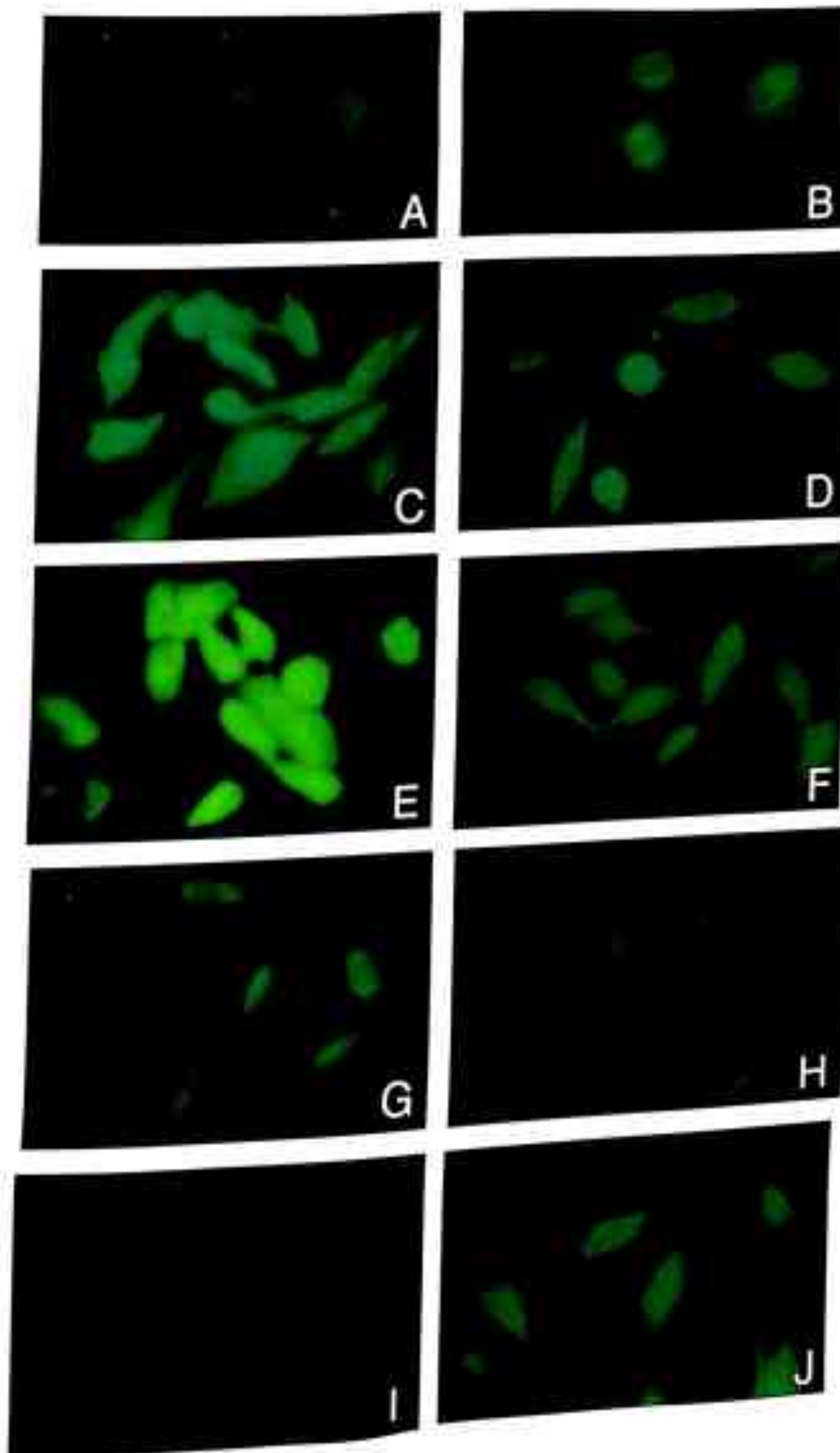
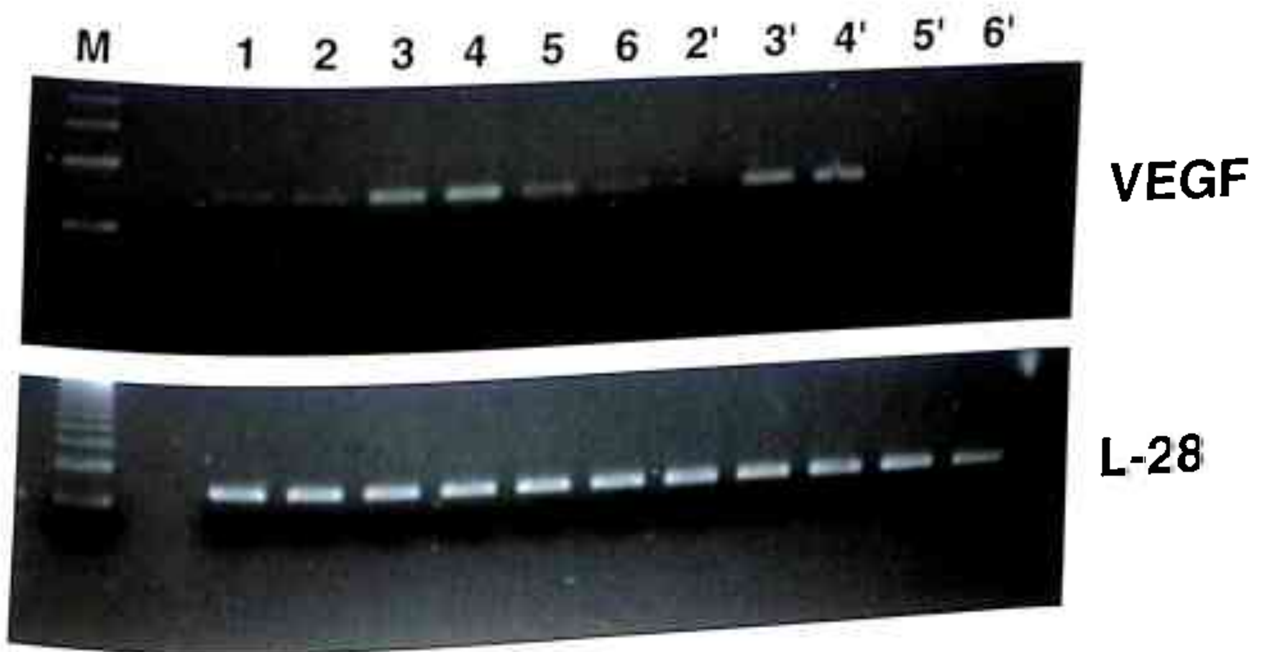

A
B
C
D
E
F
G
H
I
J

Fig.14: Dose response for VEGF in Glioma cells: Glioma cells were treated with different concentrations of picroliv (0-100 μg/ml) for 12 and 24 h time periods during normoxia. Lanes: M. marker, 1 and 6 represent untreated controls after 12 and 24 h, 2-6 and 2'-6' denote 0.5, 1, 10, 50 and 100 μg doses per ml of picroliv at 12 and 24 h time points respectively.


M
1
2
3
4
5
6
2'
3'
4'
5'
6'
VEGF
L-28

Effect of picroliv on tyrosine phosphorylation and kinase activity

The tyrosine phosphorylation state of cellular proteins was examined by immunoprecipitation of whole cell lysates with monoclonal anti-phosphotyrosine antibodies. This was followed by SDS-PAGE with subsequent anti-phosphotyrosine immunoblotting. The 80 kd protein was maximally phosphorylated at tyrosine residue in Glioma cells (Fig.15A). The phosphorylation was reduced when cells were subjected to hypoxia and reoxygenation (lane 3) as compared to hypoxia alone (lane 2). Picroliv treatment induced the tyrosine phosphorylation of 170 kd and 60 kd proteins during normoxia (lane 4). There was no change in the phosphorylation of 80 kd protein when the cells were pretreated with picroliv followed by hypoxia (lane 6) compared to hypoxia alone (lane 2), whereas the phosphorylation of other proteins was significantly reduced when the cells were subjected to reoxygenation (lane 5). Picroliv treatment dephosphorylated the proteins when the cells were simultaneously treated with picroliv and hypoxia (lane 7), and reoxygenation resulted in phosphorylation of these proteins (lane 8). Tyrosine residues were phosphorylated when the cells were subjected to hypoxia, and treated with picroliv during reoxygenation (lane 9).

Hypoxia induced the kinase activity of tyrosine phosphorylated proteins (80 kd, 63 kd, 48 kd and 32 kd) (Fig.16, lane 2) compared to untreated cells (lane 1) and on reoxygenation, kinase activity was reduced (lane 3). Pretreatment of cells with picroliv showed a basal level of kinase activity (lane 4), however, a reduction in the kinase activity was seen during hypoxia and reoxygenation (lanes 5 and 6) compared to hypoxia alone. An inhibition of kinase activity of tyrosine

phosphorylated protein was seen in cells subjected simultaneously to picroliv treatment and hypoxia, and subsequent reoxygenation (lane 7 and 8). The kinase activity was also reduced when cells were subjected to picroliv treatment during reoxygenation (lane 9) compared to hypoxia alone.

Modulation of PKC by picroliv in Glioma cells

PKC antibody used in this study identifies the PKC $\alpha/\beta 1/\beta 2$ isoforms (79-80 kD), however, two isoforms of PKC were recognized by this antibody in Glioma cells (Fig.17). PKC is activated during hypoxia (lane 2) compared to normoxia (lane 1). The expression level of PKC was decreased when the cells were subjected to hypoxia followed by reoxygenation (lane 3) compared to hypoxia alone (lane 2). Picroliv treatment did not reduce the level of PKC, however, one of the isoforms (low molecular weight) of PKC was significantly reduced (lane 4). Pretreatment of picroliv followed by hypoxia reduced the expression of one of the isoforms of PKC (high molecular weight) (lane 5), and upon reoxygenation, this isoform was completely reduced with an increase in the other isoform (lane 6). Simultaneous treatment with picroliv and hypoxia, and followed by reoxygenation completely inhibited the PKC (lane 7 and 8). The higher molecular weight isoform of PKC was moderately expressed when the cells were subjected to picroliv treatment during reoxygenation (lane 9). The quantitative estimations of various genes and proteins analysed are represented in Table 3.

Fig.15: Protein tyrosine phosphorylation in Glioma cells: Cell lysate was immunoprecipitated with agarose conjugated monoclonal antiphosphotyrosine antibody (Clone 4G10), analyzed on 10% SDS-PAGE, Western blotted and the blot was incubated with monoclonal anti-phosphotyrosine antibody. ECL was used for the detection of tyrosine phosphorylated proteins. The molecular size of proteins are indicated in kilo daltons. Lanes 1-9 represent treatments as mentioned in study design in methods section.

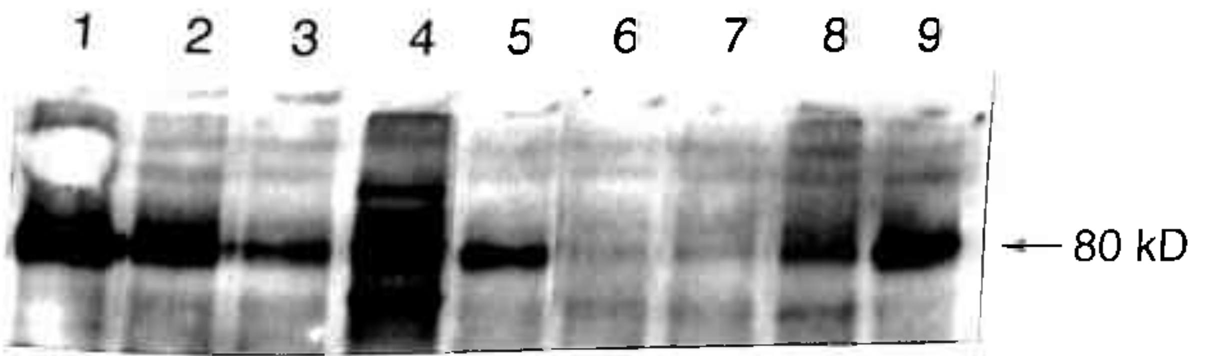

1
2
3
4
5
6
7
8
9
80 kD

Fig.16: Kinase activity of tyrosine phosphorylated proteins: Kinase activity in lysates from picroliv treated and untreated glioma cells was performed using monoclonal agarose conjugated anti-phosphotyrosine antibody (4G10 clone) and $\alpha-^{32}P$-ATP. The proteins were resolved on 10% SDS-polyacrylamide gel, and autoradiography was performed. The molecular size of proteins is indicated in kilo daltons. Lanes 1-9 represent treatments as mentioned in study design in methods section.

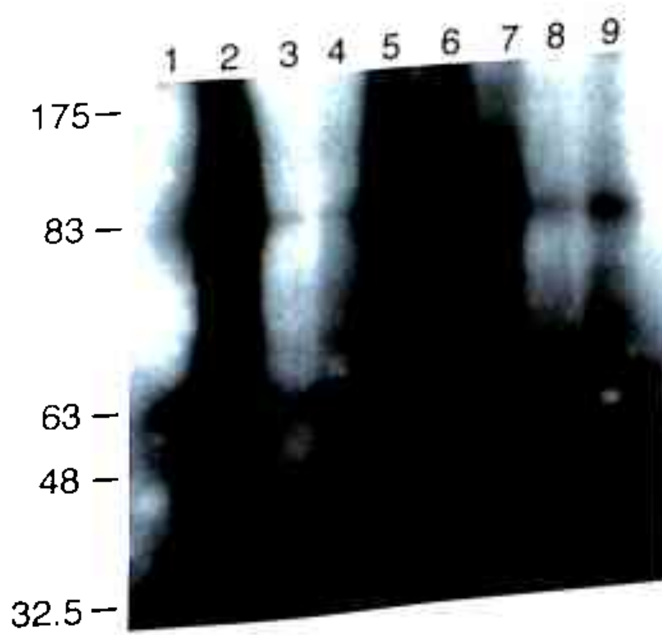

1 2 3 4 5 6 7 8 9
175
83
63
48
32.5

Fig.17: Effect of picroliv on PKC in Glioma cells: The lysates made in RIPA buffer were immunoprecipitated using PKC monoclonal antibody and the immune complex was analyzed on 10% SDS-PAGE, transferred and immunoblotting was performed using the same PKC antibody. PKC was detected using ECL. The treatment details (1-9) are same as described in study design of methods section.

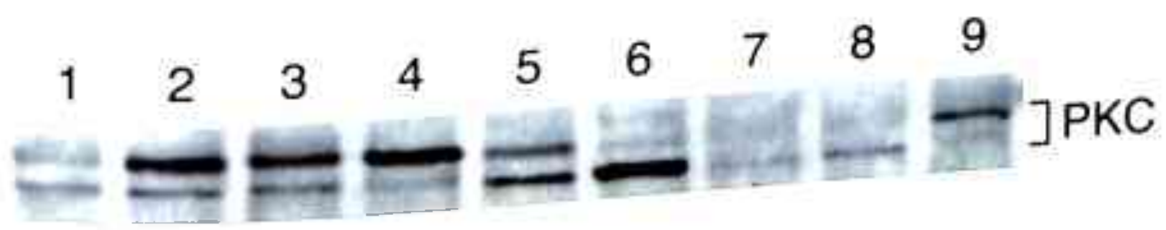

1
2
3
4
5
6
7
8
9
PKC

TABLE 3: <u>*Quantitation of the levels of expression of various genes during hypoxia/reoxygenation and picroliv treatments*</u>*.

Cell line	Gene	Controls				Experimental groups treated with picroliv				
		Normoxia (1)	Hypoxia (2)	Hypoxia + reoxygen-ation (3)	Picroliv 24 h (4)	Pre treat-ment + hypoxia (5)	Pre treatment + hypoxia + reoxygenation (6)	Simul. picroliv & hypoxia (7)	Simul. picroliv & hypoxia + reoxygenation (8)	Post hypoxia treatment (9)
Glioma	VEGF	1	5.9	4.1	6.0	5.8	2.6	0.5	1.0	4.8
	HIF-1α	1	3.0	1.9	3.4	3.0	1.9	0.5	1.5	2.4
	HIF-1β	1	3.0	2.5	5.2	4.4	2.5	0.8	0.9	3.1
	PKC α	1	3.8	3.5	4.2	2.7	0.9	0.4	0.5	2.8
	PKC β	1	1.2	0.7	0.2	2.6	4.3	0.2	0.4	0.2
HUVEC	VEGF	1	5.0	3.6	5.9	5.2	1.7	4.7	1.6	0.8
	HIF-1α	1	2.7	2.3	3.7	3.1	1.4	3.5	1.8	1.4
	HIF-1β	1	3.0	4.0	6.9	5.4	1.5	6.8	2.4	1.3
Hep 3B	VEGF	1	8.5	7.6	7.3	8.7	2.2	7.0	1.8	2.2
	HIF-1α	1	2.5	1.6	2.1	1.7	1.0	1.5	1.0	0.7
	HIF-1β	1	2.6	1.5	2.5	1.2	0.5	1.8	0.4	0.4

* Relative values calculated in comparison to control (normoxia) for each gene individually.
The numbers in parenthesis represent the treatment number as described in material and methods.

B. Effect of picroliv on Angiogenesis:

Enhanced tube formation in endothelial cells by picroliv

To evaluate picroliv in a physiological context, we performed angiogenesis assay using a matrigel model. The effect of picroliv on tube formation in the presence of 2% serum is shown in Fig.18. Endothelial cells, when cultured on a matrigel substrate, align with one another and form an intricate network of tube like capillary structures. Tube formation was promoted in endothelial cells when treated with 1 and 5 μg/ml of picroliv. A dose dependent increase in number and area of the tubes was observed. *Undifferentiated cells (shown by arrows) were more in control (A) than in picroliv treatments (B, C & D).* Photographs of the tubes were scanned and the lengths of capillary-like structures were measured using NIH image program (Version 1.59). Quantitation of angiogenesis is presented in Fig.19.

Picroliv elicits angiogenic response from rat aortic rings

Aortic rings embedded in matrigel gave rise to microvessels in the presence of picroliv alone or picroliv and ECGS (Fig.20). Picroliv by itself enhanced capillary formation (Fig.20 B,C&D) as compared to the untreated control (Fig.20A). The microvessels increased in number and length upon treatment with 0.5 and 1 μg/ml of picroliv. Enhanced sprouting and branch formation of the tube like structures were witnessed in treatments with 0.5 and 1 μg/ml of picroliv along with ECGS (Fig.20F&G) in comparison to ECGS control (Fig.20E).

Fig.18: Enhancement of tube formation in endothelial cells by picroliv: HUVEC cells were pre-treated with picroliv (0, 0.5, 1, 5 μg/ml) for 24 h and plated ($3X10^4$ cells/well) on matrigel pre-coated plates in media containing 2% serum. The cultures were left over night on matrigel to let the tubes sharpen. Tubes were then fixed in formalin, stained with Giemsa blue stain and photographed. A. Untreated control, B. 0.5 μg/ml Picroliv, C. 1 μg/ml Picroliv, D. 5 μg/ml Picroliv.

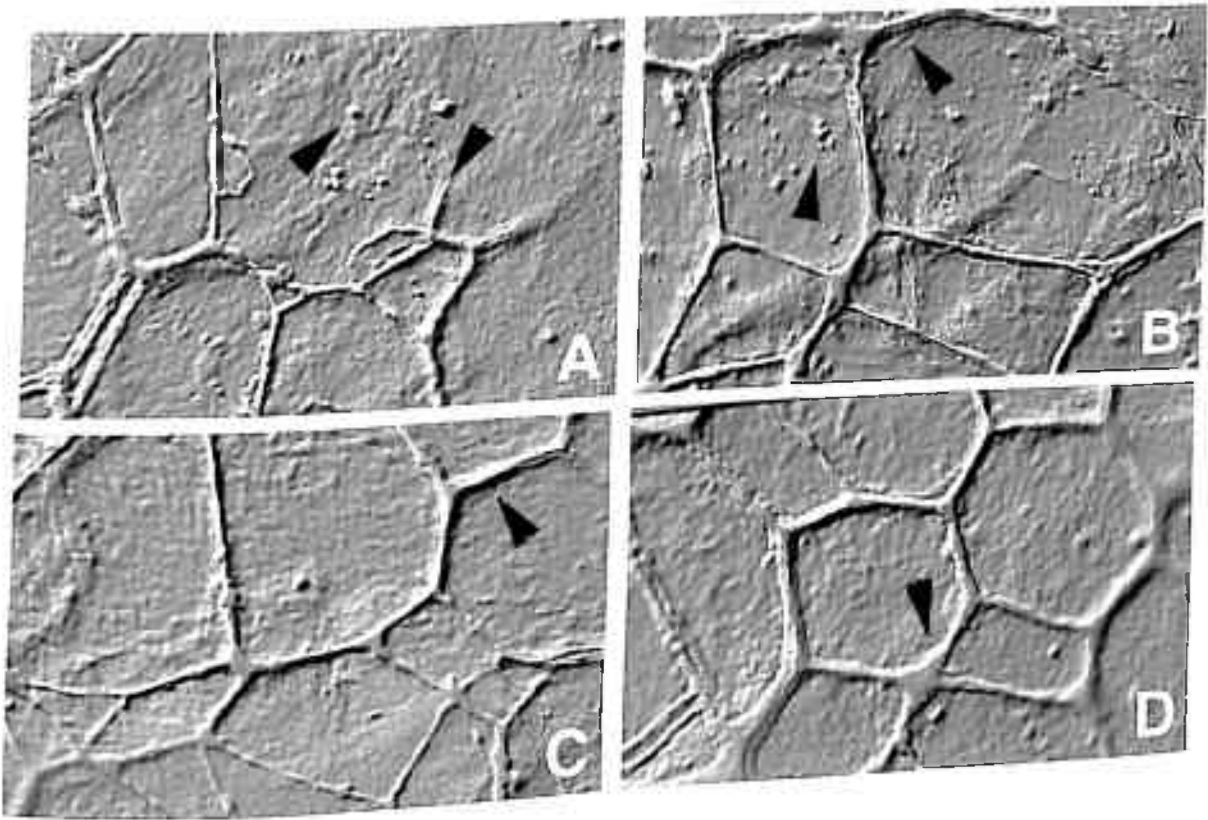

A
B
C
D

Fig.19: Quantitation of effect of varying doses of picroliv on tube formation: The tubes formed in the matrigel assay were quantitated by scanning the pictures in Adobe Photoshop. The scans were imported into NIH image program (version 1.59) and mean tube length for each scan was determined. The graph depicts an estimation of tube length in picroliv treated and untreated cells on matrigel.

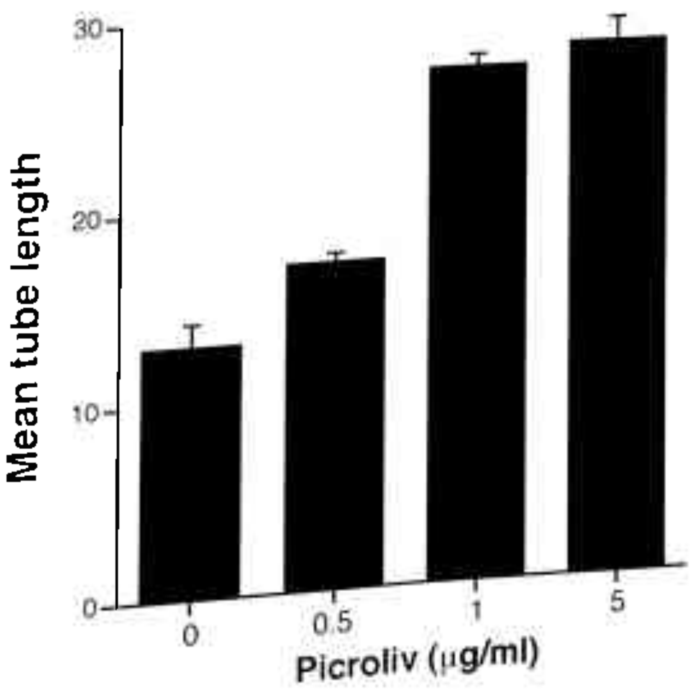
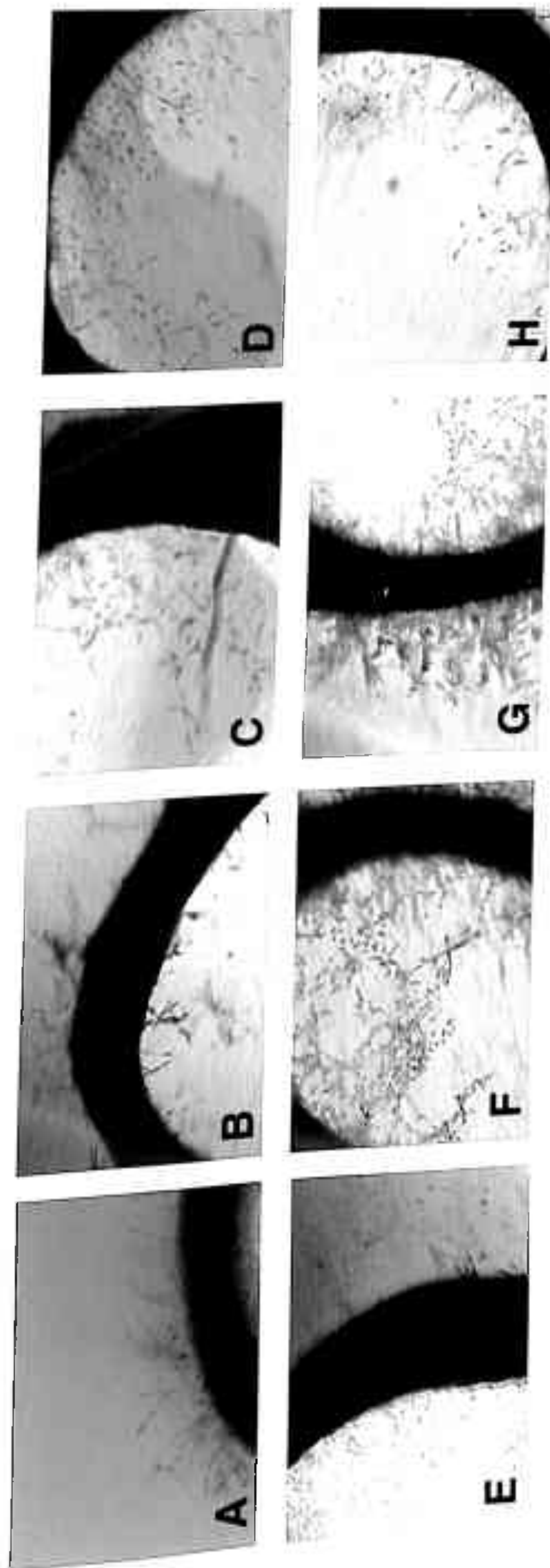

Mean tube length
30
20
10
0
0
0.5
1
5
Picroliv (μg/ml)

Fig.20: Effect of picroliv on rat aorta model of angiogenesis: The ability of picroliv to enhance angiogenesis in an *ex vivo* rat aorta model was determined by embedding the aortic rings on matrigel and culturing them in SFM basal growth medium in the presence of picroliv alone or picroliv and ECGS. The top panel (A-D) shows rings in the absence of ECGS and the bottom panel (E-H) depicts rings in the presence of ECGS. A. untreated control, B. Picroliv 0.5 μg/ml, C. Picroliv 1 μg/ml, D. Picroliv 5 μg/ml, E. EGCS control, F. ECGS + Picroliv 0.5 μg/ml, G. ECGS + Picroliv 1 μg/ml, H. ECGS + Picroliv 5 μg/ml.


A
B
C
D
E
F
G
H

Alteration of angiogenesis related gene expression by picroliv

To explore possible mechanisms for angiogenic stimulation of HUVEC by picroliv, various genes involved in the complex process of angiogeneis were analyzed. Gene expression was observed using PCR amplification of reverse transcribed HUVEC mRNAs using respective primers. As shown in Fig.21, there was minimal basal level expression of VEGF, Flt-1, KDR and MMP-9 in unstimulated HUVEC cells. Upon picroliv treatment with doses ranging from 0.5 - 5 μg/ml, there was a marked increase in the expression of VEGF, KDR, Flt-1 and MMP-9. We also observed a moderate increase in MMP-1 mRNA when treated with 1 μg/ml of picroliv (Table 3). Picroliv treatment had no significant change on Flt-4 levels. Densitometric analysis was performed using NIH image analysis software (Version 1.59) to measure the mRNA signals of these genes with L-28, house keeping gene, as a reference for relative RNA loading. The data is shown in Table 4.

Effect of picroliv on proteolytic activity

To determine the effect of picroliv on proteinases, we used a zymographic assay to visualize the proteinases expressed by HUVEC grown on plastic in the presence of picroliv (0, 0.5, 1 and 5 μg/ml). HUVEC secreted distinct proteinases of mol. wt. 72-kD and 53-kD (Fig.22). Gelatinolytic activity was also observed at mol. wt. 80 kD and 40 kD, whose characterization has not been documented in literature. The results showed that 72 kD and 53 kD proteinase activities were

enhanced upon treatment with 0.5 and 1 µg/ml of picroliv (lanes 2 and 3) as compared to untreated control (lane 1). the proteolytic activity at 80 kD showed a moderate increase with 0.5, 1 and 5 µg/ml of picroliv.

Effect of picroliv on neovascularization in wounds

Multiple cross-sections of hematoxylin and eosin sained sections of picroliv-treated and control wound tissues of rats were examined for formation of granulation tissue, fibrogenesis and neovascularization. A high power magnification of the dermal region of the wound showing granulation tissue is represented in Fig.23. Picroliv-treated wound shows extensive neovascularization and exhibited mature granulation tissue (B) as compared to the untreated control, which shows loose reticular tissue (A).

Fig.21: Modulation of genes involved in angiogenesis by picroliv: mRNA expression of genes involved in angiogenesis was analysed by RT-PCR. Picture shows representative amplification products for different genes, electrophoresed on a 2% agarose gel. Lanes are 1. Control, 2. 0.5 μg/ml of picroliv, 3. 1 μg/ml of picroliv, 4. 5 μg/ml of picroliv.

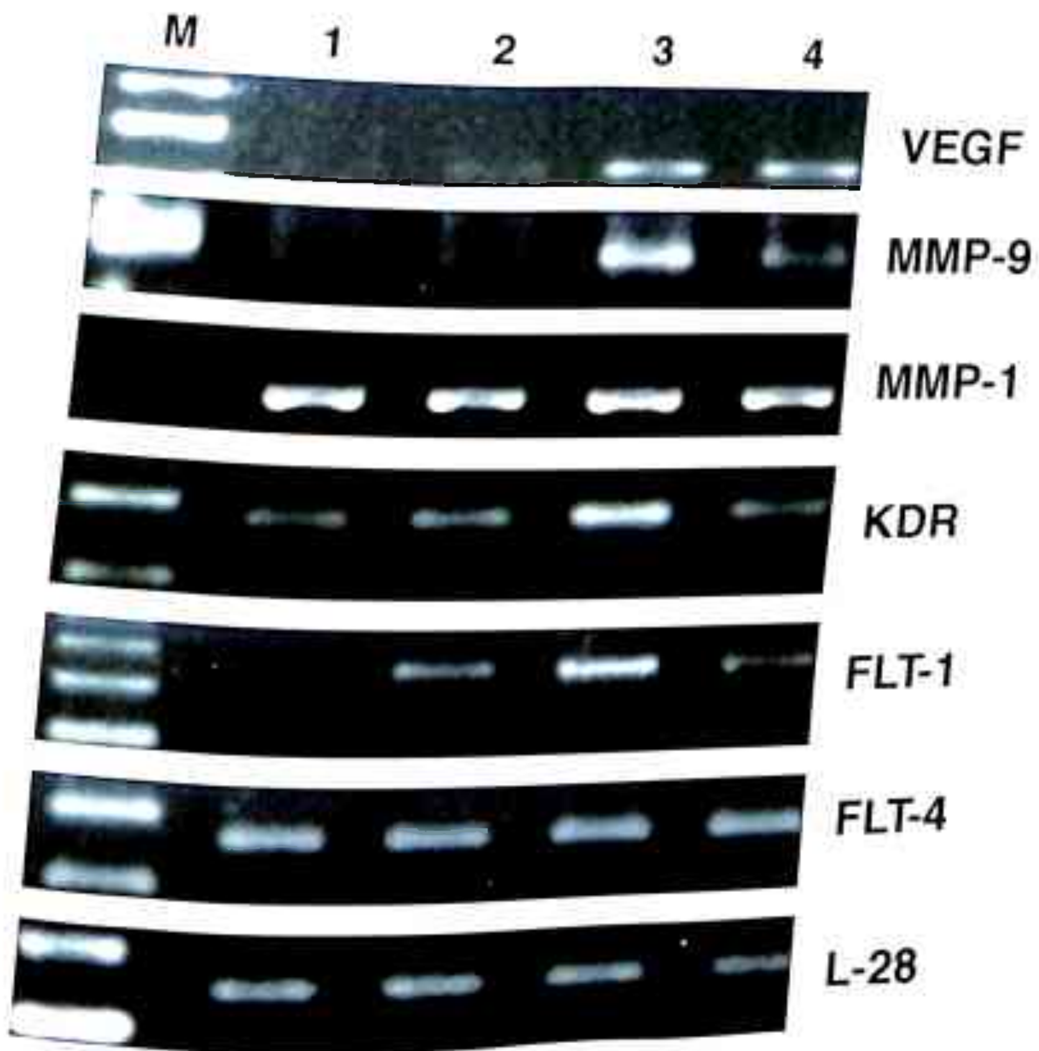

M
1
2
3
4
VEGF
MMP-9
MMP-1
KDR
FLT-1
FLT-4
L-28

Table 4: Quantitative estimation of angiogenesis-related gene expression

Gene	Control	Picroliv 0.5 μg/ml	Picroliv 1 μg/ml	Picroliv 5 μg/ml
VEGF	1	1.78	8.1	8.4
MMP-9	1	1.6	7.9	3.3
MMP-1	1	1.1	1.2	1.1
KDR	1	1.3	3.1	1.4
FLT-1	1	6	10	4.7
FLT-4	1	0.8	1	1.1

Relative values calculated in comparison to untreated control for each gene individually are shown in the table above. Quantitative estimations were done by densitometric analysis of PCR products using NIH image gel plotting macros. The numbers represent the ratio of area under the curve of treated and untreated lanes.

Fig.22: Zymogram analysis of the gelatinolytic activities: Conditioned media of HUVEC treated with picroliv was harvested after 24 h treatment and subjected to non-reducing SDS-PAGE through a 10% acrylamide resolving gel containing gelatin. The gels were renatured and incubated overnight at 37°C and stained with coomassie blue. Bands of gelatinolytic activity are indicated by pale bands in the gel at the indicated molecular weights (arrow heads). Lanes: 1. Control, 2. 0.5 μg/ml of picroliv, 3. 1 μg/ml of picroliv, 4. 5 μg/ml of picroliv.

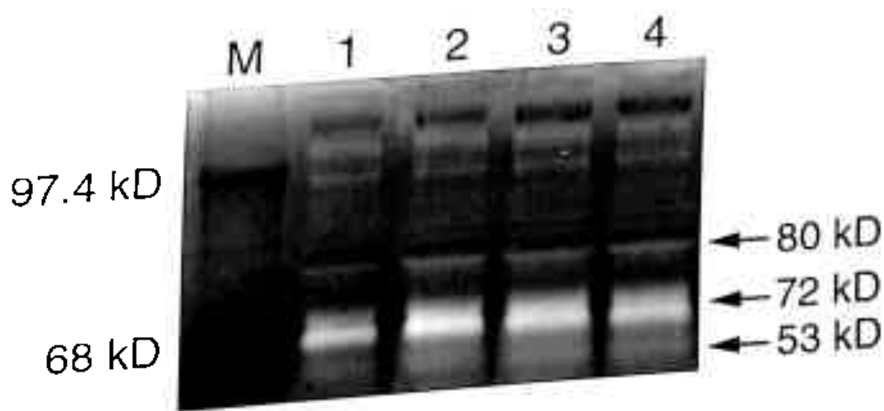

M
1
2
3
4
97.4 kD
68 kD
80 kD
72 kD
53 kD

Fig.23: High power magnification of the dermal region of the wound: A. Untreated wound shows loose reticular tissue with minimal neovacularization, B. Picroliv treated wound exhibits matured granulation tissue with ample fibrogenesis, and neovascularization.

B

A

C. *Effect of picroliv on renal IRI:*

Modulation of MDA levels by picroliv

Tissue malonaldialdehyde (MDA) levels directly correlate with lipid peroxidation due to free radical damage following ischemia reperfusion injury. MDA levels measured treated and untreated tissues were expressed as μ moles/mg protein of tissue homogenates. Picroliv treatment, *per se*, had no effect on the basal MDA levels, as the MDA levels in sham, picroliv treated (3.000 ± 0.431 μM/mg protein) and untreated (2.889 ± 0.615 μM/mg protein) rats were similar. There was an increase in the MDA levels following 60 min ischemia followed by 5, 60, 120 or 240 minutes of reperfusion as compared to sham controls (Fig.24). MDA levels increased progressively as the duration of reperfusion increased and were maximum at 240 min reperfusion. Picroliv pretreatment resulted in a significant attenuation in the free radical mediated lipid peroxidation, in the reperfused tissues, as observed by decrease in the MDA levels (Fig.24).

Effect of picroliv on gluatathione redeox cycle

Biochemical studies revealed that during IRI, the levels of reduced glutathione (GSH) reduced, specially following reperfusion. Picroliv treatment by itself exhibited a tendency to moderately increase GSH levels in comparison to the untreated controls. Furthermore, attenuated GSH levels, following IRI, were found to be improved in kidney samples obtained from picroliv pretreated rats (Fig.25).

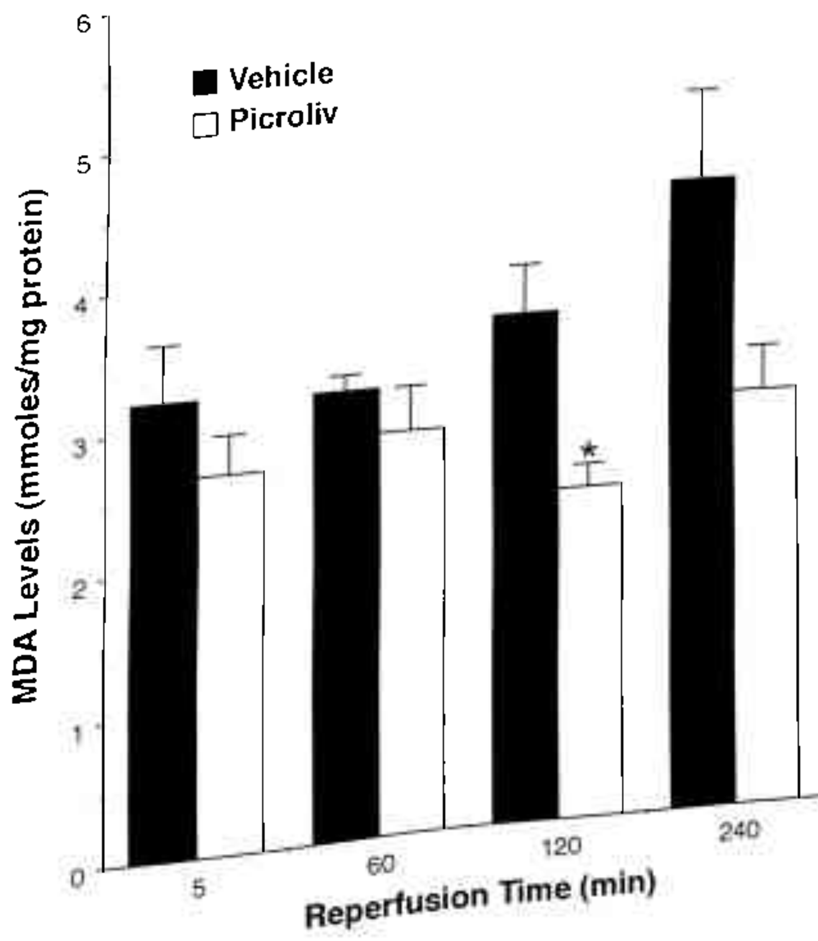
In order to have a complete picture of the glutathione redox cycle, enzyme

kinetic studies were performed to assess the activities of glutathione reductase (GR, the enzyme responsible for the replenishing the decreased GSH pool) and glutathione peroxidase (Gpx, enzyme responsible for scavenging hydrogen peroxides and lipid peroxides). We observed that following IRI, there was no convincing change in the GR activity with or without picroliv (Table 5). Gpx activity decreased by 10%, 16%, 24%, and 18% following 5, 60, 120 and 240 minute of reperfusion as compared to sham controls. Picroliv treatment enhanced Gpx activity by 47.6%, 13.3%, 23.0% and 12.63% at similar time points (Table 4).

Alteration in Nitric Oxide Release

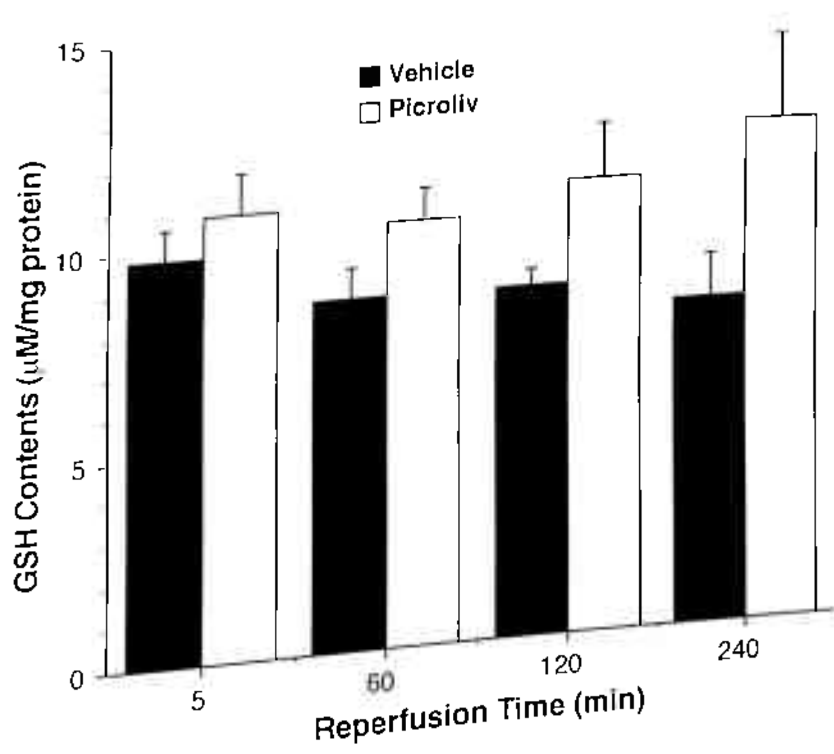
Total nitrite contents were estimated to assess the nitric oxide release from kidneys obtained from vehicle and picroliv treated rats with or without IRI. There was a correlation between duration of reperfusion and renal nitrite levels. Total nitrite contents in vehicle treated rats increased with the reperfusion time and attained a maximum at 120 min (1.3906 n moles/mg protein). Picroliv down-modulates the total nitric oxide release in our studies and shows a trend to attenuate the release by 36 % at the 120 min reperfusion time point (Fig.26).

Fig.24: Alterations in lipid peroxidation levels by picroliv following IRI.: Malonaldialdehyde (MDA) levels were measured in 10 % homogenates of kidney samples from rats subjected to 60 min ischemia and varying times of reperfusion. Solid bars represent MDA levels of vehicle treated rat kidney samples and the hollow bars are that of picroliv pre-treated rats. * $p<0.05$ as compared to untreated rat kidney sample.



Vehicle
Picroliv
MDA Levels (mmoles/mg protein)
Reperfusion Time (min)
0
1
2
3
4
5
6
5
60
120
240
*

Fig.25: Modulation of GSH levels by picroliv following IRI: GSH levels were measured in kidney sample homogenates from vehicle (solid bars) and picroliv pre-treated (hollow bars) rats. * $p<0.05$ as compared to corresponding vehicle treated group.



Vehicle
Picroliv
GSH Contents (μM/mg protein)
15
10
5
0
5
60
120
240
Reperfusion Time (min)

Table 5: Alterations in activity of Glutathione reductase (GR) and Glutathione peroxidase (Gpx).

	GR (mUnits/min/mg protein)		GPx (Units/min/mg protein)	
	IRI (Lt)	Control (Rt)	IRI (Lt)	Control (Rt)
Vi60, R5	1146.94 ± 260.84	1010.43 ± 133.81	3.867 ± 0.362	4.281 ± 0.470
Pi60, R5	952.62 ± 92.88	1029.22 ± 104.49	4.683 ± 0.067*	3.293 ± 0.240
Vi60, R60	882.60 ± 182.31	1073.81 ± 85.26	3.269 ± 0.262	4.322 ± 0.482
Pi60, R60	1180.40 ± 35.56	1235.77 ± 154.87	4.874 ± 0.485	4.301 ± 0.107
Vi60, R120	1091.64 ± 72.95	1118.30 ± 44.80	3.571 ± 0.394	4.726 ± 0.714
Pi60, R120	1009.77 ± 52.71	1027.51 ± 106.21	4.563 ± 0.587*	3.732 ± 0.320
Vi60, R240	1097.47 ± 45.84	1183.89 ± 74.90	4.083 ± 0.715	5.000 ± 0.670
Pi60, R240	1177.21 ± 80.68	1158.33 ± 75.50	4.281 ± 0.282	3.801 ± 0.463

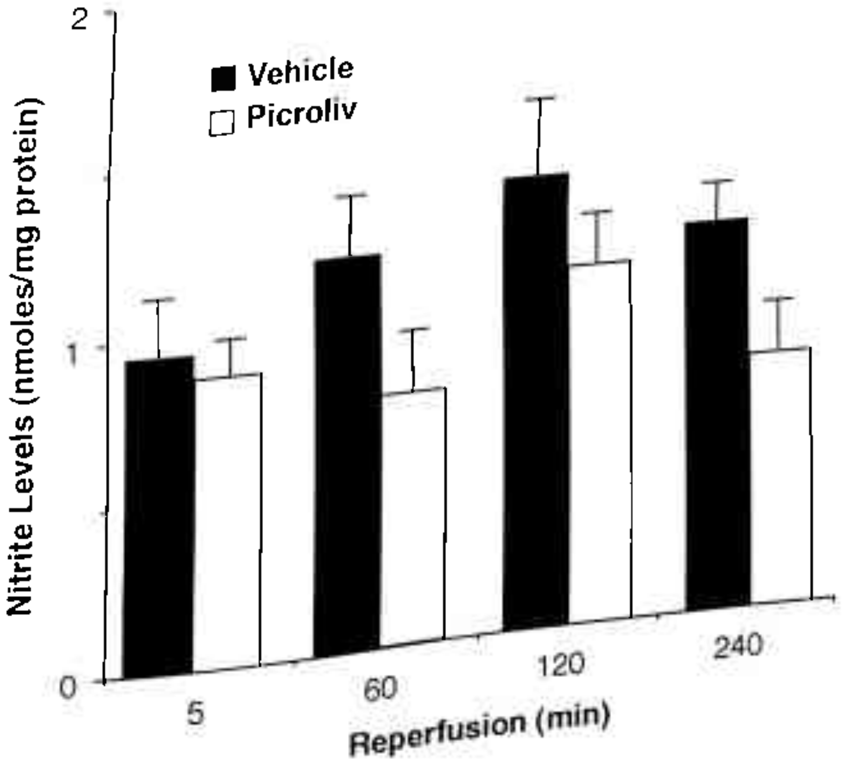
Ischemia was for 60 min and reperfusion time varied (5, 60 and 240 min). Left kidneys(Lt) were the experimental groups and the right kidneys (Rt) were controls with no IRI.

Following IRI there was no significant change in the GR activity with or without Picroliv. GPx activity was moderately decreased by 10%, 16%, 24%, and 18% following 5, 60, 120 and 240 minute of reperfusion. Picroliv treatment resulted in an increase of GPx activity by 47.6%, 13.3%, 23.0% and 12.63% at similar time points.

* $p<0.05$ as compared to their respective untreated (vehicle;Lt) IRI kidneys.

Fig.26: Effect of picroliv on nitrite contents following ischemia reperfusion injury:

Values represent the nitrite contents estimated using the modified Griess reagent and are represented as mean $\pm$ SE. Solid and hollow bars denote untreated and picroliv-treated samples respectively.


Vehicle
Picroliv
Nitrite Levels (nmoles/mg protein)
2
1
0
5
60
120
240
Reperfusion (min)

Effect of picroliv on Superoxide Dismutase

The pattern of renal Cu,Zn-SOD immunolocalization is shown in Fig.27. Picroliv treatment by itself resulted in noticeable augmentation in the expression of SOD protein in the tubular and glomerular regions of the kidney as compared to the untreated control thereby suggesting an enhanced presence of the enzyme or better antioxidant status in the tissues. Furthermore, picroliv appears to maintain an increased SOD expression in rats subjected to 60 minute ischemia followed by 120 minute reperfusion (Fig.27).

Effect of picroliv on ICAM-1 and CD-18 expression following renal IRI

Immunohistochemical studies of paraffin embedded sections show that following IRI, the expression of ICAM-1 is up-regulated mainly in the glomerular region which was best seen in sections obtained from rats that were subjected to sixty minute ischemia and 120 min reperfusion. The expression of this adhesion molecule was diminished in kidney sections illustrating glomerulus (Fig.28) from picroliv treated groups. Similarly, the expression of CD-18 was suppressed in picroliv treated groups (Fig.29). This indicates that picroliv has the potential to reduce the expression of adhesion molecules, thus protecting the tissues experiencing ischemic insult.

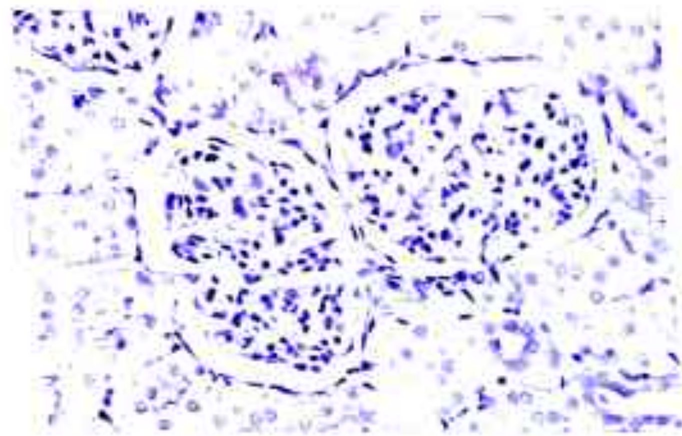
Modulation of apoptosis by picroliv

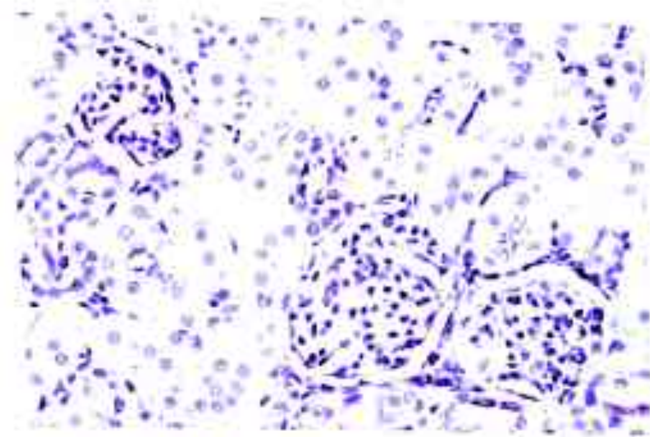
The *in situ* end labeling technique that labels the large number of DNA ends in oligonucleosomes generated within the apoptotic nuclei, was used on renal

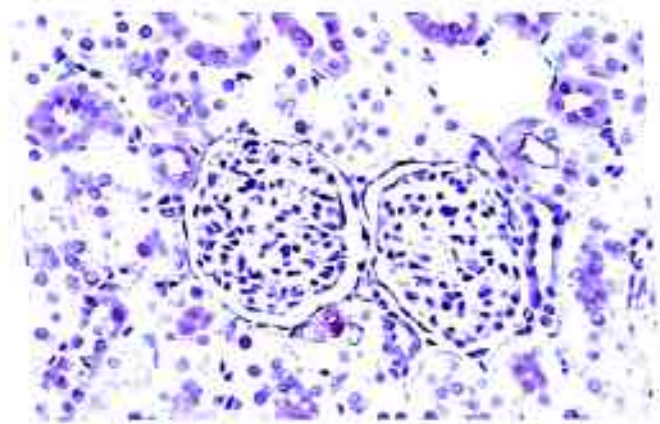
sections obtained from animals fed with or without picroliv during IRI. Apoptotic injury increased with reperfusion duration and was maximum in 60 min of ischemia and 240 min of reperfusion tissues. Picroliv fed animals showed a significantly lesser number of apoptotic positive nuclei within the glomeruli and tubular epithelial cells as compared to untreated control (Fig.30).

Fig.27: SOD expression in kidney sections: Immunohistochemistry was performed on kidney sections from treated and untreated groups using Quick Universal Kit (Vector laboratories) and polyclonal anti-Cu/Zn SOD (Calbiochem-Novabiochem Corporation). The brown DAB stain indicates SOD expression in the kidney tubules.

Not Ligated
Ligated
Vehicle
Picroliv







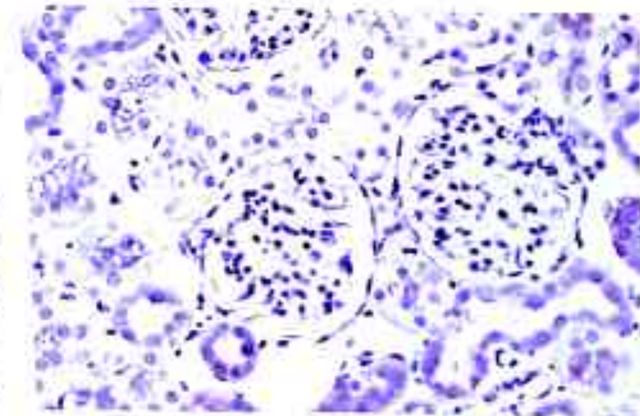
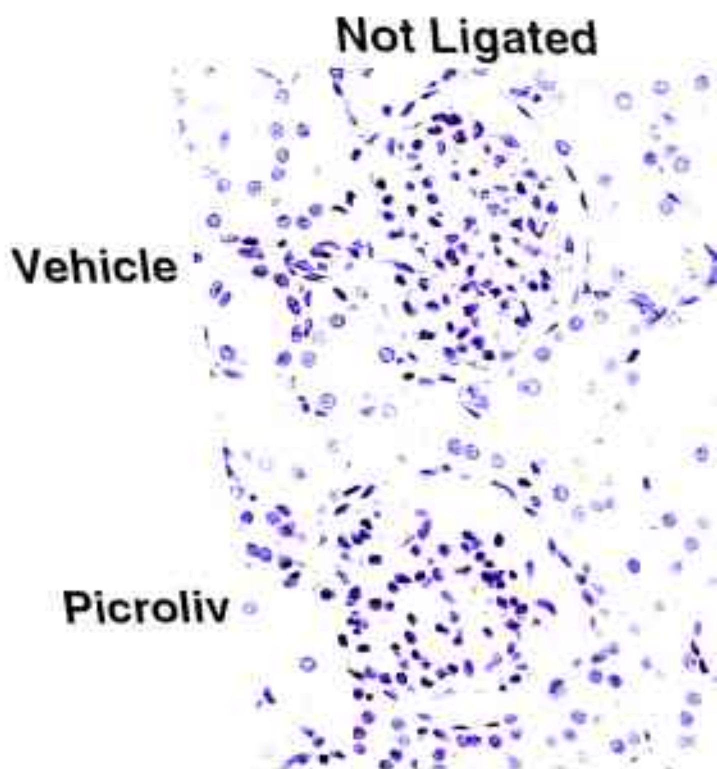


Fig.28: Expression of ICAM-1 in renal IRI: Anti-ICAM-1 monoclonal antibody was used for immunohistochemical staining of kidney sections from treated and untreated groups. The brown DAB staining represents ICAM-1 expression.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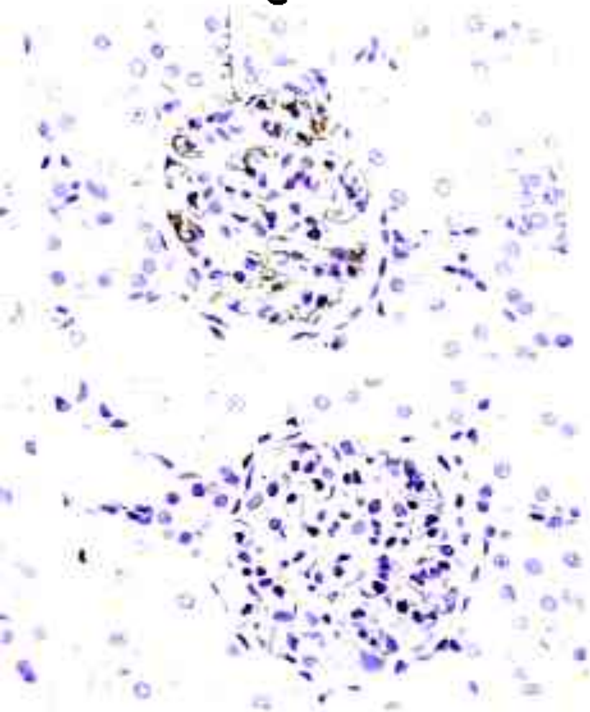

Fig.29: Expression of CD-18 in kidney sections: Anti-CD-18 monoclonal antibody was used for immunohistochemical staining of kidney sections from treated and untreated groups.

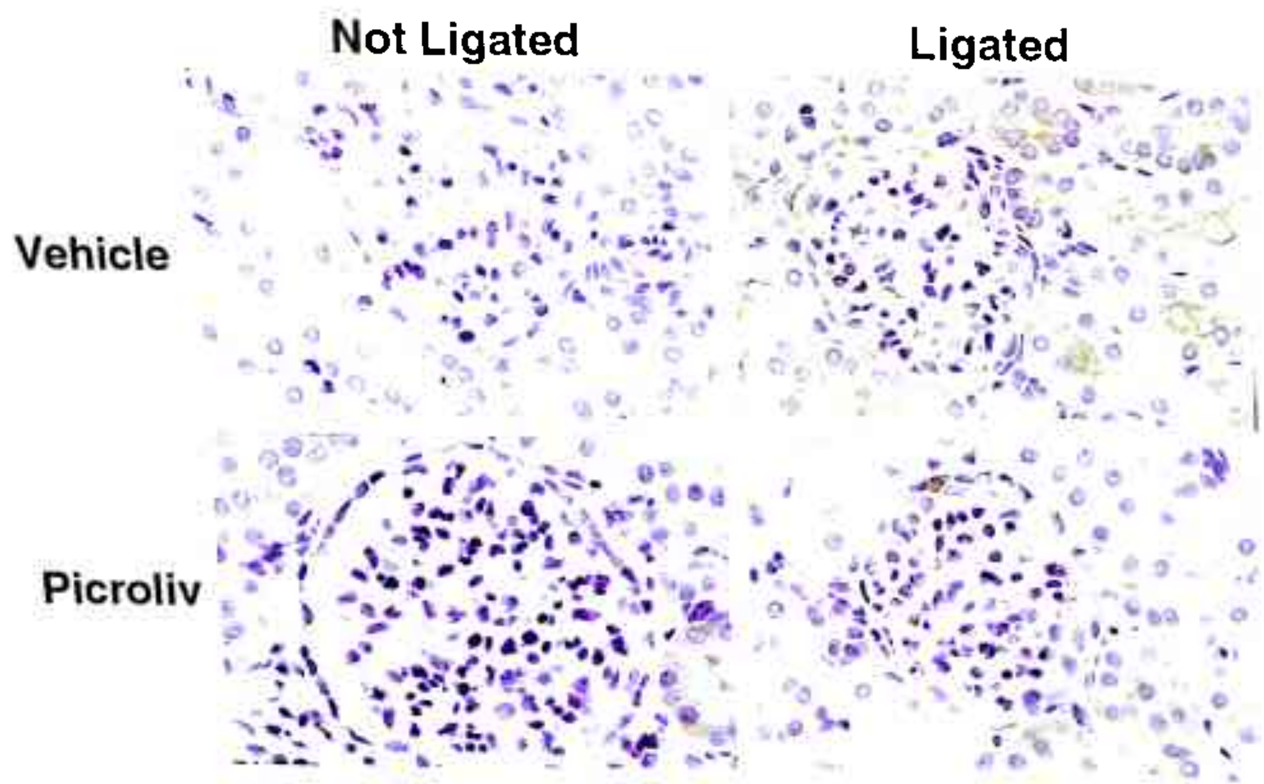
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Fig.30: TUNEL (terminal deoxynucleotidyl transferase mediated d-UTP nick end labeling) staining in renal sections: Apoptotic nuclei were determined using Apoptag kit (Oncor lab) in kidney sections from vehicle and picroliv treated ligated sections.

Vehicle

Picroliv

DISCUSSION

Our principal aim was to evaluate the potential of picroliv in ameliorating cellular and tissue injury following hypoxia-reoxygenation (*in vitro*) and ischemia-reperfusion (*in vivo*) injuries, with a view to develop this agent for clinical use. We approached the pathophysiology of hypoxia/ischemia-induced insult as a continuum of events and intended to identify those parameters that lend themselves to modulation under these conditions. Our major finding of the study is that picroliv, a non-toxic pharmacological agent, could prevent the deleterious effects of IRI.

Picroliv mediates protection against hypoxic injury in cell lines.

Our first goal was to investigate picroliv in an *in vitro* frame of reference for hypoxic injury in cell lines. It is only recently that we started to understand and dissect the mechanism by which a hypoxic signal elicits a specific response from different types of cells. Given the varying sensitivity of different cell lines to low oxygen tension, researchers are trying to delineate the molecular basis that orchestrates the hypoxic induction of glycolytic enzymes, VEGF and EPO. HIF-1 has been envisioned as a universal regulator for these hypoxia inducible genes. Keeping in mind the extended pathologic effects and physiologic imbalance created by hypoxic/ischemic disorders, we set out to investigate picroliv, a natural product against these injuries.

Picroliv is a plant extract and has been documented in Ayurveda, an ancient field of medicine. Picroliv is a very versatile drug, with a number of properties including hepatoprotective, antioxidant and immunomodulatory functions (Dhawan,

1995). Tandon et al.(1995) have shown that picroliv protects against myocardial ischemia induced by coronary artery ligation. It has been demonstrated to prevent the increase in serum marker enzymes during myocardial infarction. We therefore chose to test picroliv in the molecular context of hypoxic injuries. In this study, HUVEC (vein endothelial cells), Glioma (brain epithelial cells) and Hep 3B (hepatocarcinoma cells), cell lines of different tissue origin, were included to account for cellular variabilities.

Reoxygenation causes more damage than benefit to hypoxic cells (Shing Wu et al., 1998) and results in enormous production of reactive oxygen species (ROS) (Fellmann etal., 1997) and lipid peroxidation (Horakova et al., 1997). ROS generated during reoxygenation were demonstrated to increase the VEGF expression *in vitro* as well as *in vivo* systems (Kuroki et al., 1996). On those lines we postulated that an antioxidant would be able to reduce the hypoxic induction of VEGF. Since picroliv possesses strong antioxidant properties, a reduction in ROS production that is expected may in turn result in reduced VEGF expression during reoxygenation. As illustrated in our experiments, picroliv treatment reduced VEGF expression considerably during reoxygenation in HUVEC and Hep 3B cell lines. Glioma cells exhibit a different response from that of HUVEC and Hep 3B cells and this could be related to the differences in their abilities to respond to environmental stimuli. While it is a well-known fact that varied cells respond differently to hypoxia (Marti et al., 1998), the mechanisms underlying these differences are not known. It has been speculated that the ability of different cells to rapidly change their anti-oxidant levels in response to environmental stimuli may account for the observed

differences. Such variations were prominent in our experiments with Glioma cells. Although all the cell lines apparently contain the same general hypoxic pathway, the end response to picroliv in each cell type was individually tailored. VEGF is involved in pathological conditions such as, myocardial infarction (ischemic disorders) and tumors. Anti-VEGF antibodies are being tested as therapeutic bullets for many angiogenic disorders. Inhibition of VEGF by picroliv could prove to be effective in clinical conditions such as those mentioned above.

VEGF exerts its bological acitivities through its receptors KDR, Flt-1, and Flt-4 (Neufeld et al., 1999). To better understand the effect of picroliv on the activity of VEGF, we analyzed the expression of VEGF receptors following various treatments. It was found that picroliv modulated receptor expression in the same pattern as VEGF in HUVEC, suggesting the same mode of regulation of these genes. However, receptor expression was absent in Glioma and Hep 3B cells.

Hypoxia/reoxygenation have several biological effects that cause cellular damages. It results in cessation of aerobic respiration, decreased cellular ATP levels, increased calcium levels in mitochondria and changes in membrane lipids and enzymatic activities (Watson et al., 1984, White et al., 1984). The resulting membrane damage caused during the hypoxic exposure (either chemical or reduced oxygen concentration) is measured by the release of LDH. The decrease in LDH released during hypoxia by picroliv and the viability studies by MTT assay indicate the protective action of picroliv against hypoxic injury. This protection may be related to the membrane stabilizing properties of picroliv (Dhawan et al., 1995).

There is a growing body of evidence that hypoxia regulates the transcription

signal transduction as well as in cell proliferation, differentiation and function. Activators of PKC including PMA and 1-oleoyl-2-acetylglycerol stimulate the expression of growth factors including VEGF, EPO, EGF and PDGF in endothelial cells (Rozengurt et al., 1984). It has been shown that aminopurine and sodium fluoride, inhibitors of serine/threonine kinases and phosphatases, interfered with the hypoxic induction of HIF-1 DNA binding activity and the expression of HIF-1α and HIF-1β subunits, suggesting that PKC is involved in gene regulation during hypoxia (Wang et al., 1995). The reduced expression of VEGF and PKC during simultaneous treatment with picroliv and hypoxia in Glioma cells (Table 2) suggests that picroliv may be regulating the hypoxia-inducible genes through PKC signalling.

Hypoxia is known to induce a tyrosine kinase cascade that results in the activation of genes (Hayashi et al., 1996) and is critical in signaling triggered by the growth factors. Recently, it has been demonstrated that tyrosine-specific protein phosphorylation is involved in mediating pathological changes associated with hypoxia/ischemia (Braunton et al., 1998). Protein tyrosine kinase inhibitors, herbimycin-A, methyl 2,5-dihydroxycinnamate and tyrphostin, markedly blocked hypoxia-induced phosphotyrosine (Koroma et al., 1995, Hagar et al.,1997). We observed a selective inhibition of protein tyrosine kinase activity by picroliv during hypoxia leading to tyrosine-dephosphorylation of the proteins. The VEGF expression was completely inhibited when Glioma cells were simultaneously subjected to picroliv and hypoxia treatment, and was correlated with the tyrosine dephosphorylation of proteins during hypoxia. We suggest that the phosphorylation patterns of these proteins can be related to the regulation of VEGF expression.

This effect of picroliv may be important in situations where marked upregulation of VEGF was demonstrated such as in a vast majority of tumors and other pathological conditions like proliferative retinopathies and rheumatoid arthritis. Studies are underway to pin-point the specific proteins that are involved in the phophorylation cascade, which culminates in hypoxic gene regulation.

Picroliv enhances angiogenesis.

Our second major finding in this study is that picroliv enhances angiogenesis in both *in vivo* and *ex vivo* models. Given the mounting evidence that angiogenesis has remarkable physiological and pathological significance, we hypothesized that a pharmacological agent with the ability to enhance this process would be of great clinical relevance.

Lack of vascular supply and the resultant reduction in tissue oxygen tension, as in hypoxia, often leads to neovascularization in order to satisfy the vital needs of the tissue (Adair et al., 1990). Examples include the compensatory development of collateral blood vessels in ischemic tissues that are otherwise quiescent for angiogenesis and angiogenesis associated with the healing of hypoxic wounds (Knighton et al., 1983). Angiogenesis, the formation of new blood vessels from pre-existing ones, is a complex multi-step process requiring endothelial cell migration, proliferation, proteolytic activity, and morphogenesis. Endothelial cells are critical to this process and form new blood vessels in response to angoigenic factors secreted by a variety of cell types (Risau, 1990). Endothelial cells that line the blood vessels are anatomically positioned at the interface of the blood and tissue exchange, and thus, they are especially influenced by hypoxia. The elucidation of

the mechanism by which blood vessels regulate angiogenesis and enables the study of the angiogenic activity of the arterial wall and the effect of a pharmacologic intervention on its regulation. Our experiments reveal that both enhancement of tube formation in a matrigel model and capillary network development on a rat aorta model by picroliv are dose dependent.

VEGF has been implicated in angiogenesis. This growth factor strongly induced by hypoxia is a potent endothelial-cell-apecific mitogen and angiogenic factor that plays a central role in neovascular responses that accompany a number of physiological and pathological processes (Benjamin et al., 1997). The angiogenic property of VEGF has been attributed to several distinct functions associated with this cytokine. VEGF is a permeability enhancing factor that influences the egress of plasma proteins and cells that both directly and indirectly stimulate angiogenesis (Mandriota et al., 1997). Recent studies suggest that VEGF may also function as a survival factor for endothelial cells (Watanabe et al., 1997) by inducing them to produce a scaffold of matrix molecules that maximize cell adherence and proliferation. Thus an agent that induces the production of VEGF, like picroliv, would be an indirect stimulator of angiogenesis.

VEGF regulates angiogenesis in the rat aorta model (Nicosia et al., 1997). In their report, the researchers claim that VEGF plays a significant role in the autocrine/paracrine mechanisms that regulate the angiogenic response of the rat aorta to injury. Since picroliv enhances VEGF, the formation of capillary like structures in the rat aorta model by this agent may be mediated by VEGF. AP-1 transcription factors play a prominent role in upregulating hypoxia inducible genes

such as VEGF. The VEGF promoter features four AP-1 binding sites (Tischer et al., 1991). Its trans-activation the nucleus and binding to the promoter region of VEGF stimulates VEGF transcription. Picroliv, in our EMSA studies, elevated the trans-activation levels of AP-1 (data not shown) and thus could be enhancing VEGF, which in turn augments angiogenesis *in vitro* on matrigel.

Our laboratory has shown that picroliv enhances insulin-like growth factor-1 (IGF-1) in tissues (unpublished data). This raised the interesting possibility of involvement of IGF-1 in angiogenesis. Nicosia et al (1994) have suggested that IGF-1 promotes rat aortic angiogenesis and thus IGF-1 could be partly responsible for picroliv mediated angiogenesis. Researchers have long been speculating the importance of tyrosine kinases and their phosphorylation in angiogenesis. Martiny-Baron et al (1995), in their experiments have reflected the role of c-src tyrosine kinase in promoting angiogenesis with VEGF as the down stream target. Our phosphorylation studies indicate that picroliv phosphorylates a pp60 Kda band which we believe may be c-src. This introduces the possible signal transduction pathways that picroliv might regulate bringing about the witnessed changes in angiogenesis. Nitric oxide, a potent inorganic vasodilator, is synthesized by endothelial cells from arginine and is known to inhibit adhesive cell to cell interactions. This interaction accounts for the inhibition of angiogenesis and tube formation in the matrigel assay system by NO (Pipili-Synetos et al., 1994). We report in our *in vivo* system that picroliv down-modulates NO and could thus be preventing NO mediated inhibition of tube formation on matrigel in our experiments.

Angiogenesis signal is manifested by the binding of VEGF to its receptor

tyrosine kinases, Flt-1 (DeVries et al., 1992), Flt-4 (Quinn et al., 1993) and KDR (Terman et al., 1992) present on endothelial cells. There is evidence that the VEGF/VEGF receptor system plays a crucial part in vasculogenesis and angiogenesis (Quinn et al., 1993). Flt-1 and KDR bind VEGF with high affinity, appear to be expressed exclusively on endothelial cells, and mediate at least part of the cellular responses to VEGF, such as tube formation (Millauer et al., 1993). Flt-4 is not a high affinity receptor of VEGF and may not be involved in VEGF mediated cellular processes (Pajusola et al., 1994). We have shown using mRNA analysis that picroliv upregulates Flt-1 and KDR, but not Flt-4.

One event that plays a key role in angiogenic process is the re-organization of extracellular matrix, which is a complex and dynamic network assembled outside of the cell incorporating specific secreted glycoproteins and proteoglycans. It is a prerequisite for angiogenesis that endothelial cells aquire an invasive phenotype; they can penetrate the basement membrane and interstitial matrix. This involves proteolytic degradation and is acoomplished by activation of cellular proteases. Proteolytic enzymes of all four classes (serine, cysteine, aspartyl, and metallo) have been implicated in the angiogenic process and have been subject of intensive research for the past decade (Mignatti and Rifkin, 1993). MMP-1, MMP-3 and MMP-9 are stimulated by a host of growth factors and cytokines including interleukin-1, transforming growth factor-β, and tumor necrosis factor-α (Fabunmi et al., 1996). Wang et al (1998) demonstrated that VEGF enhances MMP-1, -3, and -9 expression in vascular smooth muscle cells. The authors also imply a role of Flt-1 in the upregulation of MMP-1 and MMP-9. As mentioned before, picroliv

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increased Flt-1 mRNA levels, which may be responsible for the elevated MMP-9 expression in HUVEC. VEGF has been shown to induce MMP-1 production in HUVEC (Zucker et al., 1998). However, no change in MMP-1 expression was observed upon picroliv treatment. These results suggest that picroliv plays an important role in regulation of these protease systems and therefore may modulate the ECM-degrading abilities of endothelial cells and their subsequent invasion and migration. The cumulative effect of picroliv on VEGF, VEGF receptors and MMPs involved in angiogenesis must be viewed as an interactive process rather than as a set of solitary events.

Angiogenesis, an essential process in wound repair, leads to increased oxygenation and endothelial permeability. This improves tissue energetics, enhances the effect of recruited cytokines, and leads to better protein transport to and from the wound site. Numerous cells participate in wound angiogenesis. Platelets contribute platelet derived growth factor (PDGF), transforming growth factor (TGF-β), and insulin like growth factor (IGF-1). Fibrobalsts produce Fibrobalst growth factors (aFGF, bFGF) and macrophages, a constant feature of wounds, produce VEGF and interleukins. Overall these mechanisms appear to be complementary. Recently Nissen et al (1998) described the contribution of VEGF to the angiogenic activity within the wounds and establish an important role for VEGF as a predominate angiogenic mediator in human surgical wounds. To better understand the mechanism of wound angiogenesis, recent efforts in our laboratory have been directed towards identifying various agents that promote wound healing such as interferon and Poly-IC, an interferon inducer (Bharatiya et al., 1992).

imbalance, which initiates activation of proteases, organelle damage and culminates in cell death. As a next step to our *in vitro* studies, we studied the various mediators of injury in ischemia/reperfusion and evaluated picroliv for its ability to ameliorate or reverse this injury.

As a specific case of ischemia, we have analyzed the effect of picroliv on renal ischemia for the following reasons. Since kidneys are highly vascularized organs, it is interesting to study the impact of blood vessel obstruction and the resulting ischemia in kidneys. In recent years evidence has accumulated to support the involvement of reactive oxygen species (ROS) in renal ischemia mediated tissue injury (Galat et al., 1990) and we reasoned that picroliv, an anti-oxidant, would help relieve the renal tissues form the IR insult. Our data support this hypothesis and

functional, morphological and pathophysiological studies. Clamping the left renal artery while the right kidney acted as an internal control served as a good model. This model results exclusively in *renal ischemic injury* as opposed to others where cross-clamping the juxtarenal or suprarenal aorta is associated with an increased perioperative cardiac and pulmonary demand in addition to an ischemia insult sustained by the kidneys and possibly the visceral organs and spinal cord (Weight et al., 1998). Harvesting the left kidney after any chosen duration of reperfusion, as well as sampling the renal venous blood, gives access to tissue for analysis of the mechanisms of reperfusion injury such as free radicals and nitric oxide (Weight et al., 1996). In addition the cellular markers of injury such as lipid peroxidation and DNA degradation can be measured and this model has been suggested to be ideal to test the efficacy of such pharmacological manipulations (Weight et al., 1998) as ours.

To be effective, renal intervention methods should be initiated prior to the beginning of renal ischemia (Hughes et al., 1996). Several ill patients are susceptible to develop acute renal failure because they sustain multiple renal insults over a prolonged period of time. It would be desirable to develop prophylactic therapies for use in this group of patients to decrease the risk of sustaining renal injury (Greene and Paller, 1991). In agreement with the above observations, we have treated the rats prophylactically with picroliv with a dose of 12 mg/kg/day that was most effective in anti-oxidant studies (Rastogi et al., 1996).

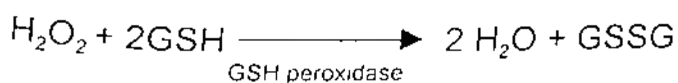
A pivotal feature of ischemia-reperfusion injury is the formation of ROS (McCord, 1985). ROS are generated during ischemia and reperfusion and cause

the recruitment of inflammatory cells and the subsequent microvascular dysfunction. Reactive oxygen species, collectively, are instrumental in impairing overall renal function (Paller, 1988) and in inducing apoptosis in renal cells (Burns et al., 1998). Antioxidant therapy has been documented to help in improvement of organ functions (Lee et al., 1997) and prevent apoptosis (Kadkhodaee et al., 1998 and Witenberg et al., 1999). The highly reactive nature of free radicals dictates that their site of action must be close to their site of formation, and thus increasing attention has been paid to free radical-mediated peroxidation of the lipid membrane. An assay for this is based on the reaction of malondialdehyde, a stable metabolite in lipid peroxidation cascade, with thiobarbituric acid (Ohkawa et al., 1979) and has been used to demonstrate lipid peroxidation following ischemia-reperfusion in the kidneys (Hauet et al., 1998). The authors of the above study have established protective effect of an anti-ischemic agent, 1-(2,3,4-trimethoxybenzyl)-piperazine dihydrochloride (TMZ), on cold storage and reperfusion of isolated perfused rat kidneys by downregulating the MDA (lipid peroxidation) levels. Known anti-oxidants such as Tocopherol (vitamin E) (Niki, 1991) posess the ability to inhibit lipid peroxidation and improve the function of canine kidneys at retransplantation after 48 h of hypothermic perfusion (Demirbas et al., 1993). Synthesized compounds such as H290/51 (*cis*-5,5a,6,106-tetrahydro-8-methoxy-6-methylindeno[2,1-6]indole) reduce lipid peroxidation and promote hypoxic kidney function in rats by decreasing the secondary radical formation produced at lipid peroxidation rather than acting as a scavenger for oxygen radicals (Sorensen et al., 1996). These studies stress the importance of inhibiting lipid peroxidation to prevent the ischemia-reperfusion

damage and furthermore suggest a role for treatment with antioxidants in clinical practice. We approached our goal of assessing the therapeutic potential of picroliv by studying the effect of picroliv on lipid peroxidation, which was measured in terms of malondialdehyde (MDA) in the renal tissues. MDA levels increased with increasing time of reperfusion following renal IRI. Picroliv reversed the increase of MDA levels to a considerable extent, thereby demonstrating its role as an antioxidant. Chander et al (1992) have provided evidence that picroliv suppressed the formation of MDA when added to rat liver microsomes in which lipid peroxidation was induced enzymatically by NADPH-ADP-Fe^{2+},. Lipid peroxidation is thought to be mediated by the reduction of iron to ferrous state which catalyzes the decomposition of fatty acid free radicals into MDA. Picroliv may play a dual role by chelating Fe^{2+} ions (Chander et al., 1992) and scavenging the free radicals from fatty hydroperoxides so as to inhibit the reaction of lipid peroxidation. A role of iron has been suggested in renal hypoxic or ischemic injury (Paller and Hedlund, 1994). Data implicate endothelial iron in intracellular formation of hydroxyl radical through Fenton reaction involving neutrophil- derived H_2O_2. H_2O_2 is capable of reacting with transition metals such as Fe^{2+} to form highly reactive hydroxyl radical (OH). Infusion of iron chelator deferoxamine during postischemic reperfusion of the kidney improved glomerular filteration rate and reduced histologic injury in the tissues (Paller and Hedlund, 1988). The potential of picroliv to scavenge free radicals correlates well with its iron chelating property.

A well developed antioxidant defense system composed of enzymatic components (superoxide dismutase, catalase, glutathione peroxidase and

glutathione reductase) and non-enzymatic components (glutathione, ascorbic acid, β-carotene, α-tocopherol and urate among others) has been demonstrated in most tissues (Chen et al., 1993, Barnard et al., 1993). Without the provisions for protection from oxidative injury, as in the case of ischemia, dysregulation, permanent cell injury and/or cell death can occur (Mathews et al., 1994). Glutahione (GSH), Glutathione peroxidase (GPx) and Glutahione reductase (GR) are three major players in the glutathione redox system and help maintain steady state in cells by keeping the radical levels low. H_2O_2, one of the deleterious byproducts of the respiratory burst during reperfusion reacts with glutathione in the presence of glutathione peroxidase to form water and glutathione disulfide (GSSG).



$$H_2O_2 + 2GSH \xrightarrow[\text{GSH peroxidase}]{} 2\,H_2O + GSSG$$

Glutathione reductase helps replinish the GSH pool by reducing GSSG to GSH (Smith et al., 1992). Since GSH functions in the removal of free radicals and protects cells from oxidative stress, we investigated the fate of this GSH pool in the ischemic kidney upon picroliv treatment. This cellular bioantioxidant, GSH, is known to be depleted following an ischemic insult in rat kidneys(Scaduto et al Am J Physiol 1998). Picroliv pretreated rats exhibited better GSH contents than their respective controls, indicating that it helps in replenishing the GSH pool. This modulation by picroliv may be attributed to its antioxidant activity. Allopurinol, an inhibitor of the enzyme xanthine oxidase, exhibits protective effect on renal failure by enhancing the GSH levels (Alatas et al., 1996). Xanthine oxidase catalyzes the conversion of xanthine (formed by degradation of ATP during ischemia) to superoxide and uric

following renal IRI (Singh et al., 1993). Use of SOD mimics (Baker et al., 1998), polyethylene glycol conjugated SOD (PEG-SOD) (Galinanes et al., 1992), and other such formulations (Razack et al., 1997) have been shown to exhibit significant protection against ROS mediated injuries. Picroliv enhanced SOD expression during 60 min ischemia followed by 120 min reperfusion, thus presenting a better antioxidant status within the tissue.

Although the role of NO has been controversial ever since it was discovered, there are now ample reports indicating that NO, as a free radical, contributes further to hypoxia and reoxygenation injury (Paller, Renal Failure, 1998). Peroxynitrite radical is formed by the interaction of nitric oxide and superoxide radical and has been reported to be much more damaging than either of them alone (Pryor & Squadrito, 1995 and Brunelli et al 1995). Nitric oxide and ROS are also known to induce apoptosis (Wang et al., 1999). Protection against ischemia-reperfusion injury has been reported after pretreatment with nitric oxide synthase (NOS) inhibitors (Sethi et al., 1999). The authors have also shown that NO mediates an increase in the intracellular calcium during hypoxia-reoxygenation, which augments the ROS generation. In our experimental IRI model we found that the NO levels increased with reperfusion time, as observed by Weight et al (Br J Surgery, 1998). Picroliv treatment reversed the elevated NO levels resulting from IRI, and this observation is in accordance with that of Sharma et al (1997) who have shown that antioxidant(s) attenuate NO involved in pathophysiology of brain injury during heat stress. where antioxidant(s) attenuate NO.

For completeness, it would be worth to mention the positive effects of NO on

free radical generation during IRI. NO, at higher concentrations inhibits the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and scavenges free radicals (Seth et al., 1994). However, the mechanisms of the increase in RCS generation induced by NO are not clearly understood.

Neutrophil accumulation is one of the major causes of ischemic injury and is markedly accelerated during reperfusion (Engler et al., 1983). It has been proposed that plugging of the microvascular endothelium by activated neutroplis, even after restoration of blood flow through a previously occluded artery, leads to the "no reflow" phenomenon, which perpetuates ischemic injury (Ambrosio et al., 1989). Diverse treatments that decrease circulating neutrophils, such as the administration antineutrophil serum (Romson et al., 1983, de Lorgeril et al., 1989) and monoclonal antibodies directed against neutrophil adhesion molecules have been reported to abate postischemic myocardial injury. Such evidence is also present in the renal arena of IRI where investigators have demonstrated a positive role of monoclonal antibodies against CD11a and CD11b (Rabb et al., 1994) and ICAM-1 (Kelly et al., 1994). This growing body of evidence supports the fact that inhibition of adhesion molecules afford impressive functional and morphologic protection in this renal IRI model. An over-expression of adhesion molecules has been implicated as the critical mediator in renal ischemia (Rabb and Postler 1998). Our observations reveal that picroliv downregulates the over expression of ICAM-1 and CD-18 in glomerular and tubular regions, respectively, suggesting that picroliv treatment prevents the neutrophil recruitment to ischemic areas and would hence protect tissue from possible consequences of activated neutrophils following IRI. The vast majority of

neutrophils are known to be present in the cortex, most in glomeruli during renal ischemia (Willinger et al., 1992). This explains the overexpression of ICAM-1 and CD18 in the glomeruli in our ischemic studies.

Our understanding of the exact mechanisms of apoptosis involved in IRI is still far from complete. ROS have been shown to cause direct DNA damage (Hagar et al., 1996) and may also act indirectly by activation of intracellular endonucleases (Yao et al., 1995). Both these mechanisms would be expected to result in apoptosis in ischemis/reperfusion injury. This hypothesis is further supported by the findings of Shimizu et al. (1996), who showed that a rise in ROS is paralleled by an increase in caspase activity and leads to apoptosis in hypoxic hepatocytes. In the experimental kidney model, apoptosis has been reported as a result of ischemia (Beeri et al., 1995). DNA fragmentation in the kidney cortex was detected after reperfusion injury (Schumer et al., 1992). NO is a known cause of apoptosis in many pathological situations (Wang et al., 1999, Kim et al., 1999). Use of antioxidants has helped to rescue cells from this apoptotic cascade. Antioxidants like dimethylthiourea (DMTU, hydroxyl radical scavenger) have been successfully used to reduce DNA fragmentation following ischemia-reperfusion in isolated perfused rat kidneys (Kadkhodaee et al., 1998). There is also proof of inhibition of apoptosis by ascorbic acid (vitamin C) in leukemic cells (Witenberg et al., 1999). In comparision to vehicle treated controls, a significant reduction in the number of apoptotic nuclei was noticed in kidney sections from picroliv pretreated animals. We believe that perhaps the reduction seen in number of apoptotic nuclei was due to better antioxidant status as evident by enhanced GSH content.

increased Gpx enzyme activity, better SOD staining and decreased NO levels in picroliv fed animals. Thus, picroliv seems to preserve renal tissues from I/R injury by reducing tissue infiltration of inflammatory cells and by downregulating adhesion molecules. In addition, it also increases the free radical scavenging capability of tissues by favorably modulating important cellular redox pathways.

To summarize, the pathological consequences resulting from hypoxia/ischemia-reperfusion are numerous and well documented. A pressing need remains for the development of medical inteventions that can protect cells and tissues form dying in the absence of oxygen. The key to this development lies in understanding this highly orchestrated series of events that takes place during hypoxia/ischemia. In this report, we have shown that picroliv, a natural pharmacological agent, helps reduce the extent of injury incurred by cells and tissues from hypoxia, by modulating some of the regulatory molecules involved.

Fig.31 is a schematic representation that summarizes our findings in the current study. At the molecular level, picroliv appears to exert its effect on transcriptional regulation of hypoxia-inducible genes (VEGF, HIF-1) through phosphorylation and signalling mechanisms. It abates lipid peroxidation thus preventing membrane damage. In the presence of normal oxygen tension, picroliv enhances angiogenesis by regulating VEGF and MMPs. Picroliv scavenges reactive oxygen species and prevents free radical injury to tissues. Adhesion molecules and NO could mediate the ability of picroliv to reduce apoptosis and tissue injury. Delineation of the complete mechanism of action of picroliv would help establish its role in IRI protection. This could be accomplished by radiolabelling the

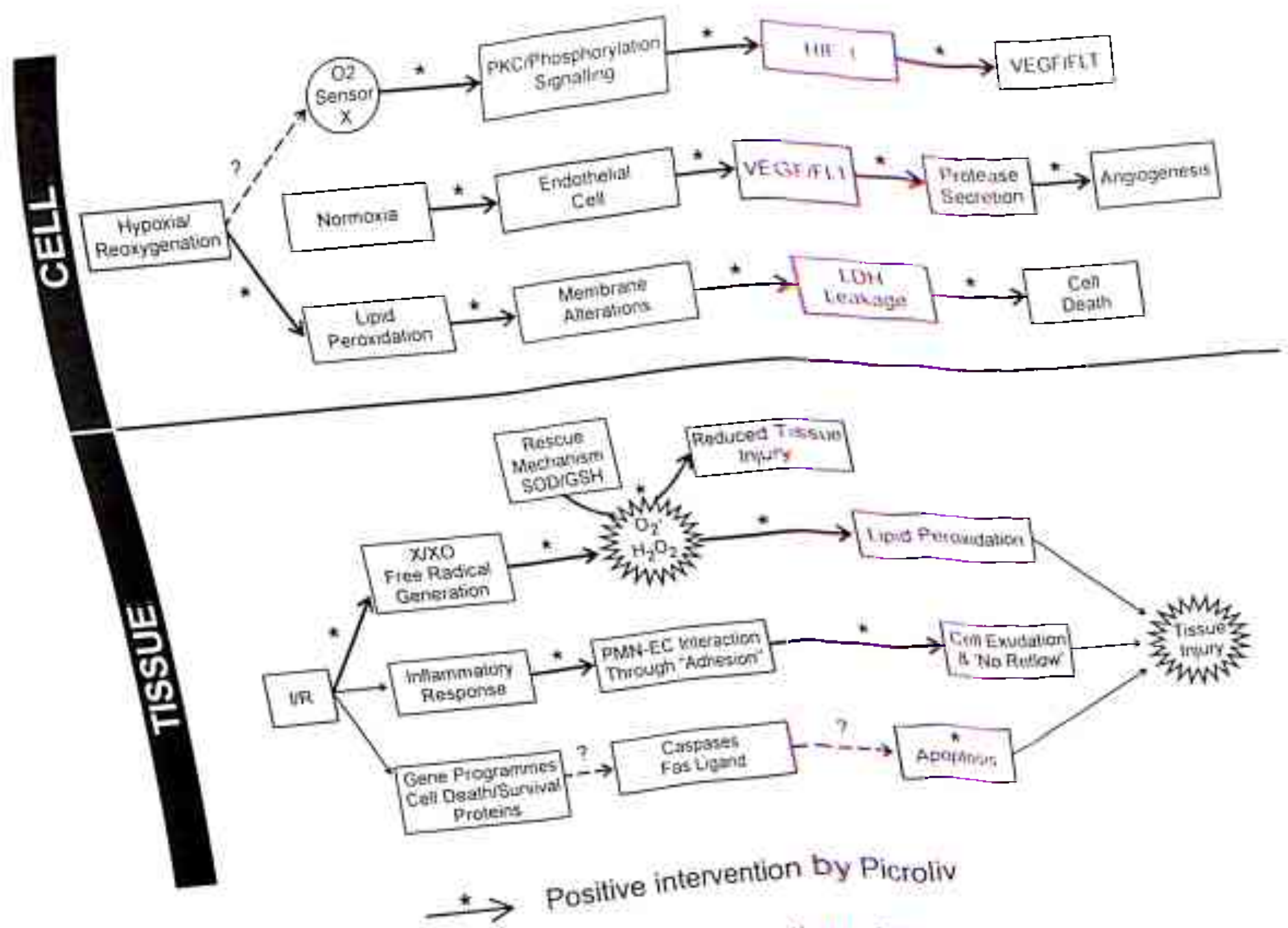


Fig.31: Mode of action of Picroliv

drug and following its path within the cell. Our studies clearly demonstrate that picroliv has the potential to be developed as an anti-IRI agent.

More studies are warranted to delve into the query: Does picroliv generate a single linear sequence of events or alternatively funciton through different pathways that lie in parallel? We are not far from explanations to the mechanisms involved in the hypoxic crisis. Be that as it may, the data presented here may lead to exciting investigations that will go a long way towards development of therapeutic interventions for hypoxia/ischemia - reoxygenation disorders.

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This is not the END;

This is not even the beginning of the END;

This is just the end of the BEGINNING...

- Winston Churchill