Investigation of the effects of chinese herb medicine on TNF-α induced apoptosis in Huvec and Jurkat cells and their antioxidative effects

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1. Introduction

1.1. The role of traditional herbal medicine

1.1.1. Plants as source of drugs

Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years. In the prehistoric times, people began to search for herbs from plants which exert healing effects. Herbal medicine is based on the premise that plants contain natural substances that can promote health and alleviate illness. The term *herbs* in herbal medicine is used loosely to refer not only to herbaceous plants but also to bark, roots, leaves, seeds, flowers and fruit of trees, shrubs and woody vines, and extracts of the same that are valued for their savory, aromatic or medicinal qualities. Today we are witnessing a great deal of public interest in the use of herbal remedies. The World Health Organization estimated that ≈ 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts or their active components. Furthermore, many Western drugs had their origin in a plant extract. Reserpine, which is widely used for the treatment of high blood pressure, was originally extracted from the plant Rauwolfia serpentina, whereas digitalis, used as a heart stimulant, was derived from the foxglove plant (Digitalis purpurea). The Chinese herb ephedra (Ma huang), which was used early on for the treatment of asthma, contains the active substance ephedrine, whereas salicylic acid (a precursor of aspirin) was obtained from willow tree bark (Salix alba) to help relieve fevers [Bruneton J, 1995].

1.1.2. Common herbal remedy

Herbal medicine is widely used today for a host of common ailments and conditions, such as anxiety, arthritis, colds, coughs, constipation, fever, headaches, infections, insomnia, intestinal disorders, premenstrual syndrome, stress, ulcers, and weakness. Some of the more popular herbs

in use today include *Echinacea*, garlic, ginseng, goldenseal, ginkgo, saw palmetto, aloe vera, and feverfew. Research continues with respect to the usefulness of ginger for motion sickness; licorice for treating ulcers; hops, passionflower, and valerian for treating insomnia; feverfew for relieving migraine headaches; peppermint oil for relieving irritable bowel syndrome; saw palmetto berries for treating benign prostatic hypertrophy; *Echinacea* for its immunostimulant properties; St John's Wort (containing hypericin) for anxiety and depression; and milk thistle (with its rich content of flavono-lignans) for protecting and restoring liver function [Dew MJ, 1984; Tyler V, 1994 and McNutt K, 1995]. Recently, several commonly used herbs have been identified as possessing cancer-preventive properties. These herbs include members of the Allium sp. (garlic, onions, and chives); members of the Labiatae (mint) family (basil, mints, oregano, rosemary, sage, and thyme); members of the Zingiberaceae family (turmeric and ginger); licorice root; green tea; flax; members of the Umbelliferae (carrot) family (anise, caraway, celery, chervil, cilantro, coriander, cumin, dill, fennel, and parsley); and tarragon [Caragay AB, 1992].

1.1.3. Research and new drug development of herbs

Due to the continuous increase of medical costs, many countries provide support to encourage scientific research on alternative therapies. Furthermore, the putative efficacy of medicinal herbs relies on empirical or anecdotal data and tradition of use, which frequently cannot satisfy the requirements of evidence-based medicine. Thus, the step back by the analysis of traditional herbal medicine and new drug development from herbs are started recently with high effort. Research interest has focused on various herbs that possess hypolipidemic, antiplatelet, antitumor, or immune-stimulating properties that may be useful adjuncts in helping reduce the risk of various diseases. In different herbs, a wide variety of active phytochemicals, including the flavonoids, terpenoids, lignans, sulfides, polyphenolics, carotenoids, coumarins, saponins, plant sterols, curcumins, and phthalides have been identified [Tyler V, 1994]. Several of these phytochemicals either inhibit nitrosation or the formation of DNA adducts or stimulate the activity of protective enzymes such as the Phase II enzyme glutathione transferase (EC 2.5.1.18) [Steinmetz KA, 1991; Caragay AB, 1992; Kikuzaki H, 1993; Bisset NG, 1994; Cuvelier ME, 1994; Ho CT, 1994; Huang MT, 1994; Lam LKT, 1994; Robbers JE, 1994; Zheng GQ, 1994; Smith TJ, 1994 and Haraguchi H, 1995]. Research has centered around the biochemical activity

of the Allium sp. and the Labiatae, Umbelliferae, and Zingiberaceae families, as well as flaxseed, licorice root, and green tea. Many of these herbs contain potent antioxidant compounds that provide significant protection against chronic diseases. These compounds may protect LDL cholesterol from oxidation [Kleijnen J, 1989; Sharma RD, 1990 and 1991; Warshafsky S, 1993; Smith TJ, 1994; Cook NC, 1996 and Manach C, 1996], inhibit lipid peroxidation [Dreosti IE, 1996 and Fuhrman B, 1997], or have antiviral or antitumor activity [Caragay AB, 1992 and Shibata S, 1994].

In the development of medicine from plants, several steps are involved: (1) identification of suitable plants to investigate; (2) isolation and identification of single chemical entities from the chosen plant; (3) identification of the pharmacological actions of those entities; (4) conduct of controlled clinical trials. The rate limiting steps appear to be (3) and (4). Many herbal medicines are not well analyzed in terms of their mechanisms of actions, toxicity, and clinical effects. Therein the challenge lies.

1.1.4. Safety of herbal medicine

Whereas some herbal products may be safe and may contain active constituents that have beneficial physiologic effects, others may be unsafe to use [Tyler V, 1994]. The Food and Drug Administration has classified several herbs as unsafe, even in small amounts, and hence they should not be used in either foods or beverages [Larkin T, 1983 and Saxe TG, 1987]. Some herbs are safe in modest amounts but they may become toxic at higher doses. For example, whereas licorice root can be used safely for treating duodenal and gastric ulcers, deaths from its excessive use have been reported. Large amounts of licorice can cause serious side effects such as hypokalemia, high blood pressure, and heart failure [Nielsen I, 1984]. Other herbs are known to be lethal. Germander, an herb used in some weight-loss programs, has been reported to cause fatal hepatitis [Mostefa-Kara N, 1992]. The Chinese herbs caowu and chuanwu are used to treat rheumatism, arthritis, bruises, and fractures. They may contain highly toxic alkaloids such as aconitine which produce neurologic, cardiovascular, and gastrointestinal disturbances. Use of these herbs can even result in death [Chan TYK, 1993].

1.2. Radix Salviae Miltiorrhizae and its components

Radix Salviae Miltiorrhizae (RSM), an important constituent of Chinese folk medicine, is the root extract of the plant Salvia Miltorrhizae Bunge (Fig. 1), which belongs to the family of Labiatae. Both injection solution and tablets made from RSM have been widely used in the Chinese community for various complaints, particularly cardiovascular and cerebrovascular, such as ischemia, myocardial infarction and thrombosis.

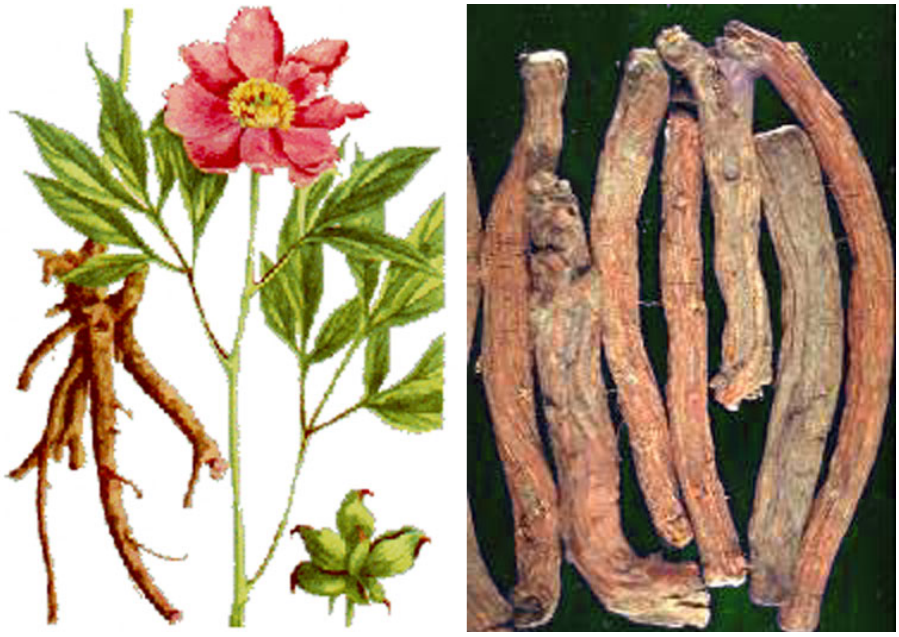


Fig. 1 The plant Salvia Miltiorrhizae Bunge and its dry root, which is being used as an important Chinese folk medicine.

Several studies within the last few years showed that RSM exhibits cardioprotective effects [Wu W, 1992; Zou ZW, 1993 and Kuang P, 1995]. Also the ability of RSM to suppress platelet aggregation and anti-thrombotic effects may contribute to improve cardio- or cerebral-circulation [Chen WZ, 1984; Li CZ, 1984 and Kuang PG, 1991]. Our unpublished data showed that RSM reduced both ischemic brain injury and leukocyte infiltration following cerebral ischemia-reperfusion, may be partially mediated via an ATP-sensitive potassium channel (K_{ATP})-linked mechanism. Seven phenolic compounds have been isolated from the water-soluble extract of RSM by Li et al. [Li NL, 1984] (Fig. 2) and have been reported to employ multiple

pharmacological activities [Liu GT, 1992]. The activities of scavenging oxygen free radical and inhibiting lipid peroxidation among the compounds were extensively studied [Li DY, 1995]. These phenolic compounds have a strong protective action against oxygen free radical induced peroxidative damage [Xi SC, 1994 and Zhang X,1994]. They are the active principle of RSM responsible for antioxidant activity and at least a part of the pharmacological bases for using RSM for the clinical treatment of certain diseases. Based on the protective effects, it has been proposed that RSM and its components may prevent apoptosis in response to pathophysiological signals. In the next paragraph, an overview about apoptosis will be provided.

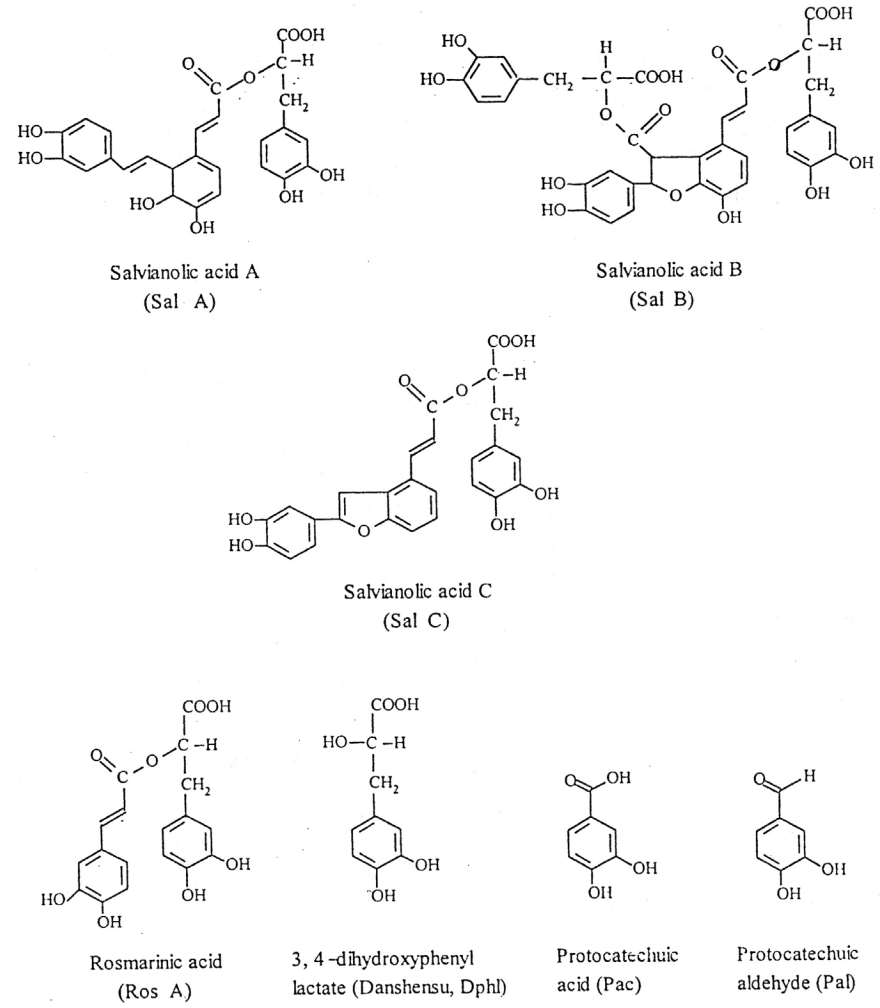


Fig. 2 Chemical structures of water-soluble components isolated from Radix Salviae Miltiorrhizae.

1.3. Apoptosis

1.3.1. Introduction

Death of cells has aroused far less interest than other basic cellular processes such as proliferation and differentiation. The relative neglect probably results at least in part from the wide prevalence of an unjustifiably circumscribed and restricted notion of cell death as a degenerative phenomenon produced by injury. This concept evolved early in the history of cellular pathology [Virchow R, 1858] and has tended to dominate thinking about both the incidence and the mechanisms of cell death ever since. In 1972 a new concept of cell death—apoptosis was firstly proposed by Kerr et al [Kerr JF, 1972]. He described that apoptosis was an active, precisely regulated, and energy requiring process that seemed to be orchestrated by a genetic program. Herence the terms *apoptosis* and *programmed cell death* were interchangeable. Apoptosis can be triggered by a variety extrinsic and intrinsic signals [Vaux DL, 1994]. This type of regulation allows for the elimination of cells that have been produced in excess, that have developed improperly, or that have sustained genetic damage.

1.3.2. The morphology of apoptosis

The morphology of cellular death is now distinguished into two types, apoptotic cell death and necrotic cell death. Necrotic cell death is a pathologic form of cell death resulting from acute cellular injury, which is typified by rapid cell swelling and lysis. In contrast, apoptotic cell death is characterized by controlled autodigestion of the cells. Cells appear to initiate their own apoptotic death through the activation of endogenous proteases. This results in cytoskeletal disruption, cell shrinkage, and membrane blebbing. Electron microscopy shows that the structural changes in apoptosis take place in two discrete stages (Fig. 3): the first comprises the formation of apoptotic bodies, the second their phagocytosis and degradation by other cells. Apoptosis also involves characteristic changes within the nucleus. The nucleus undergoes condensation as endonucleases are activated and begin to degrade nuclear DNA. In many cell types, DNA is degraded into DNA fragments the size of oligonucleosomes, whereas in others larger DNA fragments are produced. Apoptosis is also characterized by a loss of mitochondrial function. The dying cell maintains its plasma membrane integrity. However, alterations in the plasma

membrane of apoptotic cells signal neighboring phagocytic cells to engulf them and thus to complete the degradation process. Cells not immediately phagocytosed break down into smaller membrane-bound fragments called apoptotic bodies. An important feature of apoptosis is that it results in the elimination of the dying cell without induction of an inflammatory response. In contrast, necrotic cell death is associated with an early loss of cell membrane integrity, resulting in leakage of cytoplasmic contents and the induction of an inflammatory response [Wyllie AH, 1980].

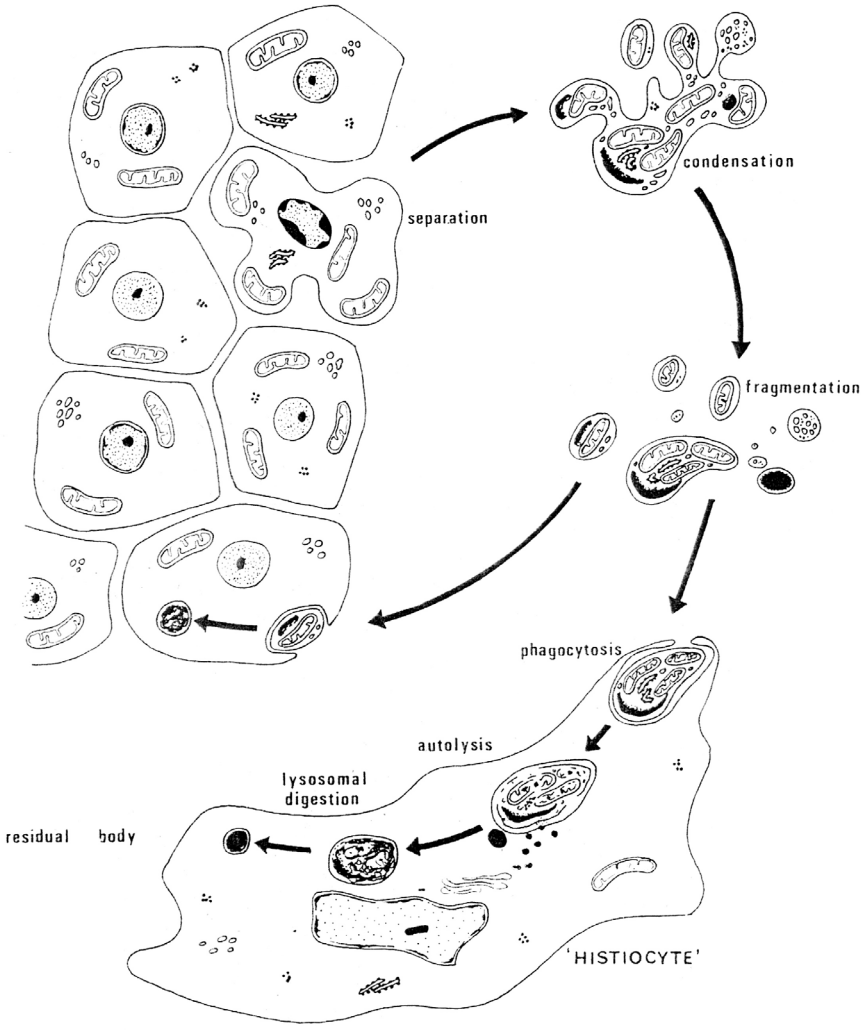


Fig. 3 Diagram to illustrate the morphological features of apoptosis.

1.3.3. Significance of apoptosis in diseases

It is now widely accepted that apoptosis is of central importance for the development and homeostasis of metazoan animals. For example, apoptosis serves as a prominent force in sculpting the developing organism [Hammer SP, 1971], as a major mechanism for the precise regulation of cell numbers [Raff MC, 1992 and 1993], and as a defense mechanism to remove unwanted and potentially dangerous cells, such as self-reactive lymphocytes [Cohen JJ, 1991], cells that have been infected by viruses [Vaux DL, 1994 and Debbas M, 1993], and tumor cells [Williams GT, 1991]. Not surprisingly, the initiation of apoptosis is carefully regulated. Many different signals that may originate either from within or from outside a cell have been shown to influence the decision between life and death. These include lineage information, cellular damage inflicted by ionizing radiation or viral infection, extracellular survival factors, cell interactions, and hormones [Raff MC, 1992 and 1993, and Steller H, 1994]. These diverse signals may act to either suppress or promote the activation of the death program, and the same signal may actually have opposing effects on different cell types [Truman JW, 1984]. Recent evidence suggests that the failure of cells to undergo apoptotic cell death might be involved in the pathogenesis of a variety of human diseases, including cancer, autoimmune diseases, and viral infections [Bursch W, 1992]. In addition, the inappropriate activation of apoptosis may also cause or contribute to a variety of diseases, including acquired immunodeficiency syndrome (AIDS) [Banda NK, 1992], neurodegenerative diseases, and ischemic stroke [Raff MC, 1993 and Martinou JC, 1994] (Fig. 4).

Diseases Associated with Inhibition of Apoptosis

1. Cancer
    - Follicular lymphomas
    - Carcinomas with p53 mutations
    - Hormone-dependent tumors
        - Breast cancer
        - Prostate cancer
        - Ovarian cancer
2. Autoimmune disorders
    - Systemic lupus erythematosus
    - Immune-mediated glomerulonephritis
3. Viral infections
    - Herpesviruses
    - Poxviruses
    - Adenoviruses

Diseases Associated with Increased Apoptosis

1. AIDS
2. Neurodegenerative disorders
   - Alzheimer's disease
   - Parkinson's disease
   - Amyotrophic lateral sclerosis
   - Retinitis pigmentosa
   - Cerebellar degeneration
3. Myelodysplastic syndromes
4. Ischemic injury
   - Myocardial infarction
   - Stroke
   - Reperfusion injury
5. Toxin-induced liver disease
   - Alcohol

Fig. 4. Diseases associated with the induction or inhibition of apoptotic cell death.

1.3.4. Regulation of cell death: therapeutic potential

The realization that apoptosis represents an active, gene-directed mechanism has fostered optimism that it may be possible to control apoptosis with the development of drugs that act against the molecular components of the death machinery. Specific therapies designed to enhance or decrease the susceptibility of individual cell types to undergo apoptosis could form the basis for treatment of a variety of human diseases.

1.4. Flow channel technique

A computer controlled flow channel system (Elias-c-) based on photometric light transmission measurements through cell monolayers was employed to investigate antioxidative capacities of natural herbal drugs and compounds [Artmann GM, 1995 and 1996]. Reactive oxygen species such as hydrogen peroxide (H_2O_2) are highly reactive molecules produced during the course of normal cellular processes involving oxygen. Oxidants have the potential to create situations of oxidative stress within cells by reacting with macromolecules causing damage to cellular structures and functions [de Haan J, 1998]. Nature has established highly effective antioxidative

defense mechanisms within cells themselves and within the lipid phase of the cell membrane [Ursini F, 1985]. In addition to these, drugs and natural healing substances, in some cases prescribed for completely different indications, act as highly effective antioxidative agents. A flow channel technique was designed to investigate antioxidative capacities of natural herbal drugs and compounds. In an automated form of flow channel system (analyzer Elias-c-), the analyzer is highly accurate and sensitive to evaluate RBC-stiffness and -relaxation time as well as RBC-EC adhesion. When RBC prepared as monolayers on a coverslip were treated with hydrogen peroxide, the stiffness enhanced time-dependently whereas the relaxation time declined [Schrier SL, 1992 and Artmann GM 1993]. Also hydrogen peroxide alters the physical state and function of the plasma membrane of endothelial cells and facilitates RBC adhesion [Block ER, 1991]. Antioxidants as shown in this study counteract these effects. In contrast to other biochemical or physical techniques to study the antioxidative capacity of drugs [Stern A, 1985 and Pincemail J, 1989], this method enables investigations on living cellular structures, i.e., above the molecular and below the animal experiment level.

Two herbs were chosen in the present study. (1) Tetramethylpyrazine (ligustrazine, TMP) is a mono-molecular metabolically active constituent of a Chinese herb Ligusticum wallichii Franchat. TMP is available commercially in China for treatment of a variety of vascular diseases, notable ischaemic stroke disease [Chen KJ, 1992], and pulmonary hypertension secondary to chronic obstructive pulmonary disease (COPD) [Peng W, 1991 and Liu SM, 1994]. TMP is known to act as an antioxidant by scavenging free radicals [Zhang ZH, 1994]. The therapeutic effects of TMP are likely to be via its action as a vasodilator [Sutter MC, 1993], inhibition of platelet aggregation [Liu SY, 1994], protection of endothelial cells against low density lipoprotein-induced damage [Li YJ, 1994]. (2) Dhpl (3,4-dihydroxyphenyl lactate), as one component of RSM was used in RBC-EC adhesion studies. Since RBC and EC could be the direct damaging targets of oxygen free radical in blood flow, and red blood cell aggregability, deformability and adherence to endothelial cells play a major role in hemodynamics, particularly in small blood vessels, as well as their impairment has been linked to microcirculatory disorders in numerous pathological states [Hovav T, 1999]. Thus RBC and EC were chosen as in vitro model systems for the flow channel assay. The efficacy of Dhpl to inhibit hydrogen peroxide

induced damage leading to RBC-EC adhesion was compared with Vitamin E, a well-known antioxidant.

1.5. Aim and importance of this study

In this thesis, the activities of two components of RSM, PAC and PAL, in cell protection from apoptosis were investigated. RSM is being used in traditional Chinese medicine as a treatment for cardiovascular and cerebrovascular diseases. Recent studies have indicated that RSM exhibited cardio- or cerebro-protective effects. However, how exactly RSM and its components exert their protective actions and the molecular basis of those effects are not well known yet. Based on the protective effects, it has been proposed that RSM and its components may prevent programmed cell death in response to pathophysiological signals. Therefore, the study was to analyze the effect of PAC and PAL on apoptosis, and to elucidate the molecular basis of their anti-apoptotic effect. In addition, a computer controlled flow channel system was employed to examine antioxidative capacity of TMP and Dhpl.

2. Materials and Methods

2.1. Buffers and Solutions

2.1.1. Routine buffers and solutions

All buffers and solutions were prepared under sterile condition by filters or autoclave.

PBS (10x)	40 g NaCl 1 g KCl 5.8 g Na_2HPO_4 1 g KH_2PO_4	pH 7.4 in 500ml d H_2O
PBS	50 ml 10x PBS	in 500ml d H_2O
HEPES buffer (10x)	40 g NaCl 1.5 g KCl 11.9 g HEPES 10 g Glucose	pH 7.55 in 500ml d H_2O
HEPES buffer	55.6 ml 10x HEPES buffer	in 500ml d H_2O
HEPES transport buffer	55.6 ml 10x HEPES buffer 4 ml Fungizone 4 ml penicillin-streptomycin solution	in 500ml d H_2O

2.1.2. Cell Culture

Collagenase I (0.2%)	0.2 g Collagenase I Store in –20°C	pH 7.55 in 100 ml 1x HEPES buffer

EDTA (0.02%)	0.02 g EDTA	pH 7.55
	1.5 ml BSA (35%)	in 100 ml PBS
Gelatin (0.2%)	0.2 g Gelatin	in 100 ml PBS

2.1.3. Transformation

Ampicillin solution	50 mg ampicillin-Na-salt per ml H_2O	
	Sterile filtration, store in –20°C	
LB-medium	10 g Bactotrypton	
	5 g Bacto-Yeast Extract	
	10 g NaCl	in 1 L d H_2O
	(for LB-plates: 15g Agar per Liter)	
Mini prep Solution I	50 mM glucose	
	25 mM Tris·Cl (pH 8.0) 10 mM EDTA (pH 8.0)	
Mini prep Solution II	10 µl 10N NaOH	
	440 µl d H_2O	
	50 µl 10% SDS	
Mini prep Solution III	5 M potassium acetate 60 ml	
	Glacial acetic acid 11.5 ml	
	H_2O 28.5 ml	

2.1.4. Western Blotting

Nonfat milk buffer (10%)	10 g milk powder	in 100 ml PBS

Antibody I (1:1000)	10 µl IκB-alpha	in 10 ml 10% milk buffer
Antibody II (1:300)	40 µl anti-rabbit-AP-IgG	in 12 ml d H_2O

2.1.5. EMSA

Nucleuprep lysis buffer I	500 µl 1M HEPES-NaOH (pH 7.9)	
	500 µl 1M KCl	
	100 µl 0.5M EDTA (pH 8.0)	
	5 µl 0.1M EGTA	in 50 ml d H_2O
Nucleuprep lysis buffer II	1 ml 1M HEPES-NaOH (pH 7.9)	
	4 ml 5M NaCl	
	10 µl 0.5M EDTA (pH 8.0)	
	500 µl 0.1M EGTA	in 50 ml d H_2O
TBE (10x)	54 g Tris	
	27.5 g Borsäure	
	40 ml 0.5M EDTA (pH 8.0)	in 500 ml d H_2O
5xEMSA binding buffer	25 mM HEPES	pH 7.8
	25 mM $MgCl_2$	
	250 mM KCl	
	1 mM EDTA	
	25 mM DTT	
	50 % glycerin	
Loading buffer	80% Formamid	
	10 mM NaOH	

	1 mM EDTA 0.1% Xylene cyanol blue 0.1% Bromphenolblau	
Gel buffer	12 ml 30% PAA 1.5 ml 10x TBE add 70 ml d H_2O 75 µl TEMED 300 µl APS	
Running buffer	12.5 ml 10x TBE	in 500 ml d H_2O

2.1.6. Flow Channel Assay

HEPES+ buffer	0.5 g glucose	in 500 ml HEPES buffer
HEPES++ buffer	0.5 g bovine albumin	in 500 ml HEPES+ buffer
100µM H_2O_2	11.4 µl 30% H_2O_2 in 10ml DMEM degas for 5 min, then 1:100 dilute in DMEM	

2.2. Chemicals

Plasmids	NF-κB *Cis*-reporter plasmid (Stratagene, Amsterdam, Netherland) was used in luciferase assay to analyse NF-κB transcriptional activity. As a control for transfection efficiency pRL-TK plasmid (Promega, Mannheim) was together used with NF-κB plasmid.

Amersham, Braunschweig	PolydIdC
Bio Rad, München	PVDF membrane
Boehringer Mannheim	RIPA buffer set
Chemicon, Hofheim/Ts	Sheep anti-rabbit IgG-alkaline phosphatase secondary antibody
Falcon, Heidelberg	Cell culture articles, ELISA-plates
Kodak, Rochester (USA)	X-Omat imaging film
Macherey-Nagel, Dueren	Nucleobond AX Kit for maxi-prep
Merck, Darmstadt	APS, Borsäure, EDTA, EGTA
Novex, Frankfurt am Main	Bluemarker, running buffer and transfer buffer for western blot
PAA, Martinsried	Penicillin-Streptomycin-L-glutamin solution, culture medium
Pierce, Bonn	BCA protein assay kit
Promega, Mannheim	Dual-Luciferase reporter assay kit NF-κB Oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGG-3')
Roche, Mannheim	Cell proliferation kit II (XTT), 10% NP-40, protease-inhibitor-cocktail tablet

Santa Cruz, Ismaning	IkBα antibody (C-21), NF-κB p50 antibody (C-19), NF-κB p65 antibody (C-20)
Serva, Heidelberg	EDTA, Nonfat milk powder, Bovine albumin
Sigma, Deisenhofen	Fetal calf serum, Dulbecco's PBS, TNF-α, ActD, MG 132, protocatechuic acid, protocatechuic aldehyde, BCIP/NBT, 30% PAA, TEMED, Vitamin E, 30% H_2O_2
Upstate, Lake Placid, USA	NF-κB inhibitory ligand

2.3. Methods

2.3.1. Cell culture

Jurkat cells (ATCC TIB-152) were maintained in RPMI 1640 supplemented with penicillin (400 U/ml), streptomycin (50 µg/ml), L-glutamine (300 µg/ml) and 10% fetal calf serum (FCS) and splitted 1:10 every three days.

Human umbilical cord vein endothelial cells (HUVEC) derived from umbilical cord were isolated as described previously [Jaffe EA, 1973]. In brief, the vein was washed with 20 ml HEPES buffer to remove the blood. After washing, the vein was incubated for 15 min at 37°C in an incubator after injecting 10 ml of collagenase solution (1 mg/ml). The vein was then rolled gently and the collagenase solution containing the cells was washed off with 20 ml Ham's F-12/IMDM medium (without serum). The complete 30 ml of cell suspension was centrifuged at 1100 U/min for 10 min without break. The pellet was resuspended and cultivated in a gelatin-coated flask with 4 ml Ham's F-12/IMDM medium supplemented with 40,000 units penicillin/streptomycin/L-glutamine solution and 20% human serum under a humidified atmosphere of 5% CO_2 at 37°C. Cells were detached for subcultivation or assay with 0.2% collagenase, 0.02% EDTA in HEPES-buffer and only used for experiments at passages 2 to 4.

Bovine aortic endothelial cells (BAEC) were isolated from aortas of freshly slaughtered cow [Zink S, 1995]. The lumen of the aortas was washed with PBS under sterile conditions and the adventitia was removed. The remaining part was immersed completely in 30 ml of dispase solution (0.5 mg/ml) for 15 min at 37°C. Afterwards the aortas were put on an aluminum tray and the BAEC were scraped off. The cells were then plated in a gelatin coated 6-well dish. BAEC were cultivated in 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma Chemical Co, Aldrich, Germany) supplemented with 10% fetal calf serum. Subcultures were harvested with trypsin and splitted at a 1:3 ratio into cell culture flasks. Cells at passage 3-12 were used for experiments.

2.3.2. Cell stimulation

In the experiments, different stimulators were applied on cells. Their concentration were respectively:

TNF-α	10 ng/ml
ActD	1 μg/ml
MG132	5 μg/ml
NF-κB inhibitory peptide	50 μg/ml
PAC	100 μM, 1 mM (for XTT HUVEC)
	50 μM, 100 μM, 500 μM, 1 mM (for XTT Jurkat)
	100 μM (for other experiments except XTT)
PAL	Same as PAC
TMP	88.5 μM
Dhpl	50 μM
Vitamin E	50 μM
H_2O_2	2 mM and 100 μM

2.3.3. Cell viability Assay (XTT Assay)

For viability assays, cells were seeded into 96-well plates at a density of 7500 cells/well in 2% serum medium. By the following day, cells had reached 80% confluence and were washed with serum-free medium. Various concentrations of PAC / PAL as indicated were applied to the cells either 2 hours before TNF-α treatment (10ng/ml) or simultaneously. PAC/PAL was either left on the cells during TNF-α treatment or washed away before. For MG132 treatment, MG132 (5 μg/ml) was applied 30 min before PAC/PAL addition. After cell treatment, 50μl of the XTT labeling mixture (final XTT concentration 0.3mg/ml), prepared as manufacturer's description, was added to each well. The microtiter plate was incubated for 4 h in a humidified atmosphere. The spectrophotometrical absorbance of the samples was measured using a ELISA reader. The wavelength to measure absorbance of the formazan product was between 450 and 500 nm.

2.3.4. Morphology

NF-κB inhibitory peptide is a cell-permeable peptide which carries a functional cargo representing the nuclear localization sequence of NF-κB p50. It can be applied to inhibit nuclear translocation of NF-κB in a concentration-dependent manner [Lin YZ, 1995]. 45,000 Huvec cells per slide were plated onto 4-well slide coated with 0.2% gelatin and allowed to attach overnight. At the following day, the nearly confluent cells were pre-incubated with PAC (100 μM) for 2 hours and then exposed in TNF-α (10 ng/ml) in the presence of ActD (1 μg/ml) for another 2 hours. NF-κB inhibitory peptide (50 μg/ml) was added to the cells 15 min prior to PAC stimulation. Morphology of the cells was analyzed under contrast microscope.

2.3.5. Transformation

Bacterial transformation is the process by which bacterial cells take up naked DNA molecules or plasmid DNA. If the foreign DNA has an origin of replication recognized by the host cell DNA polymerases, the bacteria will replicate the foreign DNA along with their own DNA. When transformation is coupled with antibiotic selection techniques, bacteria can be induced to uptake certain DNA molecules, and those bacteria can be selected for that incorporation. Bacteria which

are able to uptake DNA are called "competent" and are made so by treatment with calcium chloride in the early log phase of growth. The bacterial cell membrane is permeable to chloride ions, but is non-permeable to calcium ions. As the chloride ions enter the cell, water molecules accompany the charged particle. This influx of water causes the cells to swell and is necessary for the uptake of DNA. The exact mechanism of this uptake is unknown. It is known, however, that the calcium chloride treatment can be followed by heat. When E. coli are subjected to 42°C heat, a set of genes are expressed which aid the bacteria in surviving at such temperatures. This set of genes are called the heat shock genes. The heat shock step is necessary for the uptake of DNA.

100 µl competent bacteria were combined with 0.3 ~1 µl plasmid (NF-κB -luc plasmid: 1.56 µg/µl, pRL-TK plasmid: 1.99 µg/µl) and first incubated for 30 min on ice and then incubated at 42°C for 90 seconds. After the heat shock step, the mixture was resuspended in 1 ml LB and incubated at 37°C for 30~60 min in a shaking incubator. The transformed mixture was centrifuged at 3000 U/min for 5 min. The appropriate volume of the pellet suspension was spreaded gently over the surface of LB- ampicillin –agar plate and incubated over night.

2.3.6. Mini-Prep

For the isolation of plasmid-DNA the standard alkaline lysis purification method was used [Sambrook J, 1989]. The standard method for the miniprep isolation of plasmid DNA includes the same general strategy as the large scale isolation. Generally, smaller aliquots of antibiotic containing liquid media inoculated with plasmid-containing cell colonies are incubated in a 37°C shaker for 12-16 hours. After collecting the plasmid containing cells by centrifugation, the cell pellet is resuspended in a hypotonic sucrose buffer. The cells are successively incubated with an RNAase-lysis buffer, alkaline detergent, and sodium acetate. The lysate is cleared of precipitated proteins and membranes by centrifugation, and the plasmid DNA is recovered from the supernatant by isopropanol precipitation.

To perform mini-prep, a single Bacterial colony was transferred into 5 ml LB-ampicillin medium in a loosely capped 15 ml tube and incubated overnight at 37°C with vigorous shaking. 1.5 ml of

the culture was centrifuged at 12000 g for 5 min. The bacterial pellet was resuspended in 100 µl ice-cold Solution I and incubated no longer than 5 min on ice. 200 µl of freshly prepared Solution II was added by inverting the tube rapidly 5 times. 150 ml Solution III was immediately added and the tube was vortexed for 10 seconds, and then stored on ice for 3~5 min. The contents were centrifuged and the proteins in the supernatant was extracted with an equal volume of phenol:chloroform (1:1). 900 µl ethanol was used to precipitate the plasmid DNA. The dry DNA pellet was dissolved in 10 µl H_2O with RNAase (100 µg/ml).

2.3.7. Maxi-Prep

Large-scale preparations of plasmid DNA was preformed by using Maxi-Prep-Kit from Macherey-Nagel (Dueren). The procedure was following the manufacturer's description. The concentration of the plasmid DNA was determinated by measuring its OD_{260} value.

2.3.8. Gel electrophoresis to analyze plasmid DNA

To analyze the DNA by cleavage with restriction enzymes, 1 µl of the DNA solution was removed from Mini /Maxi-prep and added to a fresh microfuge tube containing 1 unit of the desired restriction enzyme, 1µl of the 10x restriction enzyme buffer and water. The total volume was 10 µl in the microfuge. The reaction was incubated for 1 h at 37°C, and then loaded in a 0.8% Agarose gel. Electrophoresis was carried out at 120 V for 45 min. At the end of the run, the gel was removed from the tank and soaked in ethidium bromide solution for 20 min staining and then analyzed.

2.3.9. Transient transfection

2x 10^6 cells were transiently transfected with 5µg NF-κB -luc plasmid using the electroporation method according to the manufacturer's instructions. Briefly, the cells were harvested and centrifuged at 300g x 10 min. The cell pellet was resuspended in 100 µl PBS to a concentration of 2x 10^7 and kept on ice. Each 2x 10^6 cells were combined with 5 µg NF-κB -luc plasmid and

0.2 µg pRL-TK as a control for transfection efficiency in 100 µl solution B, then the sample was transferred immediately into a 2 mm cuvette. The cuvette was inserted into the cuvette holder and program 3A was applied. After transfection, the cuvette was immediately rinsed with 400 µl complete medium. Then 2x 10^6 transfected cells were divided into 2 wells of a 6-well plate. After transfection the cells were cultivated for 24 hours and then 100 µM PAC was applied for various periods of time. The cells were harvested by incubation in passive lysis buffer. Firefly and Renilla luciferase activities were determined using the DualLuc substrates.

2.3.10. Preparation of whole cell lysates

$4x10^5$ Huvec or $1x10^6$ cells grown in T-25 flasks were treated with reagents for the times indicated, washed twice with ice-cold PBS, and harvested in 100µl RIPA cell lysis buffer containing 50mM Tris-HCl, 150mM NaCl, 1% Nonidet P40, 0.5% Natriumdesoxycholat, 0.1% SDS and protease-inhibitor-cocktail tablet. The cells were incubated for 15 min on ice and then centrifuged at 15,000 g for 10 min. The supernatant was recovered. Protein concentration was determinated by using BCA protein assay kit.

2.3.11. Western blotting

8 µg per each lane of RIPA-cell lysate was separated on a 4-12% SDS-PAGE gradient gels and subsequently blotted onto a PVDF membrane. After blocking for 1 hour with 10% nonfat dry milk/ PBS, the membrane was incubated for 16 hours with the specific antibody against IkB-α (1:1000). Then the membrane was washed three times in PBS, incubated for 1 hour with an alkaline phosphatase-coupled secondary antibody (anti-rabbit) and then washed again. For detection NBT / BCIP staining was used.

2.3.12. EMSA

2.3.12.1. Preparation of nuclear lysates

To analyze nuclear DNA-binding proteins such as transcription factors, nuclear protein need to be isolated from the cells. After cell stimulation, the cells were collected by scraping from T-75 flasks and washed 2 times with PBS. The cells were then added with 400 µl Lysis buffer I and incubated for 15 min on ice. 25 µl 10% NP-40 were added to the cells and mixed by short vigorous shaking. The cells were then centrifuged (10000 U/min, 2 min) and the pellet was resuspended with 50µl lysis buffer II and incubated with shaking for 15 min at 4°C. Finally the contents were centrifuged at 4°C 13000 U/min for 10 min and the supernatant containing nuclear protein (1-2 µg/µl) was aliquoted. Nuclear extracts were either used immediately or stored at –70°C. The protein content of the extract was measured by using BCA protein assay kit.

2.3.12.2. EMSA

DNA-binding proteins can be measured quantitatively with the electrophoretic mobility shift assay (EMSA), which also is referred to as the gel-shift or band-shift assay. In this assay, the electrophoretic mobility of a radiolabeled DNA fragment is determined in the presence and absence of a sequence-specific DNA-binding protein. Protein binding generally reduces the mobility of a DNA fragment, causing a shift in the location of the fragment band detected by autoradiography.

Gel shifts were performed as described previously [Bosserhoff AK, 1996]. 1 ng (10000cpm) ^{32}P-end-labeled double –stranded NF-κB oligonucleotide was incubated with 4-6 µg nuclear extract, 1 µl ploy-dIdC and 4 µl 5xEMSA buffer in room temperature for 10 min. Samples were loaded in a 6% native PAA gel. Competition experiments were performed using 50 fold excess of the unlabeled binding site, for supershifting experiments anti-p50 and anti-p65 antibodies were used. The gel was dried by Whatman-paper and autoradiographed over night.

2.3.13. Flow Channel Assay

2.3.13.1. Chinese medicine compounds

Tetramethylpyrazine (ligustrazine, TMP) is the active constituent of the Chinese herb Ligusticum wallichii Franchat. TMP used in this study was available as injection ligustrazini hydrochloride (20 mg/ml, sterile injection, M: 226 g/mol, Fourth Pharmaceutical Co., Beijing, China). TMP (20 mg/l equals 88.5 μM) was dissolved in HEPES buffer and it did not affect the osmolarity of the buffer.

Dhpl (3,4-dihydroxyphenyl lactate, M: 197.17 g/mol) is a water-soluble component of RSM. Dhpl was synthesized by Shanghai Medical University, Dept. of Pharmacology and purchased as sterile injection (10 mg/ml). The purity was higher than 95% as quantitated by spectrum analysis. To study its effect on RBC-EC adhesion, a concentration of 50 μM Dhpl was chosen.

2.3.13.2. Antioxidative capacity of Tetramethylpyrazine to RBC

Blood and RBC monolayer preparation

About 100 μl of blood was withdrawn from the finger tips or ear lobe of young healthy donors with heparinized micropipettes and dispersed in 10 ml of HEPES. RBC were harvested after centrifugation at 3500 rpm for 10 min. The RBC sediment was withdrawn and washed for three times with HEPES. A 20 μl aliquot was re-suspended in 50 μl HEPES. 20 μl of this final RBC suspension were pipetted onto the bottom of the flow chamber and allowed to settle for 10 minutes. After flushing away non-adhered RBC with HEPES+, the RBC monolayer preparation was ready for use.

Apparatus

The automated Elias-c-analyzer (Fig. 5) consists of a microscope, a computer-controlled pump, a flow chamber and a microprocessor unit. The stainless steel flow chamber forms a flow channel with a rectangular cross-section (width, 14mm; length, 55 mm). The assembly of the chamber forms a flow channel with a height of 0.228 ± 0.009 mm. A pulsatile reduced suction pump is

controlled by computer and produced a stationary maximum wall shear stress of 3 Pa. For relaxation time measurements, flow stoppage is accomplished within 10ms by closure of a micro-valve [Artmann GM, 1995 and 1996].

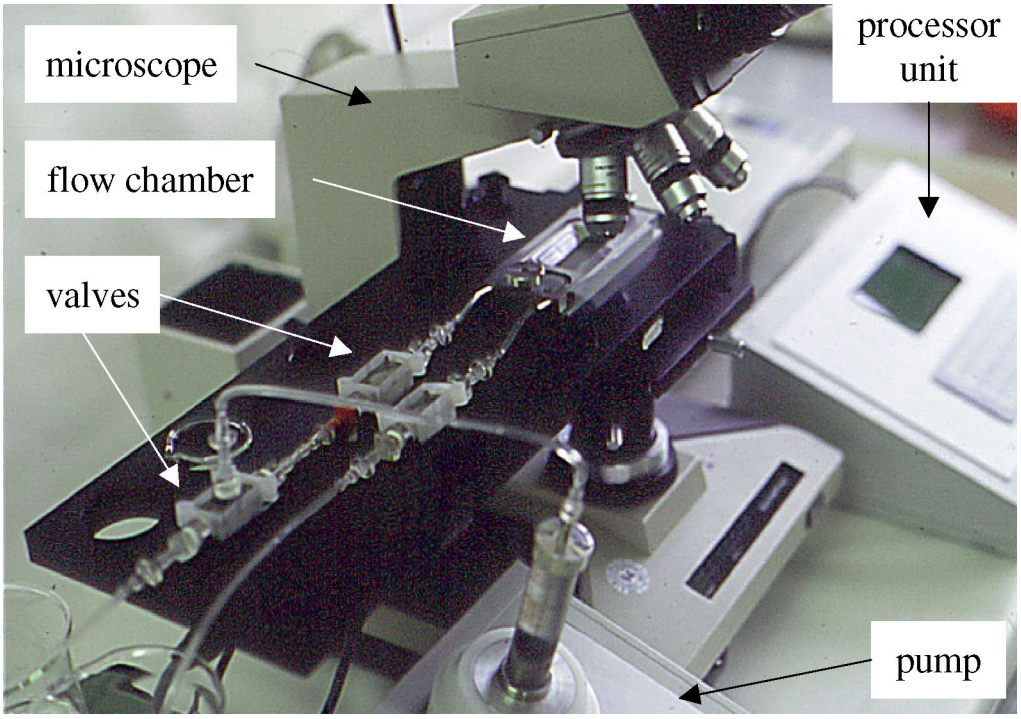


Fig. 5 Original photograph of the automated Elias-c-analyzer based on the Microscopic Photometric Monolayer Technique.

RBC stiffness and relaxation time measurements

RBC stiffness (in Pa) is defined as the ratio of 0.25 Pa wall shear stress to RBC elongation at 0.25 Pa. The relaxation time (in ms) is calculated from the dynamic shape recovery curves after flow stoppage. The relaxation time is taken when the photometrical signal has declined to 50% of the previous RBC elongation at 2.0 Pa shear stress [Artmann GM, 1995].

Time sequence and experimental conditions

RBC stiffness and relaxation time were recorded subsequently with the same RBC mono- layer for each donor. The total investigation time per monolayer was 72 min. Exposure times and sequences of exposure to H_2O_2 and TMP, respectively, are given in Fig. 6.

total time	2’	22’	42’	72’
time per step	2 min	20 min	20 min	30 min
controls	HEPES++	HEPES++	HEPES++	HEPES++
H_2O_2 (2 mM)	HEPES++	H_2O_2	HEPES++	HEPES++
TMP (88.5µM)	HEPES++	H_2O_2	TMP	TMP

Fig.6 Time protocol and steps of exposure to hydrogen peroxide and TMP, respectively.

2.3.13.3 Effects of Dhpl and Vitamin E on RBC-EC adhesion

Dhpl and Vitamin E preparation and EC treatments

Hydrogen peroxide (H_2O_2, 30%) and +-α-Tocopherol acetate (Vitamin E) were purchased from Sigma. Vitamin E was first dissolved in 98% ethanol as stock solution, with a final ethanol concentration of 0.1% in culture medium. Vitamin E and Dhpl were freshly prepared at 50 µM in cell culture medium prior to use.

Pre-treatment studies: EC were first incubated with 50 µM Dhpl or 50 µM Vitamin E, respectively, for 1 h at 37°C at 5% CO_2 and then activated by 100 µM H_2O_2 for 30 min.

Post-treatment studies: EC were first activated with 100 µM H_2O_2 for 30 min and then incubated with 50 µM Dhpl or 50 µM Vitamin E, respectively, for 1h.

Before adhesion studies were carried out, the EC were gently rinsed for three times with DMEM without FCS.

RBC preparation and treatment

Blood samples were withdrawn from the fingertip of healthy human donors. The RBC were centrifuged, washed 3 times with DMEM and resuspended in DMEM (30% hematocrit). Before the adhesion test, the RBC were treated with 100 µM H_2O_2 for 30min.

Evaluation of RBC – EC adhesion

The EC which were cultured on microslides, were placed into a flow chamber. 20 µl of H_2O_2 treated RBC suspension was pipetted on top of the EC monolayer covering the EC layer. RBC were allowed to settle at no flow for 15 min onto the EC within the closed flow chamber. Subsequently, a wall shear stress of 0.04 Pa was applied for 5 min to wash away non-adherent RBC. The remaining RBC were counted in random fields of the EC monolayer. RBC-EC adhesion was tested at three different modes of exposure to H_2O_2:

1) only RBC were treated 2) only EC were treated 3) both EC and RBC were treated.

3. Results

3.1. Analysis of PAC on TNF-α induced apoptosis

Acute or chronic ischemia resulting from cardio- and cerebrovascular diseases leads to apoptotic cell death. Since anti-apoptotic therapies have been proposed to limit tissue damage, we have therefore investigated whether PAC, a component of the Chinese Herb RSM, could potentially protect cells from apoptosis.

3.1.1. PAC blocks TNF-α induced cytotoxicity

TNF-α is one of the most potent inducers of apoptosis [Rath PC, 1999]. Whether PAC modulates TNF-α induced apoptosis is investigated. In the presence of ActD (1 μg/ml), TNF-α (10 ng/ml, 16 hours) profoundly sensitized Huvec cells to apoptosis, whereas 2 hours pretreatment with PAC partially protected the cells from death (Fig. 7).

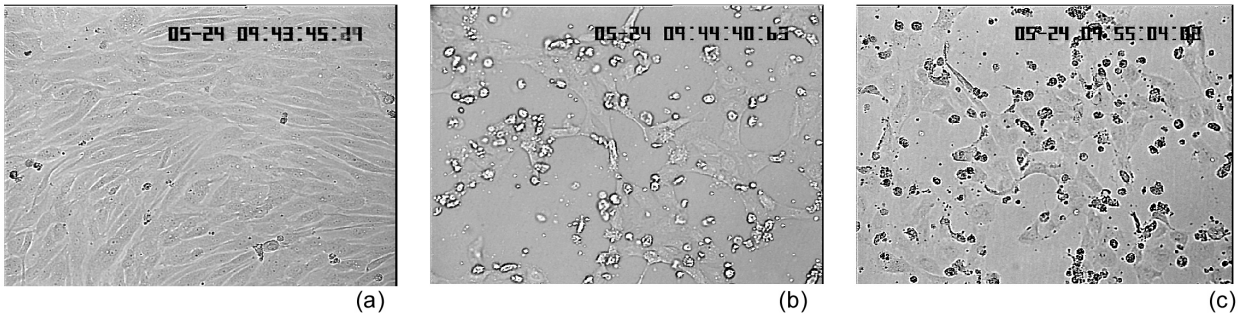


Fig. 7 Morphological analysis of the effect of PAC on TNF-α induced apoptosis in Huvec and Jurkat cells. Pretreatment of Huvec cells with PAC increased resistance to cell death caused by subsequent treatment with TNF-α (10 ng/ml) in the presence of actinomycin D (1 μg/ml). (a) normal Huvec cells after 16 hours of incubation in culture medium (magnification, ×170). (b) confluent cells were treated with TNF-α for 16 hours in the presence of actinomycin D. (c) confluent cells were pretreated with 1 mM PAC for 2 hours followed by TNF-α for 16 hours in the presence of actinomycin D.

To quantify the number of cells undergoing apoptosis, the XTT dye reduction assay was performed (Fig. 8).

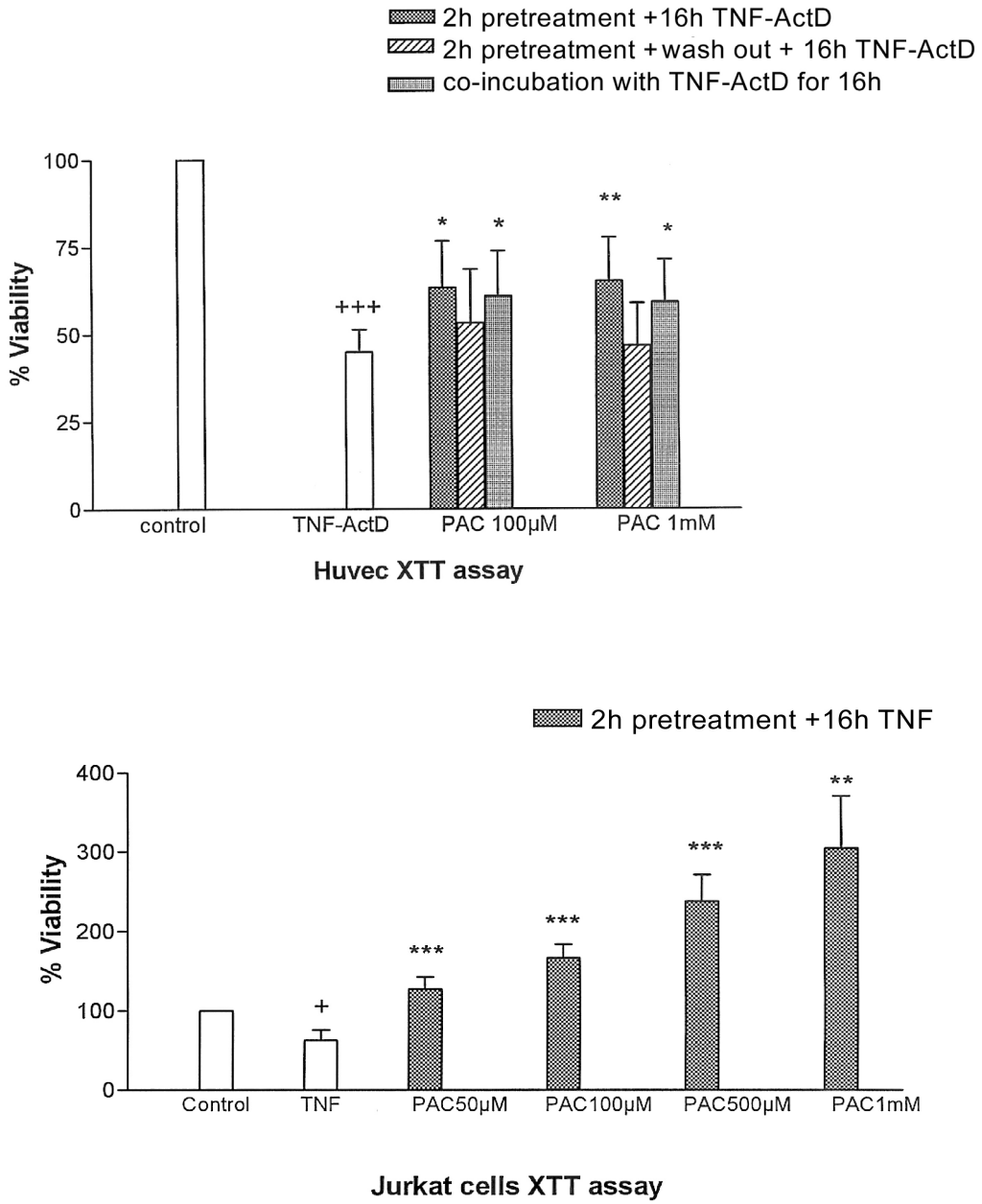


Fig. 8 Dose-dependent protection of Huvec cells (1) and Jurkat cells (2) from apoptosis was measured using XTT assays to analyze viability of the cells. PAC was applied to the cells 2 hours before TNF-α treatment (10 ng/ml) and either left on the cells during TNF-α treatment or washed away before or treated with PAC simultaneously with the TNF-α treatment. As a control non treated cells and cells treated with TNF-α alone were used. In the case of Huvec cells apoptosis was always induced by adding TNF-α together with actinomycin D (1 µg/ml). Data indicated the means of 6 and 4 individual experiments in Huvec and Jurkat cells, respectively, and each experiment was done in triplicate (+ vs. Control, * vs. TNF-α).

16 hours incubation of Huvec and Jurkat cells with TNF-α (10 ng/ml) resulted in 55% and 37% cell death, respectively. PAC was shown to inhibit TNF-α induced apoptosis in both cell types. The protection was seen when PAC was added 2 hours before or simultaneously with the TNF-α but not when PAC was washed from the cells after two hours before TNF-α treatment. Further, the effect on Jurkat cells was dose-dependent. Maximal protection by treatment with 1 mM PAC resulted in 44% more living cells in Huvec and 380% in Jurkat than TNF-α treated cells.

3.1.2. PAC activates DNA-binding ability of NF-κB proteins

Resistance to TNF-α cytotoxicity can be achieved through different signaling pathways [Guo YL, 1999]. Recently an activated form of NF-κB has been proposed to switch on transcription of yet unidentified anti-apoptotic genes and therefore in development of resistance to TNF-α [Natoli G, 1998[1]]. To analyze the molecular mechanism of PAC protection from apoptosis induced by TNF-α, activation of NF-κB was analyzed. The nuclear extracts from 100 μM PAC-treated Huvec and Jurkat cells were examined for DNA-binding ability by EMSA. The results revealed PAC to activate DNA-binding of NF-κB proteins (Fig. 9).

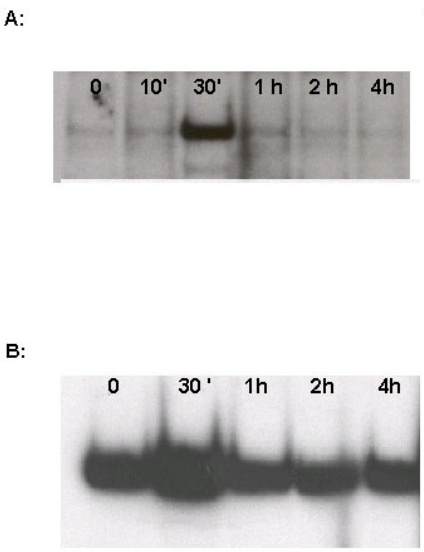


Fig. 9 EMSA analyzed DNA binding of NF-κB after PAC treatment. Huvec (A) and Jurkat cells (B) were treated with PAC (100 μM) for the indicated times. Then nuclear proteins were extracted and assayed versus a non PAC treated control.The amount of active NF-κB in the nucleus of the cells was assayed by binding to an oligonucleotide harboring the conserved NF-κB binding motif.

Maximal activation of NF-κB bandshift activity was seen within 30 min in both cell types. Supershift assays using antibodies to either the p50 or p65 subunits of NF-κB verified the specificity of the shifting NF-κB complex in both Huvec and Jurkat cells (Fig. 10).

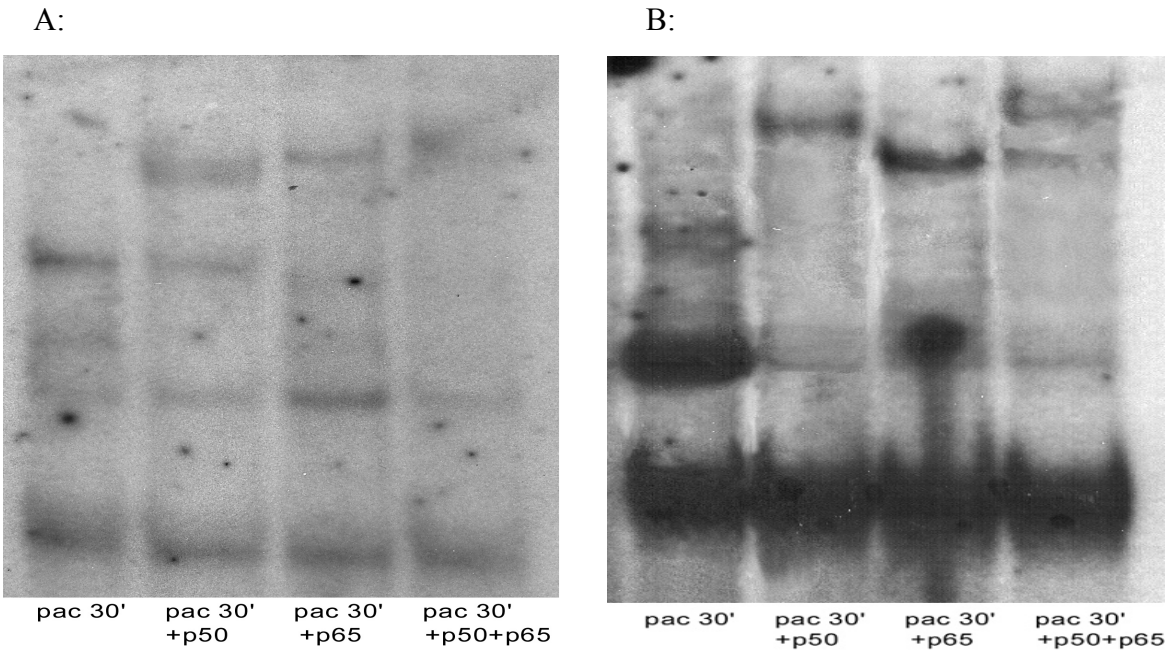


Fig.10 Supershift assay verified the specificity of the shifting NF-κB complex in both Huvec and Jurkat cells.

3.1.3. Activation of NF-κB controlled reporter gene by PAC

3.1.3.1 Purification of NF-κB luciferase reporter gene by Mini and Maxi prep

NF-κB luciferase reporter gene construct belongs to a *cis*-reporting system, which is designed for rapid assessment of the in vivo activation of many intracellular transduction pathways. Each *cis*-reporter plasmid contains the luciferase reporter gene driven by a basic promoter element (TATA box) joined to the tandem repeats of AP-1, CRE, SRE, NF-κB, p53 or SRF binding elements. Increased luciferase expression indicates either direct or indirect transcripitional activation (Fig. 11).

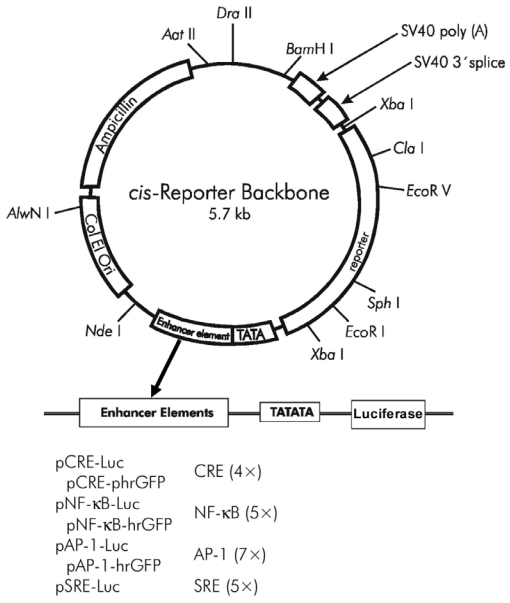


Fig.11 Diagram of cis-reporter backbone.

NF-κB *cis*-reporter plasmid was digested by XbaI after purification by Mini/ Maxi-prep. 2 bands (about 1.4 kb and 4 kb) appeared on 0.8% agarose gel. pRL-TK plasmid as a control of transfection efficiency was digested by Bgl II/ Hind III. 2 bands (760 bp and 3285 bp) appeared on 0.8% agarose gel (Fig. 12).

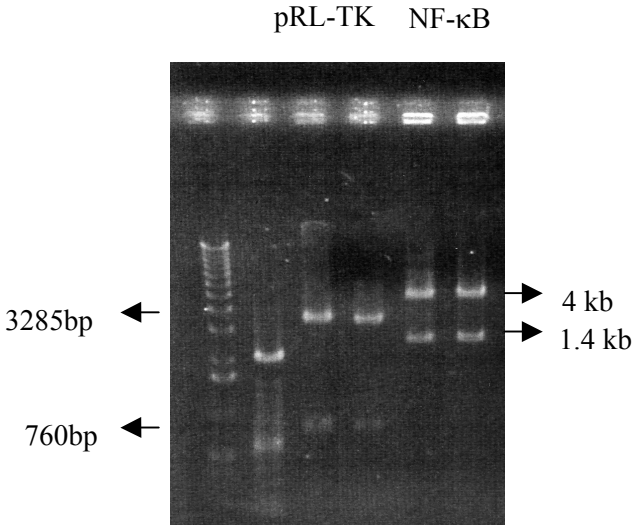


Fig. 12 Digestion of NF-κB *cis*-reporter plasmid on 0.8% agarose gel.

3.1.3.2. Effects of PAC on activation of NF-κB controlled reporter gene

NF-κB activation could be determined in luciferase assays using a trimerized NF-κB site controlling a luciferase reporter (Fig. 13).

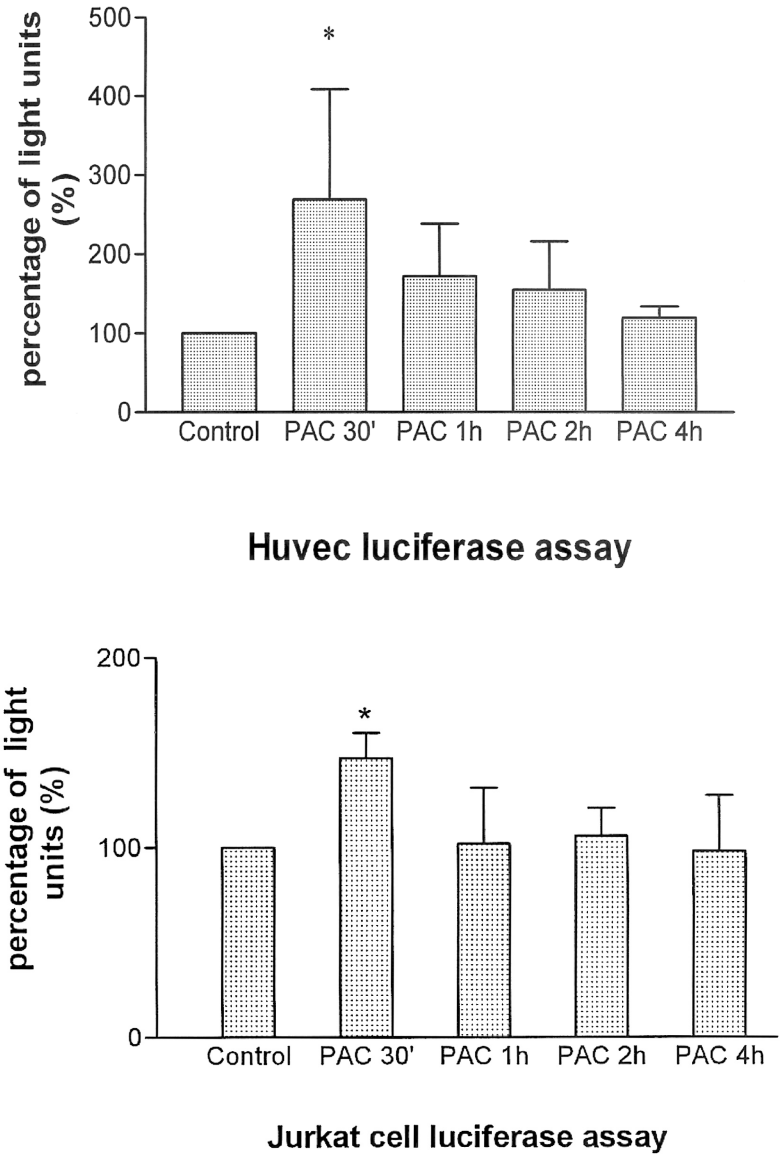


Fig. 13 Activation of NF-κB after PAC treatment was analyzed by luciferase assay. Cells (A: Huvec B: Jurkat cells) were transfected with 5μg NF-κB -luc plasmid using electroporation. As a control for transfection efficiency pRL-TK was used. 24 h after transfection, the cells were subsequently treated with PAC (100μM) for 30 min, 1 hour, 2 hours and 4 hours. Non PAC treated cells were used as control. Results show the means of duplicate in a single experiment and represent three separate experiments.

Cells were transiently transfected with a NF-κB dependent reporter, and the activity of NF-κB was then measured after PAC treatment. The results showed that the activity of NF-κB was significantly enhanced within 30 min of PAC stimulation.

3.1.4. Inhibitor of proteasome function suppresses the protective effect of PAC

The importance of NF-κB activation for PAC protection from apoptosis was further analysed by the use of MG132, a proteasome inhibitor. MG132 inhibits NF-κB activation by preventing degradation of IkB, the endogeneous inhibitor of NF-κB. Addition of MG132 in the experiments led to loss of PAC protection from apoptosis (Fig. 14A and 14B).

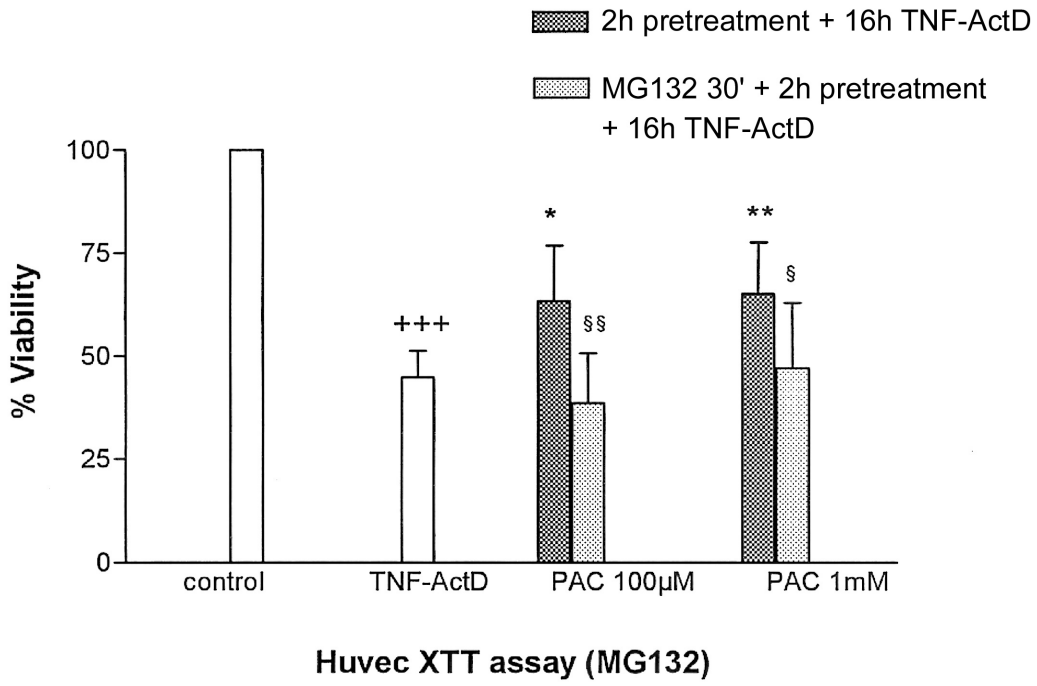


Fig. 14A Inhibition of NF-κB activation by MG132 suppressed PAC function in Huvec cells. The protective effect of PAC on TNF-α induced apoptosis was analyzed in the presence of MG132 (5 µg/ml). In XTT assays cell viability was measured after adding MG132 to the cells 30 min before PAC treatment. Viability was expressed as percentage of untreated cells. Data show the means of triplicate in a single experiment and represent 6 individual experiments (+: vs. Control, *: vs.TNF, $: vs. 2h pretreat+16h TNF).

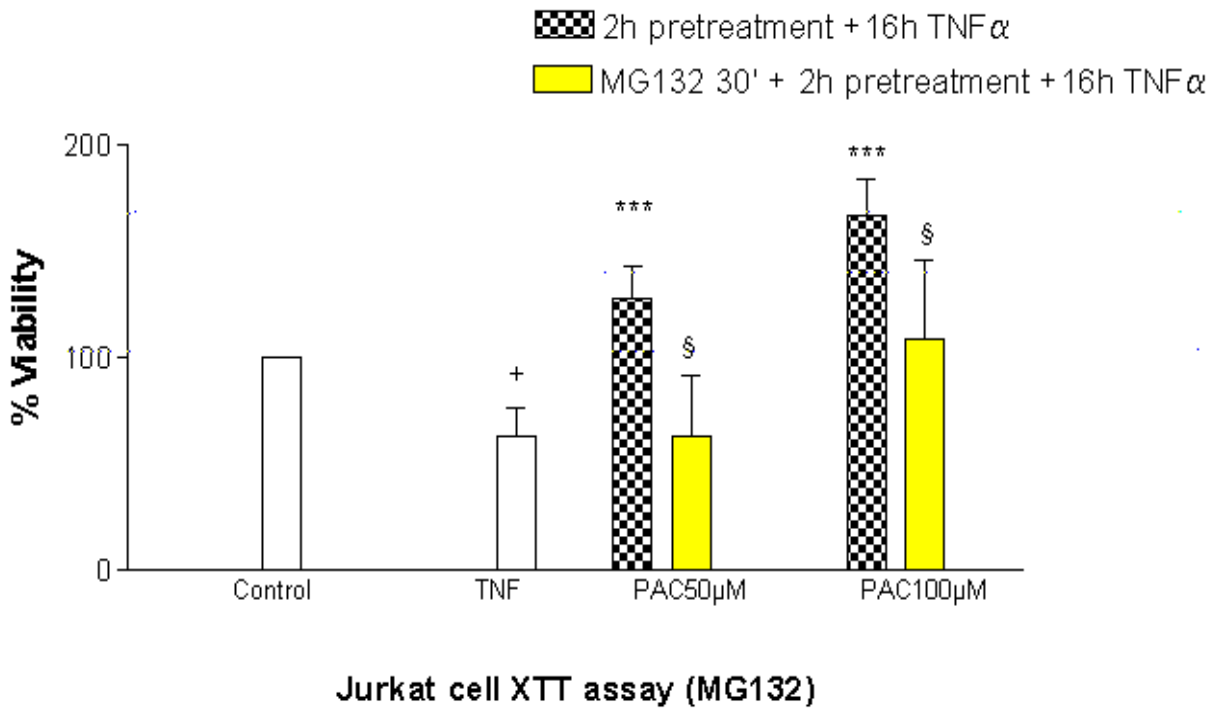


Fig. 14B Inhibition of NF-κB activation by MG132 suppressed PAC function in Jurkat cells. The protective effect of PAC on TNF-α induced apoptosis was analyzed in the presence of MG132 (5 μg/ml). In XTT assays cell viability was measured after adding MG132 to the cells 30 min before PAC treatment. Viability was expressed as percentage of untreated cells. Data show the means of triplicate in a single experiment and represent 4 individual experiments (+: vs. Control, *: vs.TNF, $: vs. 2h pretreat+16h TNF).

3.1.5. Inhibition of NF-κB activation by NF-κB inhibitory peptide blocks PAC function

NF-κB inhibitory peptide, a cell-permeable peptide bearing a functional domain of NF-κB p50 (Fig. 15), was reported to inhibite nuclear translocation of NF-κB in cultured endothelial cells stimulated with different agonists [Li YZ ,1995]. Therefore, we examined whether activation of NF-κB by PAC could be blocked by NF-κB inhibitory peptide.

^{1}AAVALLPAVLLALLAP**VQRKRQKLMP**^{26}

Fig.15 Sequence of NF-κB inhibitory peptide (single-letter amino acid code). The nuclear localization sequence of NF-κB p50 is printed in bold face.

The results indicated that NF-κB inhibitory peptide inhibited PAC protection from TNF-α induced apoptosis (Fig. 16). Taken together these results provided clear evidence that TNF-α induced apoptosis was not suppressed by PAC when NF-κB activation was inhibited.

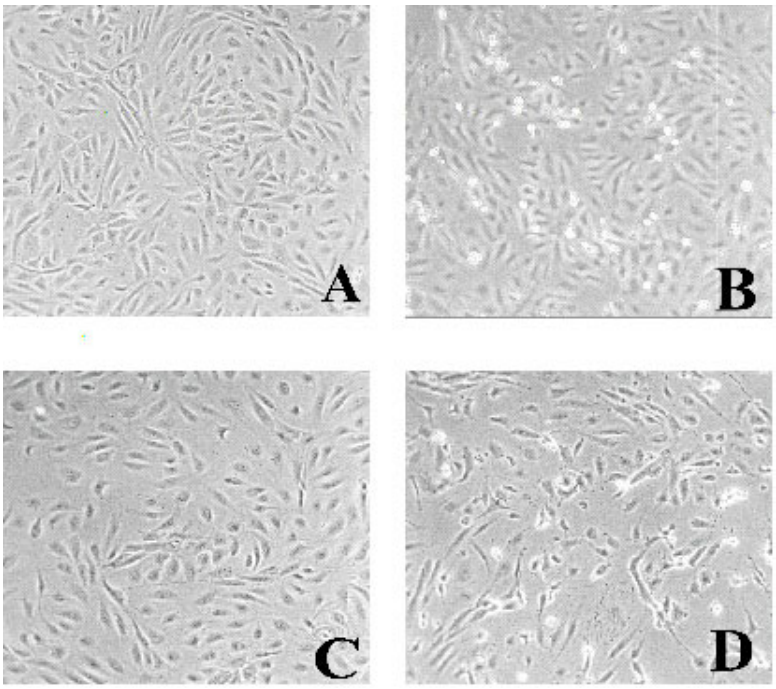
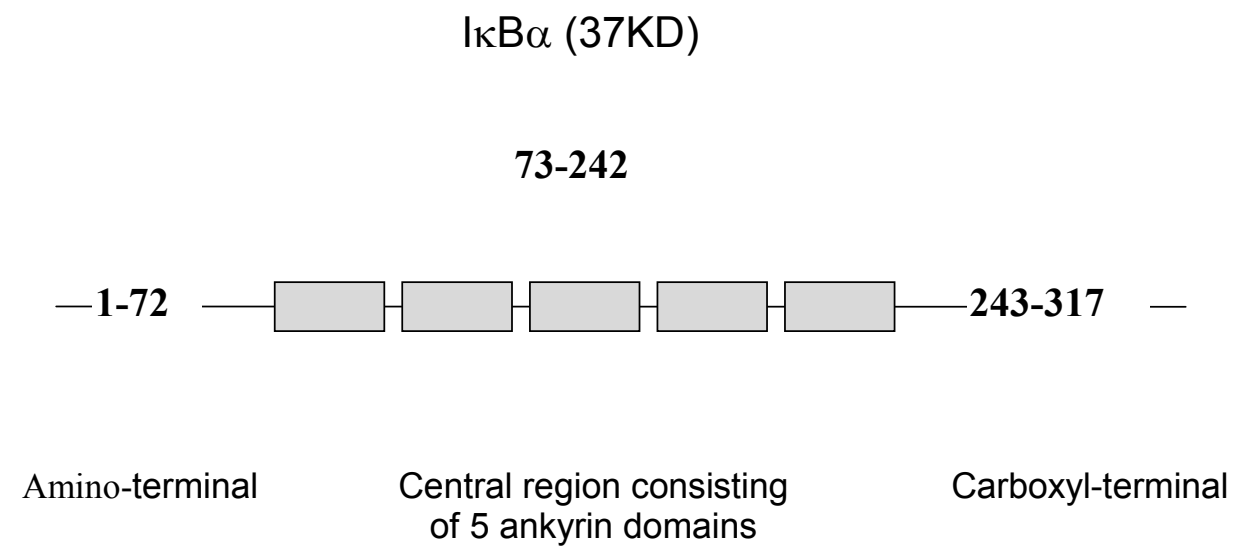


Fig. 16 Inhibition of NF-κB activation by NF-κB inhibitory peptide suppressed the anti-apoptotic effect of PAC. Nearly confluent Huvec cells were pre-treated with PAC (100μM) for 2 hours and then exposed to TNF-α (10 ng/ml) in the presence of actinomycin D (1 μg/ml) for 2 hours. NF-κB inhibitory peptide (50 μg/ml) was added to the cells 15 min prior to PAC stimulation. Morphology of the cells was analyzed under contrast microscope. (A): non treated Huvec. (B): Huvec cells were treated with TNF-α (10ng/ml) in the presence of actinomycin D (1 μg/ml) for 2 hours. (C): Huvec cells were pre-treated with PAC (100μM) for 2 hours and subsequently exposed to TNF-α (10 ng/ml) in the presence of actinomycin D (1 μg/ml) for 2 hours. (D): NF-κB inhibitory peptide (50 μg/ml) was added to the Huvec cells 15 min prior to PAC stimulation, and then the cells were treated as indicated for C.

3.1.6. PAC activates NF-κB through degradation of IkBα

Next we aimed to analyze the molecular pathway of NF-κB activation by PAC. Translocation of NF-κB into the nucleus is proceeded by phosphorylation and then ubiquitination-dependent degradation of IkB. To determine whether the protective action of PAC was due to an effect on IkBα degradation, Huvec and Jurkat cells were treated with 100 μM PAC for the times indicated and then subjected to immunoblotting. A 37 kD protein (Fig. 17) was readily detected in cytoplasmic extracts from unstimulated cells with peptide anti-serum raised against the carboxyl terminus of IkBα.



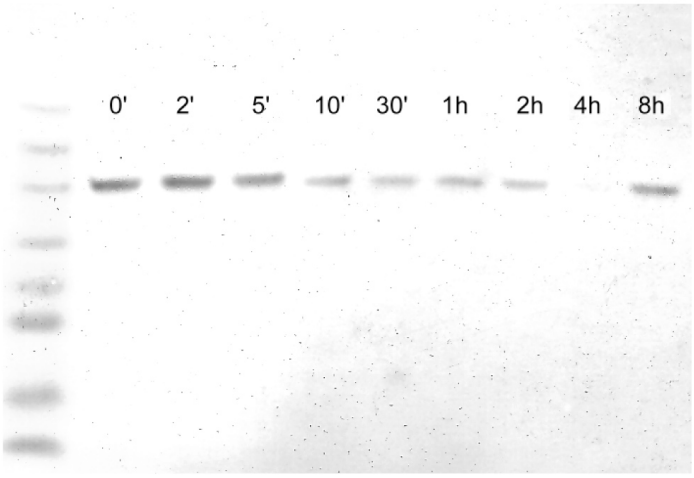
Origin single-letter amino acid code of IκBα

1	mfqaaerpqe	wamegprdgl	kkerllddrh	dsgldsmkde	eyeqmvkelq	eirlepqevp
61	rgsepwkqql	tedgdsflhl	aiiheekalt	mevirqvkgd	laflnfqnnl	qqtplhlavi
121	tnqpeiaeal	lgagcdpelr	dfrgntplhl	aceqgclasv	gvltqscttp	hlhsilkatn
181	ynghtclhla	sihgylgive	llvslgadvn	aqepcngrta	lhlavdlqnp	dlvsllkcg
241	advnrvtyqg	yspyqltwgr	pstriqqqlg	gltlenlqml	pesedeesyd	teseftefte
301	delpyddcvf	ggqrltl				

Fig. 17 Structure and sequence of IkBα.

Degradation of IkBα was noted within 10 min of PAC treatment and IkBα almost completely disappeared within 4 hours and 1 hour of PAC stimulation in Huvec and Jurkat cells, respectively. IkBα protein reappeared thereafter in the cytoplasm (Fig. 18). These findings correspond to the increase in DNA binding of NF-κB and suggest that PAC activates NF-κB by inducing IkBα degradation and thereby leads to rapid translocation of NF-κB from the cytoplasm into the nucleus.

A



B

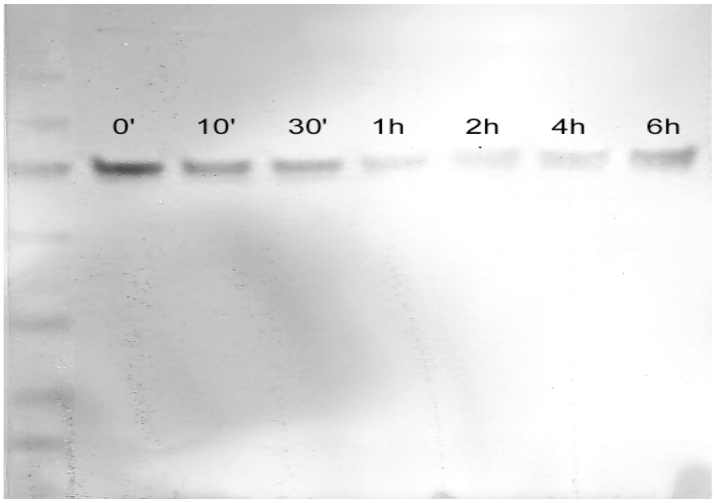


Fig. 18 IkBα degradation after PAC treatment was determined by western blotting. $4x10^5$ Huvec (A) and $1x10^6$ Jurkat cells (B) were treated with 100μM PAC for the time indicated and not treated as control. After treatment, the cells were harvested and whole cell extracts (8 μg/lane) were subjected to immunoblot for IkBα analysis.

3.2. Analysis of PAL on TNF-α induced apoptosis

In order to compare with PAC, another component of RSM – PAL was analyzed for its effect on TNF-α induced apoptosis. In in vitro XTT assay, PAL was shown to protect Huvec and Jurkat cells from TNF-α cytotoxicity (Fig. 19 A, B). In morphological studies, TNF-α profoundly sensitized Huvec cells to apoptosis in the presence of ActD, whereas 2 hours pretreatment of PAL effectively protected the cells from death (Fig. 20).

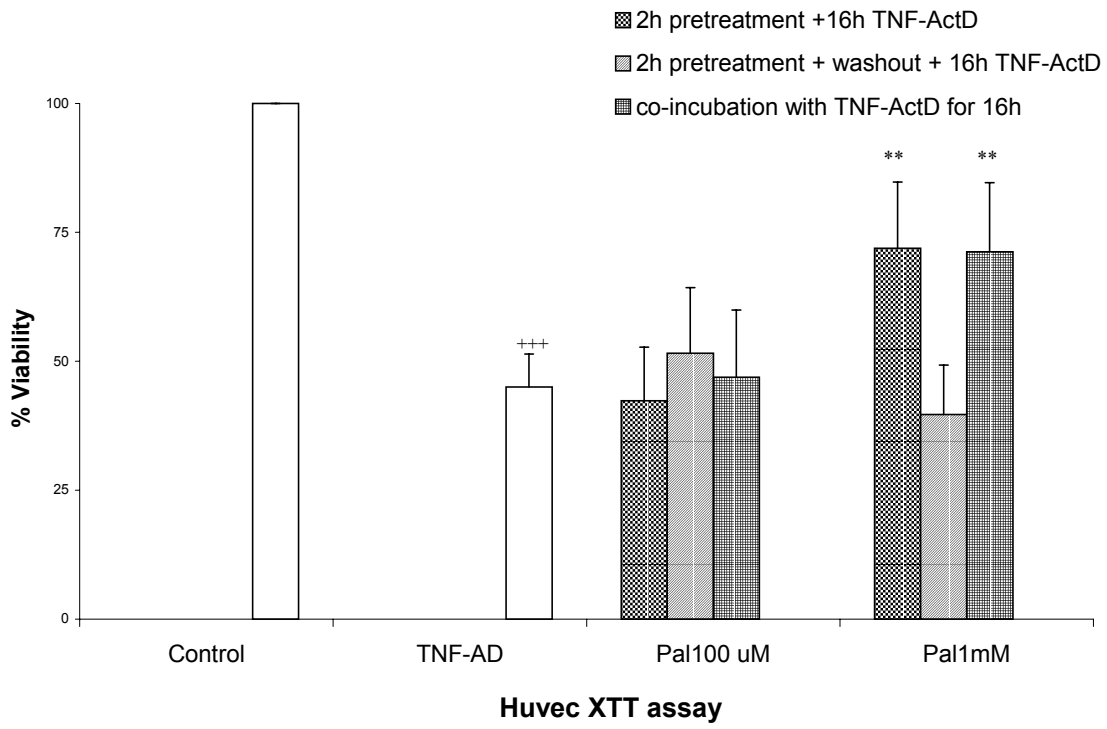


Fig. 19A Dose-dependent protection of Huvec cells from apoptosis was measured using XTT assay to analyze viability of the cells. PAL was applied to the cells 2 hours before TNF-α treatment (10 ng/ml) and either left on the cells during TNF-α treatment or washed away before or treated with PAL simultaneously with the TNF-α treatment. As a control non treated cells and cells treated with TNF-α alone were used. In the case of Huvec cells apoptosis was always induced by adding TNF-α together with actinomycin D (1 µg/ml). Data indicat the means of 6 individual experiments and each experiment was done in triplicate (+: vs. Control, *: vs. TNF-α).

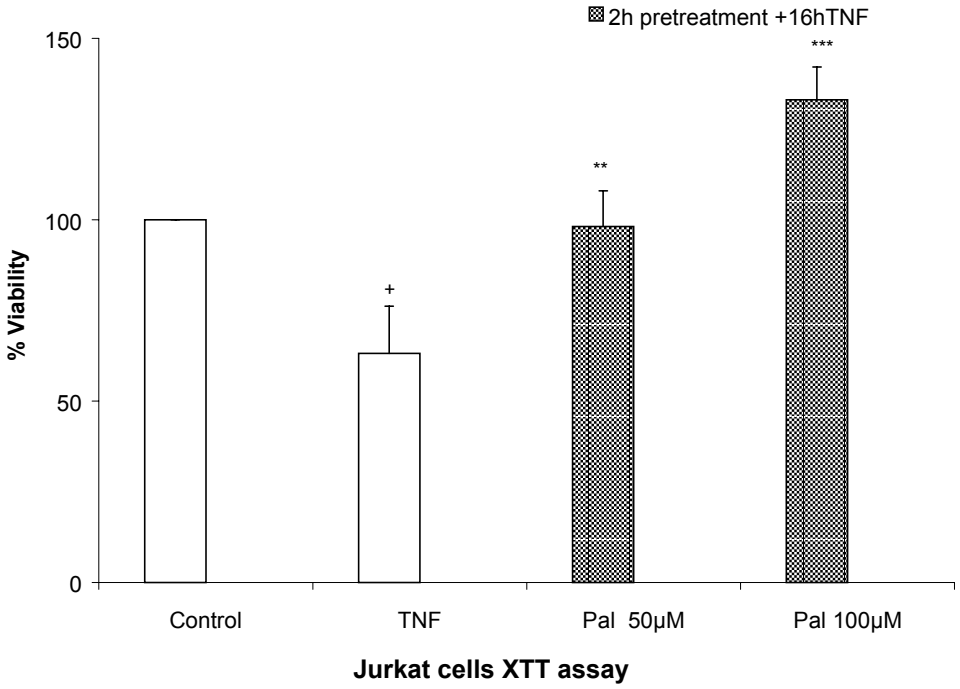
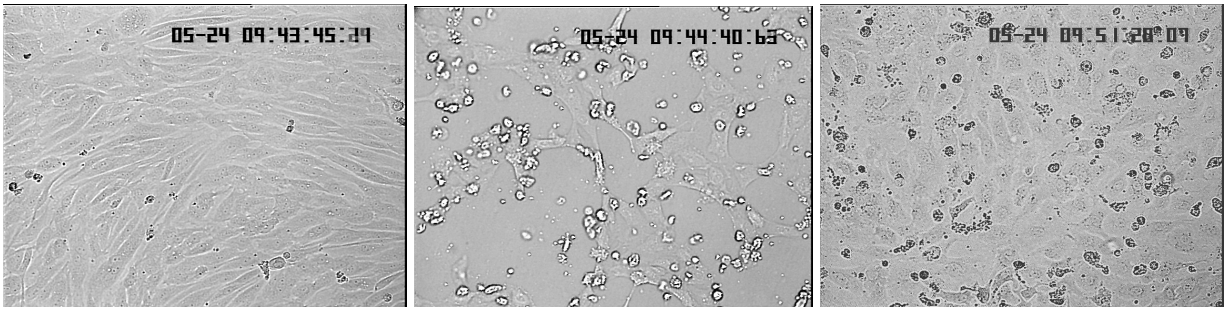


Fig. 19B Dose-dependent protection of Jurkat cells from apoptosis was measured using XTT assay to analyze viability of the cells. PAL was applied to the cells 2 hours before TNF-α treatment (10 ng/ml) and either left on the cells during TNF-α treatment or washed away before or treated with PAL simultaneously with the TNF-α treatment. As a control non treated cells and cells treated with TNF-α alone were used. Data indicate the means of 4 individual experiments and each experiment was done in triplicate (+: vs. Control, *: vs. TNF-α).



(a) (b) (c)

Fig.20 Morphological analysis of the effect of PAL on TNF-α induced apoptosis in Huvec cells. Pretreatment of Huvec cells with PAL increased resistance to cell death caused by subsequent treatment with TNF-α (10 ng/ml) in the presence of actinomycin D (1 µg/ml) (a) normal Huvec cells after 16 hours of incubation in culture medium (magnification, ×170). (b) confluent cells were treated with TNF-α for 16 hours in the presence of actinomycin D. (c) confluent cells were pretreated with 1mM PAL for 2 hours followed by TNF-α for 16 hours in the presence of actinomycin D.

However, the protective effect of PAL was not suppressed by MG132, the inhibitor of IkBα degradation (Fig. 21), suggesting a different regulatory mechanism.

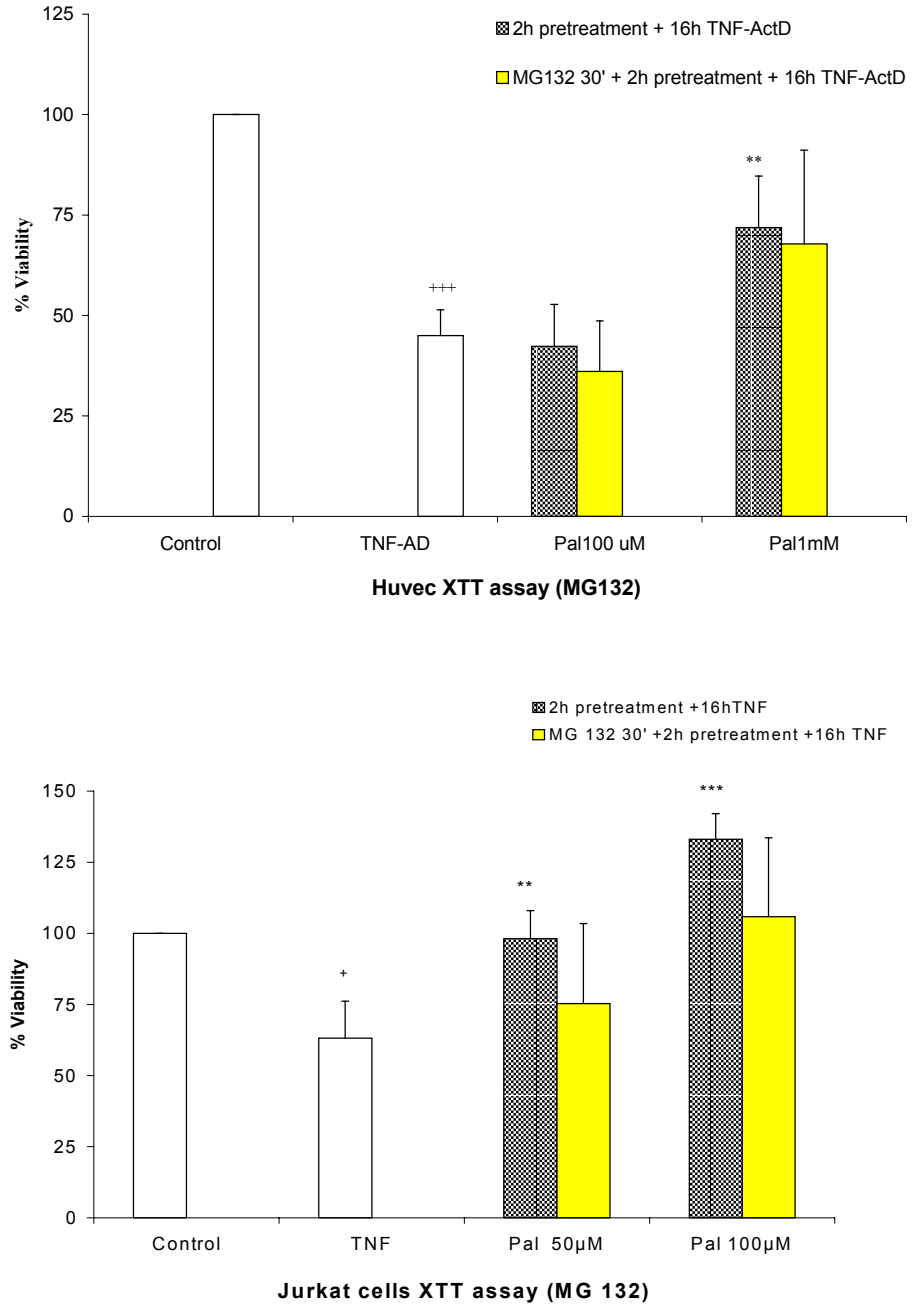
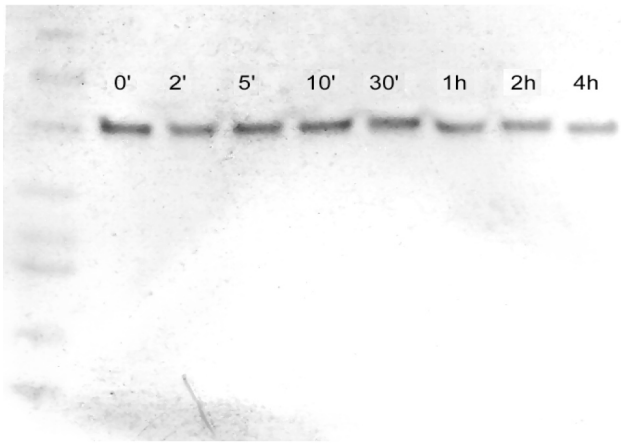


Fig. 21 MG132 did not suppress PAL protective effect. The protective effect of PAL on TNF-α induced apoptosis was further analyzed by the use of MG132 (5 µg/ml). In XTT assay cell viability (Huvec and Jurkat cells) was measured after adding MG132 to the cells 30 min before PAL treatment. Viability was expressed as percentage of untreated cells. Data show the means of triplicate in a single experiment and represent 6 and 4 individual experiments in Huvec and Jurkat cells, respectively (+: vs. Control, *: vs.TNF).

Furthermore, PAL did not induce IkBα degradation (Fig. 22), again suggesting a different mode of action.

A



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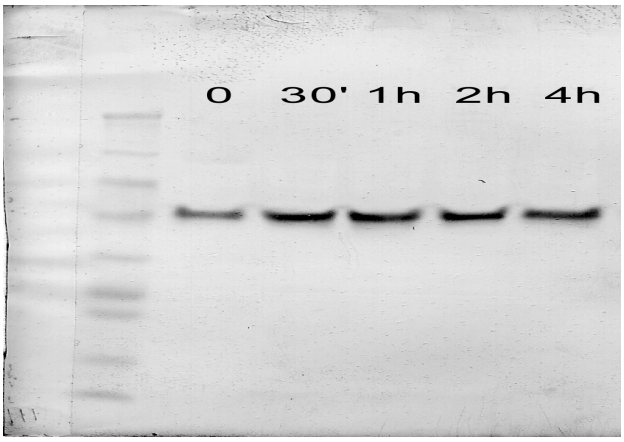

Fig. 22 PAL did not induce IkBα degradation. $4x10^5$ Huvec (A) and $1x10^6$ Jurkat cells (B) were treated with 100 μM PAL or not treated as control. After treatment, the cells were harvested and whole cell extracts (8 μg/lane) were subjected to immunoblot for analysis IkBα degradation.

Taken together these results suggested that the anti-apoptotic action of PAL may be medicated by other signaling pathways than activation of NF-κB.

3.3. Results of flow channel assay

3.3.1. Antioxidative capacity of Tetramethylpyrazine to RBC

When RBC were incubated with HEPES++ buffer over a total period of 72min (control group), no significant changes of stiffness and relaxation time, respectively, were observed (Fig. 23).

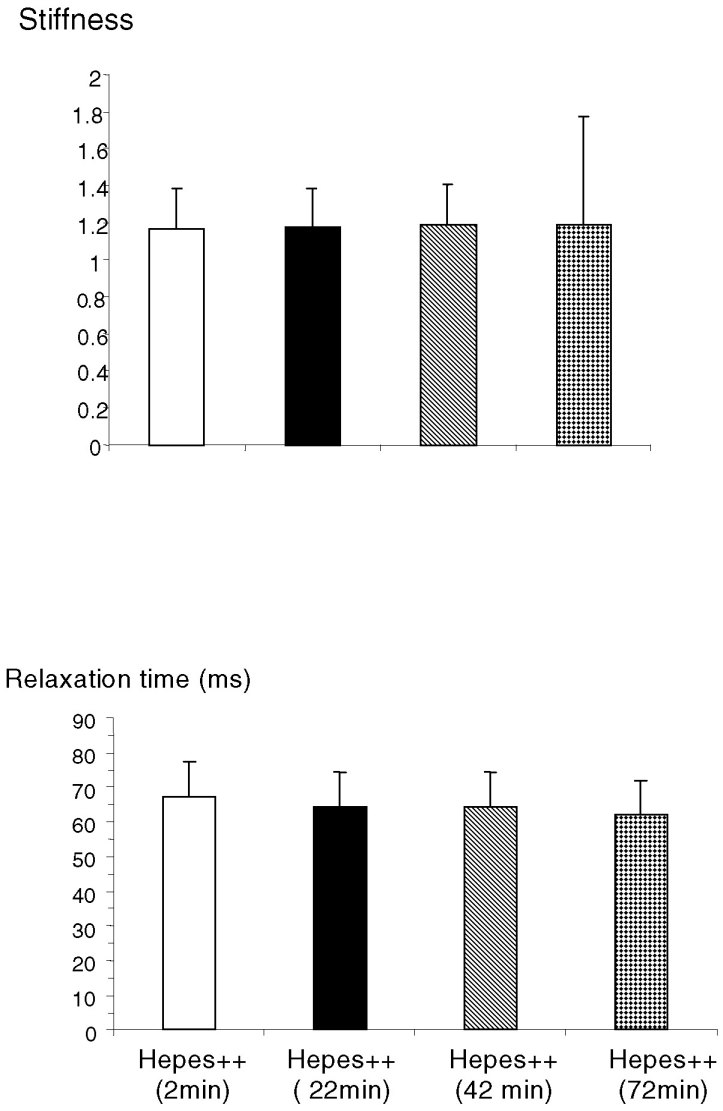

Fig. 23 The effect of HEPES++ buffer on RBC stiffness and relaxation time over 72 min (control group, N=6). No significant changes of stiffness and relaxation time were observed.

Previous experiments showed that RBC stiffness and relaxation time did not change due to exposure to HEPES++ with 88.5 µM TMP. Following the H_2O_2 treatment at 2 mM for 20 min, RBC became significantly stiffer and, at the same time, relaxation time was reduced as compared to control (Fig. 24). RBC stiffening (Fig. 24, upper panel). as well as relaxation time shortening (Fig. 24, lower panel), could not be reversed by subsequent re-incubation with HEPES++. These results indicated that the "damaging" effect of H_2O_2 on stiffness and relaxation time was irreversible within 50 min of re-incubation in HEPES++ .

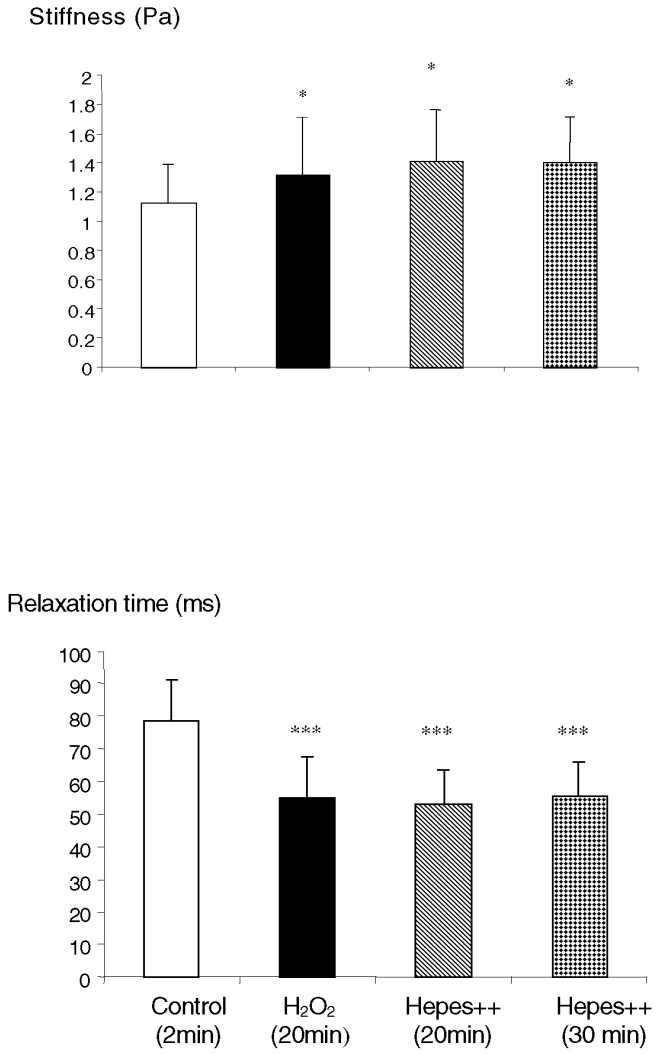

Fig. 24 RBC stiffness and relaxation time were markedly changed after exposing RBC to 2 mM H_2O_2 over a period of 20 min. Re-incubation of RBC in HEPES++ did not reverse the H_2O_2-induced changes in stiffness and relaxation time. (*: $p < 0.05$ vs. control, ***: $p < 0.001$ vs. control. n=13.).

TMP did not show any effect on RBC stiffness after the RBC had been pre-damaged with H_2O_2 (Fig. 25, upper panel). However, when "damaged" RBC were post-treated for 20 min with TMP, the hydrogen peroxide-induced reduction in relaxation time returned to control levels (Fig. 25, lower panel).

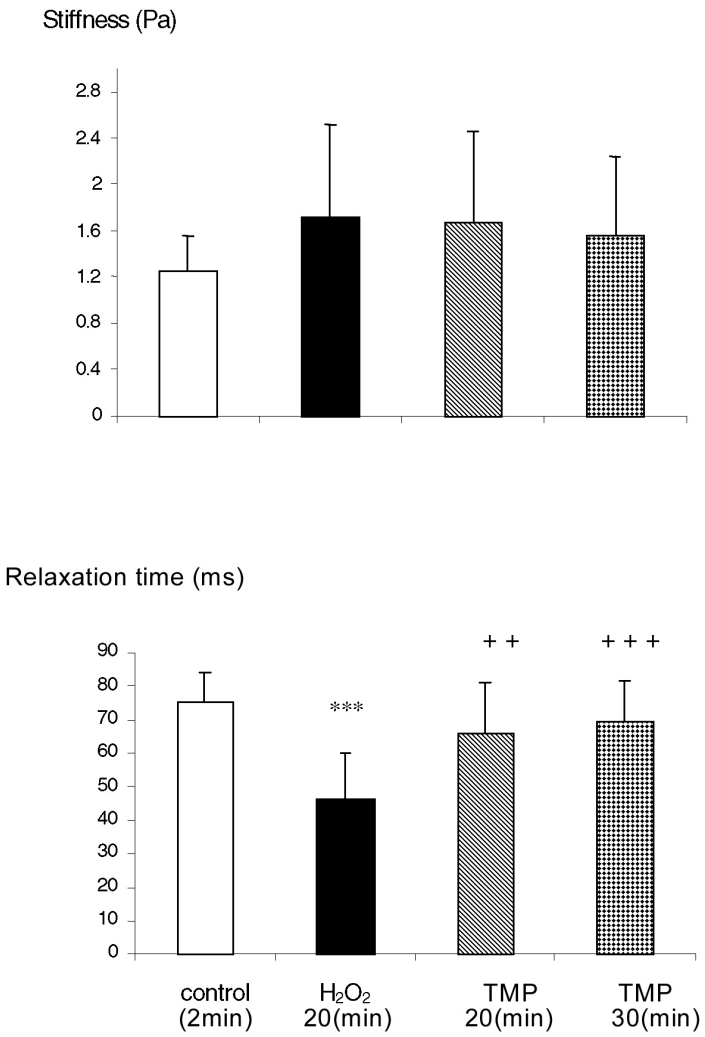

Fig. 25 Tetramethylpyrazine (TMP, 88.5 μM) effectively reverted H_2O_2-induced relaxation time shortening. However, it did not reduce H_2O_2-enhanced RBC stiffening (*: $p < 0.05$ vs. control, +: $p < 0.05$ vs. H_2O_2. n=12.).

3.3.2. RBC-EC adhesion tests

Prior to the red blood cells (RBC)-endothelial cells (EC) adhesion test, the effects of Dhpl on RBC stiffness and relaxation time were investigated (Fig. 26). When RBC were incubated with 1 mM Dhpl for 1 hour, only about 2% increase of stiffness was observed. Simultaneously, the relaxation time was 10% enhanced. Therefore in order to exclude the marginal effects, a concentration of 50 µM Dhpl was chosen in the RBC-EC adhesion studies.

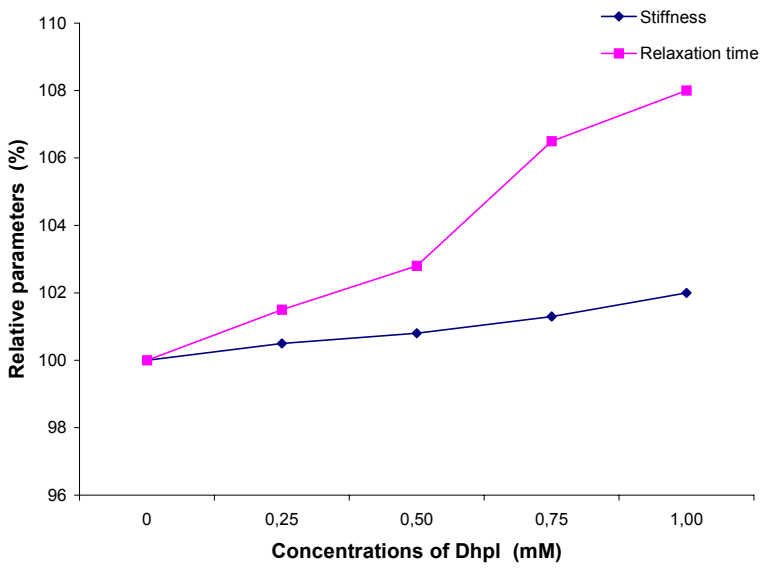

Fig. 26 The effect of Dhpl on RBC mechanical parameters (n=3).

In our RBC-EC adhesion studies, the efficacy of Dhpl to inhibit hydrogen peroxide induced damage leading to RBC-EC adhesion was compared with Vitamin E, a well-known antioxidant. When only RBC were exposed to H_2O_2, a significant RBC-EC adhesion was observed. In contrast, no enhanced adhesion was obtained when only EC were treated with H_2O_2. When both, EC and RBC, were treated with H_2O_2, the adhesion was most pronounced (Fig. 27).

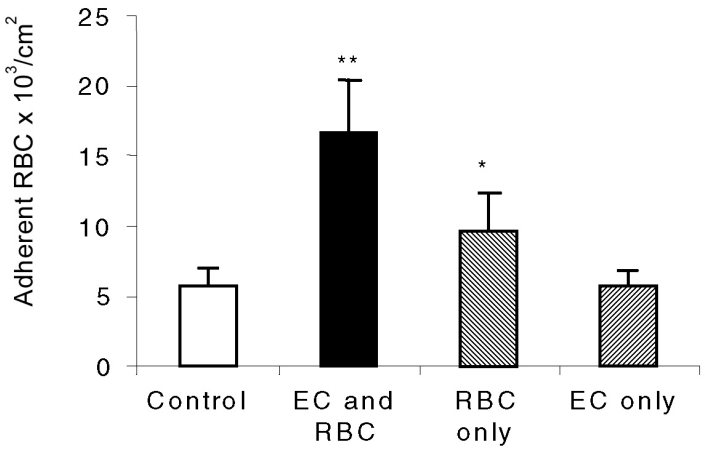

Fig. 27 RBC adhesion to Endothelial cells (control: untreated; EC and RBC: both RBC and EC were treated with H_2O_2; RBC only: only RBC were treated with H_2O_2; EC only: only EC were treated with H_2O_2). The adhesion was expressed as remaining number of RBC after applying a wall shear stress of 0.04 Pa for 5min to the EC monolayer. The adhesion was remarkably increased when both EC and RBC were treated with 100μM H_2O_2 for 30min. The data represent the mean of 5 assays (* $p<0.05$, ** $p<0.01$vs. control).

For further investigation of RBC-EC adhesion, both cell types were treated with H_2O_2. As shown in Fig. 28, Dhpl inhibited the H_2O_2 –induced RBC adhesion to EC, at about the same molar efficacy as Vitamin E. The extent of this inhibition was about the same at pre-treatment and post-treatment conditions.

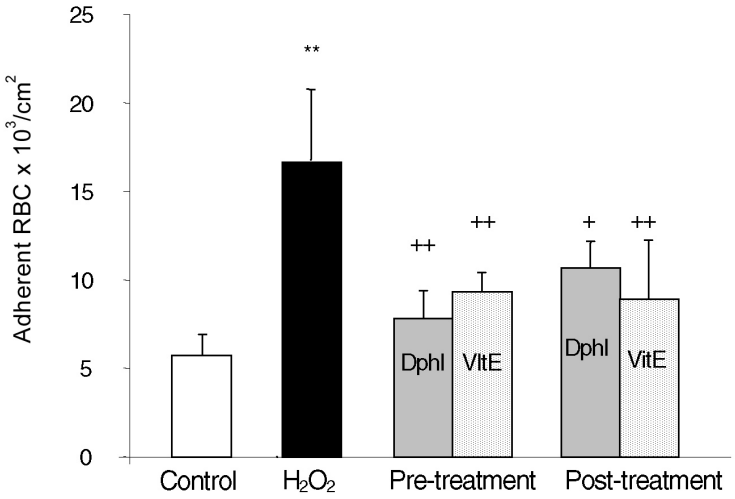

Fig. 28 Inhibitory effects of 50μM Dhpl or 50μM Vitamin E on H_2O_2 induced RBC-EC adhesion. Both RBC and EC were exposed to 100μM H_2O_2 for 30min. Dhpl and Vitamin E, respectively, were applied to EC only. Under pre-treatment conditions, the EC were incubated with Dhpl or Vitamin E, respectively, for 1h, prior to H_2O_2 treatment. Whereas under post-treatment conditions, the EC were incubated with these compounds for 1 hour after H_2O_2 treatment. The data represent the mean of 5 assays (**: $p<0.01$ vs. control, +: $p<0.05$, ++: $p<0.01$ vs. H_2O_2).

4. Discussion

New drugs to treat cardio- or cerebrovascular diseases are actually urgently needed. The step back by analysis of traditional treatments was started recently with high effort. The hope that drugs will be detected in herbs or extracts which are used for a long time in traditional medicine for example in china or rain forest populations is great. RSM, a traditional Chinese herb extract, has been widely used in Chinese community to treat cardio- or cerebrovascular diseases such as brain and myocardial infarction. In this study, we analyzed PAC and PAL, components of RSM, for their activities in protecting cells from apoptosis, and the antioxidative effect of TMP and Dhpl on red blood cells and endothelial cells.

4.1. Role of apoptosis in cardio- or cerebrovascular diseases

Two common vascular-disorders associated with cell death are myocaridal infarctions and stroke. These diseases arise primarily as a result of an acute loss of blood flow (ischemia). In both disorders, cells within the central area of ischemia appear to die rapidly as a result of necrosis. However, outside the central ischemic zone, cells die over a more protracted time period and morphologically appear to die by apoptosis [Cohen JJ, 1993]. Ischemia of both neurons and cardiac myocytes in culture results in the induction of apoptosis [Tanaka M, 1994 and Rosenbaum DM, 1994]. Agents known to be inhibitors of apoptosis in vitro have been shown to limit infarct size in these disorders [Uyama O, 1992]. So far, the most effective method of limiting infarct size is restoration of blood flow. Advances in medicine have developed a number of techniques to restore blood flow rapidly in acutely occluded blood vessels. Unfortunately, further tissue injury frequently occurs during establishment of reperfusion. Reperfusion is associated with acute increases in free radical production and increases in intracellular calcium, both potent inducers of apoptosis. The death of cardiomyocytes that occurs during reperfusion bears all the hallmarks of apoptosis [Gottlieb RA, 1994].

Our unpublished data in a rat model of focal cerebral ischemia indicated that RSM could induce a pharmacological preconditioning in the brain, thus reduced both ischemic brain injury and leukocyte infiltration following cerebral ischemia-reperfusion. The neuroprotective effect of RSM might be partially mediated via an ATP-sensitive potassium channel (K_{ATP})-linked mechanism. To our best knowledge, a very limited number of pharmacological agents have been reported to be able to induce pharmacological preconditioning in vivo. We believe that the preconditioning effects of RSM are not only limited to brain, but also to other organs or tissues. Based on the protective effects of RSM, we are interested to further investigate several components of RSM for their protective effect from apoptosis and try to elucidate the possible molecular mechanism of their anti-apoptotic effects.

4.2. Tumor necrosis factor alpha – mediator of apoptosis

TNFα is a cytokine mainly produced by activated macrophages and in smaller amounts by several other cell types. It was originally identified through its capacity to suppress hemorrhagic tumors in mice [Carswell EA, 1975]. After its isolation during the 1980s, considerable efforts were made to understand the molecular mechanisms of the various biological effects of TNFα [Aggarwal BB, 1984 and Pennica D, 1984]. In addition to its activity against transformed cells, TNFα exerts various effects on different normal cell types, including hepatocytes, myocardial cells, endothelial cells, lymphocytes, thymocytes, macrophages, polymorphonuclear neutrophils, nephrocytes, enterocytes, and Kupffer cells [Dayer JM, 1985; Gamble JR 1985; Leist M, 1994; Klosterhalfen B, 1997; Giardino I, 1998; Natoli G, 1998²; Fukuzuka D, 1999; Hase K, 1999; Messmer UK, 1999 and Niwa M, 1999], and it can elicit different biological responses [Xia P, 1999]. One of these responses is the induction of apoptosis. Although tumor cells, virally infected cells or damaged cells are frequently sensitive to TNF-α induced apoptosis, the cytotoxic effects of TNF-α on most cells are only evident if RNA or protein synthesis is inhibited, suggesting that de novo RNA or protein synthesis protects cells from TNF-α cytotoxicity, probably by induction of protective genes [Wong GH, 1989; Yonehara S, 1989; Itoh N, 1991 and Karsan A, 1996]. Recent studies have identified the protective genes that are involved in transducing TNF-α death signals [Cleveland JL, 1995 and Muzio M 1996]. The current hypothesis to explain the relation

between TNF-α and apoptosis suggests two opposite signaling pathways: TNF-α produces positive survival signals and simultaneously negative apoptotic signals. The balance of those pathways will regulate the fate of cells in response to TNF-α stimulation [Nagata S, 1997]. The molecular mechanisms of TNF-α induced cell death have been extensively studied, and recent clinical evidence has shown that serum levels of TNF-α are increased after myocardial infarction. Moreover, TNF-α directly decreases animal and human myocardial contractility in dose-dependent fashion [Cain BS,1999]. Therefore, TNF-α was used in the present studies to elicit cytotoxicity, and Huvec as well as Jurkat cells were chosen as an in vitro system to analyze cell protective action of PAC and PAL.

4.3. PAC and PAL protect cells from induction of cell death

The morphological studies have shown that TNF-α (16 h, 10 ng/ml) resulted in extensive loss of viability in Huvec cells and less cell death occurred by preincubation with 1mM PAC or PAL for 2 hours. The data provided a first impression that PAC and PAL could protect Huvec cells from TNF-α induced cytotoxicity. Further, XTT assays were performed to quantify the number of cells undergoing apoptosis. The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolic active cells [Gerlier D,1986]. In cell viability XTT assays, PAC and PAL were shown to be potent inhibitors of TNF-α induced apoptosis in both Huvec and Jurkat cells. Since XTT assays are based on the mitochondrial dehydrogenase activity of metabolic active cells, an increase in number of living cells results in an increase in the overall activity of mitochondrial dehydrogenase [Berridge MV, 1997]. These data of cell viability assays suggested that PAC and PAL may “switch on” positive signals which could protect cells from death. This positive signals might prevent the induction of apoptosis by reducing the amount or activity of crucial cell death effector proteins to harmless levels. Alternatively, they may inhibit cell death by boosting the activity of protective, anti-apoptotic proteins.

4.3.1. Transcription factor NF-κB

To a large extent, gene expression is controlled by the frequency of transcriptional initiation at the promoter. Initiation of transcription is preceded by the formation of a large nucleoprotein complex containing promoter DNA, basal transcription factors, upstream activator proteins, and a variety of other proteins. In many cases, the rate at which transcription initiates is limited by the availability or activity of the DNA-binding upstream activators. One such upstream activator is the transcription factor NF-κB, whose DNA-binding activity and nuclear/cytoplasmic distribution are controlled by the IκB inhibitor proteins. In unstimulated cells, NF-κB is held in the cytoplasm, in a form that is unable to bind DNA, by the inhibitory IκB proteins. Exposure of cells to a wide variety of stimuli results in release of the transcription factor from the IκB proteins, allowing the active DNA-binding form of the transcription factor to translocate to the nucleus, where it binds to its recognition sites in the upstream regions of a wide variety of genes that respond to immune and inflammatory response [Hay RT,1993 and Liou HC, 1993](Fig. 29).

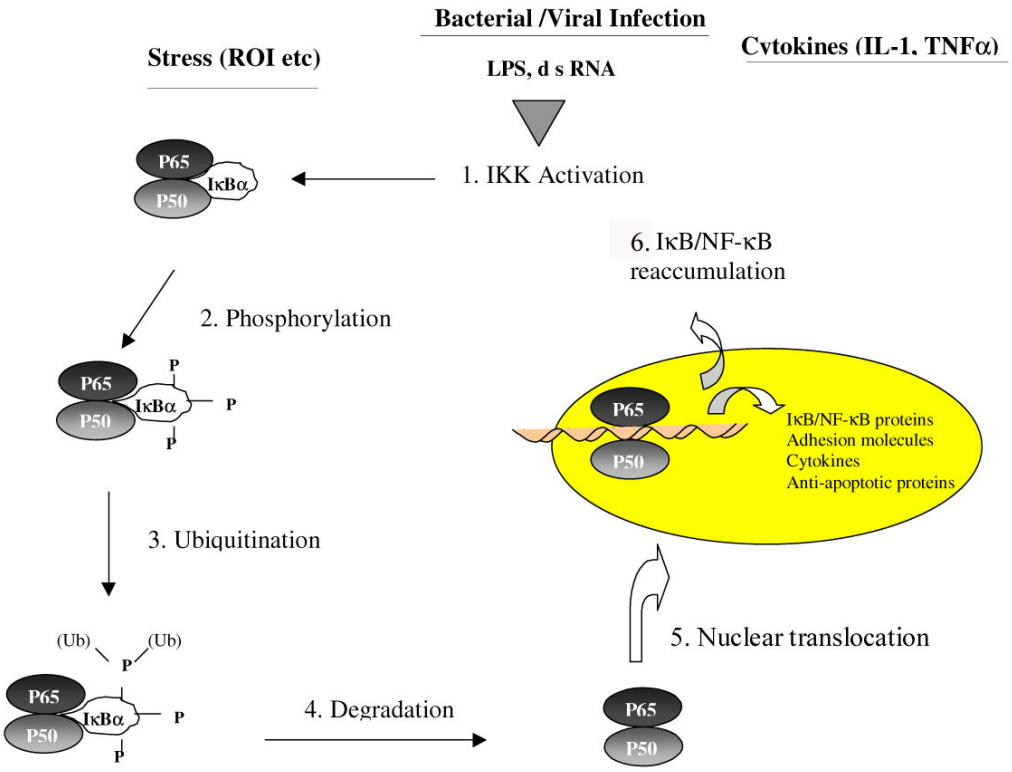

Fig. 29 NF-κB signaling pathway.

NF-κB is a dimer of proteins that share a highly conserved region known as the Rel homology domain which contains the sequences responsible for DNA binding, dimerization, and nuclear localization. The 11-bp binding site of NF-κB is recognized by the protein in an unusual way involving base and backbone contacts with the DNA over one complete helical turn [Clark L[1,2], 1989]. In humans, the family of proteins consists of p50 [Ghosh SA, 1990 and Kieran M, 1990], p52 [Bours V, 1992**;** Neri AC, 1992 and Schmid RM, 1991], p65 [Nolan GP, 1991 and Ruben SM, 1991], c-Rel [Wilhelmsen KC, 1984], and RelB [Ryseck RP, 1992]. Although it appears that almost all combinations of homo- and heterodimers can exist, the typical form of NF-κB, that is activated in response to extracellular signals, is composed of a heterodimer of p50 and p65. p50 represents the N-terminal region of a p105 precursor from which it is processed by a pathway thought to involve ubiquitinylation of the protein [Palombella VJ, 1994]. The C-terminal region removed from p105 contains multiple repeats of a 30- to 35-aminoacid sequence present in the erythrocyte protein ankyrin [Lux SE, 1990] and found in all proteins with IκB activity [Gilmore TD, 1993]. In lymphoid cells, the C-terminal region of p105 has been identified as an independent entity known as IκB γ [Inoue JI, 1992 and Liou HC, 1992] that preferentially inhibits the DNA-binding activity of p50 homodimers. In the p105 precursor molecule, the C-terminal region is thought to function as a *cis*-acting inhibitor of p105 DNA-binding activity [Henkel T,1993]. Although p50 does not possess a transcriptional activation domain, its p65 partner does have an acidic activation domain that accounts for the transcriptional activity of the NF-κB heterodimer [Fujita T, 1992 and Schmitz ML, 1991]. NF-κB activity is regulated by its association with the inhibitor subunit(s) of IkB [Baeuerle PA, 1988 and Haskill S, 1991]. The IkB family of proteins is defined by its ability to interact with NF-κB/Rel subunits and by the presence of between five and seven repeats of a 33 amino acid sequence termed the ankyrin motif [Beg AA, 1993[1]]. The ankyrin motif mediates sequestration of NF-κB in the cytoplasm, possibly in association with cytoskeletal proteins [Rosette C, 1995]. IκBα [Haskill S, 1991] is the most extensively studied protein in this family. When cells are exposed to stimuli which activate NF-κB, IκBα becomes hyperphosphorylated, detectable in immunoblots as a slowly migrating form, sensitive to phosphatase treatment [Beg AA, 1993[1]; Brown K, 1993; Cordle SR, 1993; Henkel T, 1993; Mellits KH, 1993 and Sun SC, 1993]. The hyperphosphorylation occurs at the N-terminus of the molecule and is a signal for subsequent ubiquitination and degradation by the 26S

proteasome. Neither hyperphosphorylation [Traenckner EB-M, 1994 and Alkalay I, 1995[1]] nor ubiquitination [Chen Z, 1995] impairs the ability of IκBα to associate with NF-κB. Moreover, only hyperphosphorylated IκBα is a target for degradation by an in vitro reconstituted ubiquitin-proteasome system [Alkalay I, 1995[2]]. Phosphorylation and subsequent degradation via the ubiquitin-proteasome degradation pathway are therefore key elements in NF-κB liberation and nuclear translocation [Beg AA, 1993²; Brown K, 1993; Traenckner EB-M, 1994; Alkalay I, 1995[12]; Chen Z, 1995].

Recent studies have described a role for NF-κB in blocking apoptosis which is induced by tumor necrosis factor. For instance, Baltimore's group treated cells taken from NF-κB knockout mice with TNF, and compared their response to that of cells from normal mice [Beg AA and Baltimore D, 1996]. The normal cells survived, but those lacking NF-κB died. Another proof of the essential role of NF-κB comes from experiments in which NF-κB activity is inhibited by antibodies or by an inhibitory protein that triggers apoptosis [Van-Antwerp DJ, 1996; Wang CY, 1996 and Bach FH, 1997]. Baldwin's and Verma's groups introduced into a variety of cultured tumor and nontumor cells a mutant form of IκB that acts as a "super-repressor", keeping NF-κB irreversibly shackled in the cell's cytoplasm. With NF-κB out of the picture, TNF could kill all the cell types. Therefore, it appears that NF-κB has a general role in preventing apoptosis.

4.3.2. Activation of NF-κB by PAC but not by PAL

NF-κB has been recently described as one survival signaling pathway to counteract the cytotoxicity of the apoptotic pathway and then suppress apoptotic cascades in diverse cell types. Thus, an important question was raised: whether activation of NF-κB plays a role in PAC protective effect against apoptosis? This speculation was supported by our observations in different experimental approaches.

At first, in Huvec and Jurkat cells the NF-κB pathway was efficiently activated by PAC, as evidenced by luciferase assays for measuring reporter gene and gel shift assays for measuring

DNA-binding of nuclear extracts. 30 min incubation of PAC led to strong increase of NF-κB reporter gene and DNA-binding in both cell types (Fig. 9, 13). Previous studies have demonstrated that the primary form of NF-κB is retained as a latent form in the cytoplasm by its inhibitory protein IκB. Thus our western blotting experiment further confirmed that PAC activated NF-κB due to the degradation of IκBα, the NF-κB endogenous cytosolic inhibitor. IκBα was rapidly degraded after PAC stimulation and almost completely disappeared within 4 h and 1 h of PAC stimulation in Huvec and Jurkat cells, respectively. IκBα protein reappeared thereafter in the cytoplasm (Fig. 18). Since proteolytic degradation of IκBα is essential for activation of NF-κB [Palombella VJ, 1994; Siebenlist U, 1994; Traenckner EB-M, 1994 and Lin YC, 1995], when degradation of IκBα is blocked, activation of NF-κB is prevented. Approaches to inhibit activation of NF-κB include gene therapy delivery of super-repressor IκBα or use of a variety of agents to block NF-κB function such as proteasome inhibitors. Proteasome inhibitors inhibit activation of NF-κB by blocking the degradation of IκBα and subsequent nuclear translocation of NF-κB/Rel protein [Henkel T, 1993 and Marui N, 1993]. To further determine PAC function when activation of NF-κB is blocked, MG132, a potential proteasome inhibitor, was used in this study. Treatment of cells with MG132 resulted in failure of PAC to protect from apoptosis triggered by TNFα (Fig. 14). An alternative approach to block NF-κB in this study is use of a NF-κB inhibitory peptide, which contains a cell membrane-permeable motif and nuclear localization sequence of NF-κB p50. It can directly inhibit nuclear translocation of NF-κB /Rel complexes in intact cells. Addition of NF-κB inhibitory peptide resulted in a loss of PAC protection from apoptosis (Fig. 16). Taken together, activation of NF-κB was shown to be crucial for the anti-apoptotic effect of PAC.

Recent studies have shown that NF-κB controls the expression of IκBα by means of an inducible autoregulatory pathway [Sun SC, 1993] (Fig. 30).

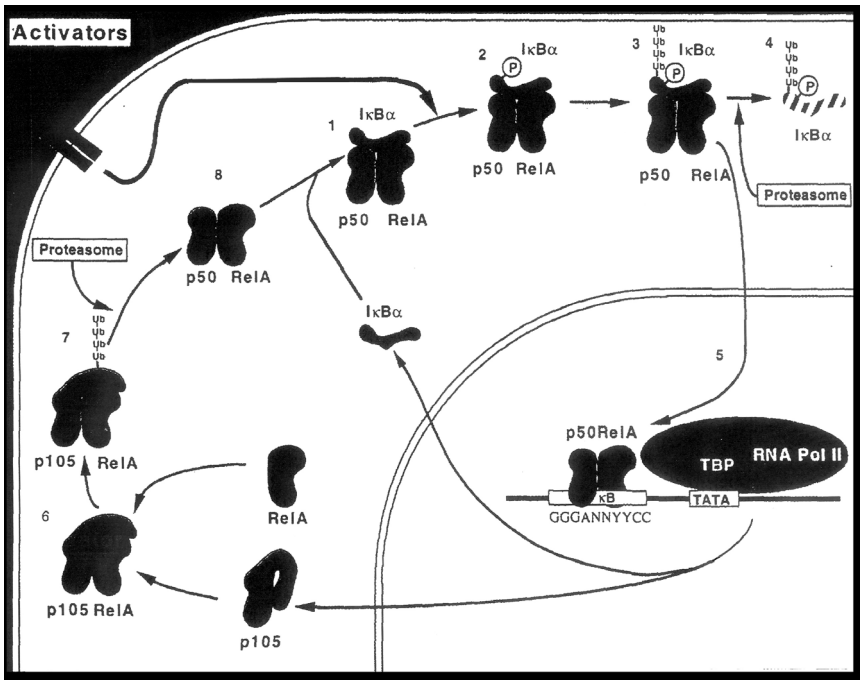

Fig. 30 Auto-regulatory pathway of the NF-κB/ IkBα. Activators of NF-κB trigger signal transduction cascades that result in hyperphosphorylation of IkBα (steps 1-2). Hyper-phosphorylation is a signal for IkBα ubiquitination (step 3) and subsequent degradation by the 26S proteasome (step 4). Following IkBα degradation, NF-κB translocates to the nucleus and activates NF-κB-dependent cytokine genes. Also activated are components of the NF-κB pathway such as IkBα, NF-κB1 (p105) and NF-κB2 (p100) (step 5). Newly synthesized IkBα can retain NF-κB in the cytoplasm or may move to the nucleus and dissociate NF-κB/DNA complexes, thereby exerting a direct inhibition on gene expression. Newly synthesized p105 and p100 proteins also retain NF-κB proteins such as Rel A in the cytoplasm (step 6). P105 and p100 are proteolytically cleaved to generate p50 and p52 respectively (step 7-8), which can localize to the nucleus or be retained in the cytoplasm, complexed to IkBα.

After degradation of IκBα, active DNA-binding form of NF-κB translocates to the nucleus, where it binds to DNA recognition sites in the promoter of a wide variety of genes, including IκBα gene, and activates gene expression. The cytoplasmic reservoir of IκBα is then completely replenished by NF-κB-induced de novo synthesis of IκBα protein [LeBail O, 1993 and Chiao PJ, 1994]. Newly synthesized IκBα protein appears transiently in the nucleus, where it terminates DNA-binding and transcription [Arenzana-Seisdedos F, 1995]. Our results of immunoblotting and gel shifts are in complete agreement with this autoregulatory signal pathway.

In this study, in order to compare it to PAC , PAL as another component of RSM was analysed for its effect on activation of NF-κB. In in vitro experiment PAL was shown to protect Huvec and Jurkat cells from cytotoxicity triggered by TNFα. Nevertheless treatment of MG132 could not

inhibit the PAL protection from apoptosis triggered by TNFα. Additionally PAL did not induce IκBα degradation (Fig. 20-22). These results imply that the anti-apoptotic action of PAL is medicated by another molecular mechanism instead of activation of NF-κB. Moreover, from the structural difference between PAC and PAL, it might suggest that the carboxylic group of PAC acts as an essential constituent to induce NF-κB activation (Fig. 2).

4.4. Investigation of antioxidative capacity of TMP and Dhpl by flow channel techniques

Two different applications of flow channel techniques were used to investigate the antioxidative capacity of natural compounds, plant extracts and in particular Chinese herbs. The Microscopic Photometric Monolayer Technique is capable to precisely quantitate RBC stiffening and relaxation time shortening due to hydrogen peroxide damage over time [Artmann GM, 1995]. This tool has been applied to investigate the anti-oxidative protective capacity of TMP. RBC treated with hydrogen peroxide exhibited significantly attenuated relaxation times. Simultaneously, the RBC stiffness increased. These changes were irreversible during re-incubation with HEPES++. Reduced overall RBC elongation after hydrogen peroxide treatment have been observed earlier based on evaluation of freely suspended RBC sheared in a cone-plate system [Hebbel RP, 1990]. Even though the cone-plate system is different in many aspects from the flow channel technique used here, the data support the validity of the hydrogen peroxide damaging model used in our experiments.

4.4.1. Antioxidative capacity of Tetramethylpyrazine to RBC

TMP, as tested for its antioxidative capacity, reversed the hydrogen peroxide-induced shortening of relaxation time. Simultaneously the RBC stiffness was not reversed and osmotic effects of TMP were excluded. Thus, TMP may have partially re-established a normal RBC membrane viscosity after H_2O_2 pre-damaging. It is known, that hydrogen peroxide enhances the interactions of hemoglobin with the RBC cytoskeleton namely with spectrin which in turn leads to an enhanced overall membrane viscosity [Rice-Evans C, 1986]. As the underlying mechanism we

hypothesize, that TMP applied to hydrogen peroxide pre-damaged RBC may partially reverse hydrogen peroxide mediated hemoglobin-spectrin complexes, thus, leading to a normalization of the membrane viscosity.

RBC stiffening is related to the elasticity of the RBC cytoskeleton. The RBC's cytoskeleton represents an extensive and complex self-associating network of proteins. Cross-linking of cytoskeletal proteins due to peroxidation in particular leads to a decrease of RBC deformability [Nicolson GL, 1973; Girotti AW, 1984; Schrier SL, 1985; Snyder LM, 1985 and Oroszlan G, 1986]. TMP did not reverse hydrogen peroxide induced RBC stiffening, indicating that TMP did not reverse hydrogen peroxide cytoskeletal crosslinkings.

4.4 2. Protection of Dhpl from H_2O_2 induced RBC-EC adhesion

In a further approach the flow channels technique was used to study RBC-EC adhesion as induced with hydrogen peroxide. A similar hydrogen peroxide based in vitro model was established previously [Wang L, 1995]. The authors reported on enhanced adhesion of RBC of patients with cerebral thrombosis to cultured human umbilical vein endothelial cells treated with H_2O_2 using a flow chamber system. In our study, the inhibitory capacity of Dhpl was compared with Vitamin E, a well-known antioxidant. Dhpl is used in Chinese traditional Medicine to cure ischemic cerebral vascular diseases and ischemic cardiovascular diseases [Zhang ZH, 1994 and Li DY, 1995]. The exposure of only RBC with hydrogen peroxide led to a significant adhesion (about two-fold) to cultured EC monolayers. Whereas, no enhanced RBC adhesion occurred when only EC were treated with hydrogen peroxide. When both EC and RBC were treated with H_2O_2 a remarkable (about three-fold) RBC-EC adhesion was observed. The results imply that modifications in surface properties due to hydrogen peroxide on both RBC and EC were required to induce a major RBC-EC adhesion. If an oxidative event in vivo occurs, it usually would affect both cell types. Thus, damaging of both RBC and EC in this in vitro model should fit best to the in vivo situation. Dhpl as well as Vitamin E both at 50 μM protected from oxidatively induced RBC-EC adhesion to the same extend. It did not matter whether the compounds were added before damaging or after, respectively. The mechanism of protection observed when the drugs

were added before hydrogen peroxide damaging might be addressed to a direct free radical scavenging effect of antioxidants dissolved in the EC membrane. Although very interesting from a clinical point of view, the protection mechanism of drugs applied after damaging must be addressed in future studies.

In summary, flow channel techniques as used here in terms of cellular bioassays could become useful tools for evaluating antioxidative active substances in particular of Chinese herbs, plant extracts and other substances. The fact, that extracts do not contain single molecules but a variety of sub-components rather underlines the need of studies on a cellular level. Of particular advantage in terms of creating an antioxidative protection scale is the use of drugs (Vitamin E) known for a specific effect as basis for efficacy comparisons.

4.5. Future perspectives

The present study provides a mechanistic explanation for the anti-apoptotic effect of PAC and indicates that this effect involves degradation of IkBα and subsequent activation of NF-κB. Further studies are required to test whether PAC could be useful in preventing apoptotic cell death in vivo resulting from cardiovascular or cerebrovascular diseases. In addition, efforts could be made in the following fields to provide futher evidence for PAC induced NF-κB activation.

- Many signal transduction pathways resulting in NF-κB activation culminate in a serine phosphorylation of IkBα on residues 32 and 36 [Brown K, 1995; Traenckner EB, 1995; DiDonato JA, 1997; Mercurio F, 1997 and Regnier CH, 1997] and trigger its ubiquitination-dependent degradation [Alkalay I[2], 1995; Chen Z, 1995 and Scherer DC, 1995]. Therefore, it could be analysed in the future whether PAC induces serine phosphorylation of IkBα.

- A specific serine-protein-kinase activity responsible for IκBα phosphorylation has been identified as a large cytoplasmic multisubunit complex (700-900 kDa); two kinase subunits (IKK1/α and IKK2/β) and a structural component (NEMO/IKKγ/IKKAP) have been cloned

[Scheidereit C and Israël A, 2000]. IKK complex could be then investigated to elucidate the mechanisms contributing to the exquisite regulation of NF-κB activity.

- Active DNA-binding form of NF-κB translocates to the nucleus, where it binds to its recognition sites in the upstream regions of a wide variety of genes that respond to immune and inflammatory signals. Therefore, such NF-κB regulative genes , e.g. ICM-1, VCAM-1 and IL-8 could be chosen to check if PAC also activates these genes.

Although PAL also shows cell protection from TNF-induced cytotoxicity, the molecular basis of this effect is not yet clear. In order to identify its mechanism, other anti-apoptotic pathways except NF-κB need to be analyzed in the future.

5. Summary

The Chinese herb, Radix Salviae Miltiorrhizae (RSM), is being used in traditional Chinese medicine as a treatment for cardiovascular and cerebrovascular diseases. Several components of the plant extract from Salvia Mitorrhiza Bunge have been determined previously, two of which are protocatechuic acid (PAC) and protocatechuic aldehyde (PAL). Since anti-apoptotic therapies have been proposed to limit tissue damage in cardiovascular and cerebrovascular diseases, PAC and PAL effects on cell protection from apoptosis were investigated in this thesis. XTT assays were first used to quantify cell viability. We found that PAC and PAL inhibited TNF-α induced apoptosis of human umbilical vein endothelial cells (Huvec) and Jurkat cells in a concentration of 100 μM and 1mM respectively, when applied 2 hours prior to TNF-α exposition. To investigate molecular consequences on cellular signal transduction pathways, NF-κB reporter gene and DNA binding activities were investigated by luciferase assay and gel shift assay. Degradation of IkBα was determined by western blotting. The molecular studies revealed that PAC activated NF-κB with a maximal effect after 30 min of treatment. Inhibition of NF-κB action by MG132 and NF-κB inhibitory peptide suppressed the anti-apoptotic effect of PAC. Further, degradation of IkBα occurred in response to PAC treatment. Our results provide evidence that activation of NF-κB plays an important role in mediating the anti-apoptotic effect of PAC on HUVEC and Jurkat cells. Nevertheless, PAL protection from apoptosis triggered by TNF-α could not prevented by the treatment of MG132. Additionally, PAL did not induce IκBα degradation. The results implied that the anti-apoptotic action of PAL may be mediated by a molecular mechanism other than activation of NF-κB.

In addition, a computer controlled flow channel system (Elias-c-) was tested in this thesis to study antioxidative capacities of herbal medicine. Effects of herbal extracts and single components on oxidatively impaired red blood cell (RBC) stiffness and relaxation time as well as on oxidative damage (H_2O_2, 2 mM) induced RBC-endothelial cell (EC) adhesion were

investigated. Following H_2O_2 treatment (20 min), RBC became significantly stiffer and the relaxation time was reduced as compared with control. These changes were irreversible after re-incubating oxidatively damaged RBC in HEPES buffer. However, when oxidatively damaged RBC were re-incubated with 88.5 μM Tetramethylpyrazine (TMP), the H_2O_2-induced reduction in relaxation time turned back to control levels whereas the RBC stiffening did not. As mechanism we hypothesize, that TMP applied to hydrogen peroxide damaged RBC may partially reverse the hydrogen peroxide mediated formation of hemoglobin-spectrin complexes possibly leading to a normalization of the membrane viscosity. In the RBC-EC adhesion tests, a maximum (about three-fold as compared to control) increase in RBC-EC adhesion was obtained when both RBC and EC were treated with H_2O_2. This increase in adhesion was almost completely inhibited by 50 μM 3,4-dihydroxyphenyl lactate (Dhpl), when applied either prior to or after treatment with H_2O_2. The mechanism of the protection may be addressed in part to a direct free radical scavenging effect of antioxidants dissolved in the EC membrane. From a methodological point of view, the Elias-c in combination with appropriate cell types and experimental designs can be seen as a cellular bioassays to test herbal extracts with high laboratory efficacy.

6. Zusammenfassung

Das in der traditionellen chinesischen Medizin verwendete Extrakt Radix Salviae Miltiorrhizae (RSM) wird zur Behandlung von Herzkreislauf und zerebrovaskulären Krankheiten benutzt. Einige Bestandteile des Pflanzenextraktes von Salviae Mitorrhiza Bunge sind bereits extrahiert und analysiert worden. Zwei dieser Komponenten sind Protocatechuic Säure (PAC) und Protocatechuic Aldehyd (PAL). Es wird angenommen, daß anti-apoptotische Therapien die Gewebeschäden bei Herzgefäßkrankheiten sowie zerebrovaskulären Krankheiten reduzieren. In dieser Arbeit wurde daher die zellprotektive Wirkung von PAC und PAL, im besonderen Hinblick auf Apoptose, untersucht. XTT-Assays wurden verwendet, um den Prozentsatz lebender Zellen unter verschiedenen Bedingungen quantitativ zu bestimmen. Menschliche Nabelschnur Endothelzellen sowie Jurkat Zellen wurden 2 Stunden mit PAC (100 μM) bzw. PAL (1 mM) inkubiert. Es konnte gezeigt werden, daß PAC und PAL die TNF-α induzierte Apoptose von menschlichen Nabelschnur Endothelzellen (Huvec) und Jurkat Zellen reduziert. Um molekulare Effekte auf die zelluläre Signaltransduktion zu untersuchen, wurden NF-κB Reportergen und DNA Bindungsaktivitäten mittels Luciferase Assays sowie Gel Shift Assays bestimmt. Die Degradation von IkBa wurde durch Western Blotts untersucht. Die molekularen Studien zeigten, daß NF-κB durch PAC aktiviert wurde. Der maximalen Effekt trat nach 30-minütiger Vorbehandlung der Zellen mit PAC auf. Hemmung der NF-κB Aktivität durch MG132 und ein NF-κB hemmendes Peptid unterdrückten den anti-apoptotischen Effekt von PAC. Weiterhin trat eine Degradation von IkBα infolge der Behandlung mit PAC auf. Unsere Ergebnisse liefern Hinweise darauf, daß die Aktivierung von NF-κB bei der anti-apoptotischen Wirkung von PAC auf Huvec und Jurkat Zellen eine wichtige Rolle spielt. Allerdings wurde die protektive Wirkung von PAL gegen Apoptose nicht von MG132 beeinflusst. Auch induzierte PAL keinen Abbau von IκBα in den Zellen. Diese Resultate deuten darauf hin, daß die anti-apoptotische Wirkung von PAL durch andere molekulare Mechanismen als durch die Aktivierung von NF-κB vermittelt wird.

Zusätzlich wurde ein computergesteuertes Flußkanalsystem zur Bestimmung zellulärer rheologischer Parameter (Elias-c -) verwendet, um die antioxidative Wirkung von chinesischen Naturheilmitteln zu untersuchen. Dabei interessierten uns die Effekte der Kräuterextrakte sowie einiger ihrer Bestandteile auf die durch oxidative Schädigung veränderten rheologischen Parameter der Zellen. Als Parameter wurde die Steifigkeit und die Relaxationszeit roter Blutkörperchen (RBC) sowie die durch oxidative Schädigung (H_2O_2, 2 mM) induzierte Adhäsion von roten Blutkörperchen Endothelzellen gemessen. Nach Inkubation der roten Blutkörperchen mit H_2O_2 (20 min), zeigten die Zellen eine signifikant höhere Steifigkeit. Weiterhin war die Relaxationszeit im Vergleich zu Kontrollversuchen reduziert. Diese Änderungen der rheologischen Parameter waren irreversibel, nachdem die oxidativ geschädigten RBCs in Hepes Puffer reinkubiert wurden. Wurden die Zellen jedoch in Puffer mit 88.5 µM Tetramethylpyrazine (TMP) reinkubiert, ging die Änderung der Relaxationszeit auf das Niveau der Kontrolle zurück, während sich kein Effekt auf die Steifheit der Zellen zeigte. Als möglichen Mechanismus nehmen wir an, daß TMP die durch Wasserstoffperoxid verursachte Bildung von Spektrin-Hömoglobin-Komplexen teilweise aufhebt, was möglicherweise zu einer Normalisierung der Membranviskosität führen könnte. In den RBC-EC Adhäsionstests wurde eine maximale (über dreifache verglichen mit der Kontrolle) Zunahme der Adhäsion, bei mit H_2O_2 behandelten roten Blutzellen und Endothelzellen festgestellt. Diese Adhäsion wurde fast vollständig durch 50 µM 3,4-dihydroxyphenyl lactate (Dhpl) inhibiert, wenn diese Komponente entweder vor oder nach der Behandlung der Zellen mit H_2O_2 zugegeben wurde. Der Mechanismus dieser protektiven Wirkung könnte teilweise einem Effekt von Dhpl als Radikalfänger von in der Zellmembran gelösten Oxidantien zugeschrieben werden. Von einem methodischen Gesichtspunkt aus kann der Elias-c im Verbindung mit bestimmten Zellenarten und experimentellen Designs als zellulärer Bioassay verwendet werden, um die Wirkung von Kräuterextrakten mit hoher Effizienz im Labor zu prüfen.

7. References

Aggarwal BB, Moffat B, Harkins RN. Human lymphotoxin: production by a lymphoblastoid cell line. Purification and initial characterisation. J. Biol. Chem. (1984) 259, 686-691.

Alkalay I[1], Yaron A, Hatzubai A, Jung S, Avarham A, Gerlitz O, Pashut-Lavon I, Ben-Neriah Y. In vivo stimulation of IκB phosphorylation is not sufficient to activate NF-κB. Mol. Cell. Biol. (1995) 15, 1294-1301.

Alkalay I[2], Yaron A, Hatzubai A, Orian A, Ciechanover A, Ben-Neriah Y. Stimulation-dependent IκBα phosphorylation marks the NF-κB inhibitor for degradation via the ubiquitin-proteasome pathway. Proc. Natl. Acad. Sci. USA (1995) 92, 10599-10603.

Arenzana-Seisdedos F, Thompson J, Rodriguez MS, Bachelerie F, Thomas D, Hay RT. Inducible nuclear expression of newly synthesized I kappa B alpha negatively regulates DNA-binding and transcriptional activities of NF-kappa B. Mol. Cell. Biol. (1995) 15(5), 2689-2696.

Artmann GM, Schikarsky C. Ginkgo Biloba extract (EGb 761) protects red blood cells from oxidative damage. Clinical Hemorheology (1993) 13(4), 529-539.

Artmann GM. Microscopic Photometric Quantification of stiffness and relaxationtime of Red Blood Cells in a flow chamber. Biorheology (1995) 32/5, 553-570.

Artmann GM, Li A, Ziemer J, Schneider G, Sahm U: A photometric method to analyze induced erythrocyte shape changes. Biorheology (1996) 33/3, 251-265.

Bach FH, Ferran C, Soares M, Wrighton CJ, Anrather J, Winkler H, Robson SC, Hancock WW. Modification of vascular responses in xenotransplantation: inflammation and apoptosis. Nat. Med. (1997) 3(9), 944-948.

Baeuerle PA, Baltimore D. IkB: a specific inhibitor of the NF-κB transcription factor. Science (1988) 242, 540–546.

Banda NK, Bernier J, Kurahara DK, Kurrle R, Haigwood N, Sekaly RP, Finkel TH. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. J. Exp. Med. (1992) 176(4), 1099-10106.

Beg AA[1], Baldwins ASJr. The IκB proteins: multifunctional regulators of Rel/NF-κB transcription factors. Genes. Devl. (1993) 7, 2064-2070.

Beg, AA[2], Finco TS, Nantermet PV, Baldwin ASJr. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IkB-a: a mechanism for NF-κB activation. Mol. Cell. Biol. (1993) 13, 3301–3310.

Beg AA, Baltimore D. An Essential Role for NF-κB in Preventing TNF-Induced Cell Death. Science (1996) 274, 782-784.

Berridge MV, Tan AS, McCoy KD, Wang R. The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. Biochemica Information (1997) 100, 15-20.

Bisset NG. Herbal drugs and phytopharmaceuticals. A handbook for practice on a scientific basis. Stuttgart, Germany. Medpharm Scientific Publishers, 1994.

Block ER. Hydrogen peroxide alters the physical state and function of the plasma membrane of pulmonary artery endothelial cells. Journal of cellular physiology (1991) 146, 362-369.

Bosserhoff AK, Hein R, Bogdahn U, Buettner R. Structure and Promoter Analysis of the Gene Encoding the Human Melanoma-inhibiting Protein MIA. J. Biol. Chem. (1996) 271, 490-495.

Bours, V, Burd PR, Brown K, Villalobos J, Park S, Ryseck R.-P, Bravo R, Kelly K, Siebenlist U. A novel mitogen-inducible gene product related to p50/p105 NF-κB participates in transactivation through a kB site. Mol. Cell. Biol. (1992) 12, 685–695.

Brown, K, Park S, Kanno T, Franzoso G, Siebenlist U. Mutual regulation of the transcriptional activator NF-κB and its inhibitor, IkB-a. Proc. Natl. Acad. Sci. USA (1993) 90, 2532–2536.

Brown K, Gerstberger S, Carlson L, Franzoso G, Siebenlist U. Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. Science (1995) 267 (5203), 1485-1488.

Bruneton J. Pharmacognosy, phytochemistry, medicinal plants. Hatton CK, translator. Paris: Lavoisier Publishers, 1995. Translation of: Pharmacognosie.

Bursch W, Oberhammer F, Schulte-Hermann R. Cell death by apoptosis and its protective role against disease. Trends Pharmacol. Sci. (1992) 13(6), 245-251.

Cain BS, Harken AH, Meldrum DR. Therapeutic strategies to reduce TNF-alpha mediated cardiac contractile depression following ischemia and reperfusion. J. Mol. Cell Cardiol. (1999) 31(5), 931-947.

Caragay AB. Cancer-preventative foods and ingredients. Food Technol. (1992) 46, 65–68.

Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. U S A. (1975) 72(9), 3666-3670.

Chan TYK, Chan JCN, Tanlinson B, Critchley JA. Chinese herbal medicines revisited: a Hong Kong perspective. Lancet (1993) 342, 1532–1534.

Chen KJ, Chen K. Ischemic stroke treated with Ligusticum chuanxiong [Review]. Clin. Med. J. Peking (1992) 105, 870-873.

Chen WZ. Pharmacology of Salvia miltiorrhiza. Acta. Pharm. Sin. (1984) 19, 876-880.

Chen Z, Hagler J, Palombella VJ, Melandri F, Scherer D, Ballard D, Maniatis T. Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. Genes. Devl. (1995) 9, 1586-1597.

Chiao PJ, Miyamoto S, Verma IM. Autoregulation of IκBα activity. Proc. Natl. Acad. Sci. USA (1994) 91, 28-32.

Clark L[1], Hay RT. Sequence requirement for specific interaction of an enhancer binding protein (EBP1) with DNA. Nucleic. Acids Res. (1989) 17, 499–515.

Clark L[2], Nicholson J, Hay RT**.** Enhancer binding protein (EBP1) makes base and backbone contacts over one complete turn of the DNA double helix. J. Mol. Biol. (1989) 206, 615–626.

Cleveland JL, Ihle JN. Contenders in FasL/TNF death signaling. Cell (1995) 81(4), 479-482.

Cohen JJ. Programmed cell death in the immune system. Adv. Immunol. (1991) 50,55-85.

Cohen JJ. Apoptosis: the physiologic pathway of cell death. Hosp. Pract. (1993) 28(12), 35-43.

Cook NC, Samman S. Flavonoids—chemistry, metabolism, cardioprotective effects, and dietary sources. J. Nutr. Biochem. (1996) 7, 66–76.

Cordle SR, Donald R, Read MA, Hawiger J**.** Lipopolysac-charide induces phosphorylation of MAD3 and activation of c-rel and re-lated NF-κB proteins in human monocytic THP-1 cells. J. Biol. Chem. (1993) 268, 11803–11810.

Cuvelier ME, Berset C, Richard H. Antioxidant constituents in sage (Salvia officinalis). J. Agric. Food Chem. (1994) 42, 665–669.

Dayer JM, Beutler B, Cerami A. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E2 production by human synovial cells and dermal fibroblasts. J. Exp. Med. (1985) 162(6), 2163-2168.

Debbas M, White E. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev. (1993) 7(4), 546-554.

Dew MJ, Evans BK, Rhodes J. Peppermint oil for the irritable bowel syndrome: a multicentre trial. Br. J. Clin. Pract. (1984) 38, 394–398.

DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature (1997) 388(6642), 548-554.

Dreosti IE. Bioactive ingredients: antioxidants and polyphenols in tea. Nutr. Rev. (1996) 54, S51-58.

Fuhrman B, Buch S, Vaya J. Licorice extract and its major polyphenol glabridin protect low-density lipoprotein against lipid peroxidation: in vitro and ex vivo studies in humans and in atherosclerotic apolipoprotein E-deficient mice. Am. J. Clin. Nutr. (1997) 66, 267-275.

Fujita T, Nolan GP, Ghosh S, Baltimore D. Independent modes of transcriptional activation by the p50 and p65 subunits of NF-κB. Genes Dev. (1992) 6, 775–787.

Fukuzuka K, Rosenberg JJ, Gaines GC, Edwards CK, Clare-Salzler M, MacKay SL, Moldawer LL, Copeland EM, Mozingo DW. Caspase-3-dependent organ apoptosis early after burn injury. Ann. Surg. (1999) 229(6), 851-858.

Gamble JR, Harlan JM, Klebanoff SJ, Vadas MA. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. Proc. Natl. Acad. Sci. U S A. (1985) 82(24), 8667-8671.

Gerlier D, Thomasset N. Use of MTT colorimetric assay to measure cell activation. J. Immunol. Methods (1986) 94(1-2), 57-63.

Ghosh, S, Gifford AM, Riviere LR, Tempst P, Nolan GP, Baltimore D. Cloning of the p50 DNA binding subunit of NF-κB: ho-mology to *rel* and *dorsal*. Cell (1990) 62, 1019–1029.

Giardino I, Fard AK, Hatchell DL, Brownlee M. Aminoguanidine inhibits reactive oxygen species formation, lipid peroxidation, and oxidant-induced apoptosis. Diabetes. (1998) 47(7), 1114-1120.

Gilmore TD, Morin PJ. The IkB proteins: members of a multifunctional family. Trends Genet. (1993) 9, 427–433.

Girotti AW, Thomas JP. Superoxide and hydrogen peroxide-dependent lipid peroxidation in intact and triton-dispersed erythrocyte membranes. Biochem. Biophys. Res. Commun. (1984) 118(2), 474-480.

Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J. Clin. Invest. (1994) 94(4), 1621-1628.

Guo YL, Kang BB, Williamson JR. Resistance to TNF-α cytotoxicity can be achieved through different signaling pathways in rat mesangial cells. A. J. P.-Cell physiology (1999) 276 (2), C435-441.

Haan Jde, Bladier C, Griffiths P, Kelner M, O'Shea RD, Cheung NS, Bronson RT, Silvestro MJ, Wild S, Zheng SS, Beart PM, Hertzog PJ, Kola I. Mice with a homozygous null mutation for the most abundant glutathione peroxidase, gpx1, show increased susceptibility to the oxidative stress-inducing agents paraquat and hydrogen peroxide. J. Biol. Chem. (1998) 273(35), 22528-22536.

Hammar SP, Mottet NK. Tetrazolium salt and electron-microscopic studies of cellular degeneration and necrosis in the interdigital areas of the developing chick limb. J. Cell Sci. (1971) 8(1), 229-251.

Haraguchi H, Saito T, Okamura N, Yagi A. Inhibition of lipid peroxidation and superoxide generation by diterpenoids from Rosmarinus officinalis. Planta. Med. (1995) 61, 333–336.

Hase K, Xiong Q, Basnet P, Namba T, Kadota S. Inhibitory effect of tetrahydroswertianolin on tumor necrosis factor-alpha-dependent hepatic apoptosis in mice. Biochem. Pharmacol. (1999) 57(12), 1431-1437.

Haskill S, Beg AA, Tompkins SM, Morris JS, Yurochko AD, Sampson-Johannes A, Mondal K, Ralph P, Baldwin ASJr. Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. Cell (1991) 65, 1281–1289.

Hay RT. Control of nuclear factor-kB DNA binding activity by inhibitor proteins containing ankyrin repeats. Biochem. Soc. Trans. (1993) 21, 926–930.

Henkel T, Zabel U, van Zee K, Muller JM, Fanning E, Baeuerle PA. Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-κB subunit. Cell (1992) 68, 1121–1133.

Henkel T, Machleidt T, Alkalay I, Krönke M, Ben-Neriah Y, Baeuerle PA. Rapid proteolysis of IkB-a is necessary for activation of transcription factor NF-κB. Nature (London) (1993) 365, 182–185.

Ho CT, Ferraro T, Chen Q, Rosen RT, Huang MT. Phytochemicals in teas and rosemary and their cancer-preventive properties. In: Huang MT, Osawa T, Ho CT, Rosen RT, eds. Food phytochemicals for cancer prevention II. Teas, spices and herbs. Washington, DC: American Chemical Society (1994) 2–19.

Hovav T, Goldfarb A, Artmann GM, Yedgar S, Barshtein G. Enhanced adherence of beta-thalassaemic erythrocytes to endothelial cells. Br. J. Haematil. (1999) 106 (1), 178-181.

Huang MT, Ferraro T, Ho CT. Cancer chemoprevention by phytochemicals in fruits and vegetables. An overview. In: Huang MT, Osawa T, Ho CT, Rosen RT, eds. Food phytochemicals for cancer prevention I. Fruits and vegetables. Washington, DC: American Chemical Society (1994) 2–16.

Inoue, JI, Kerr LD, Kakizuka A, Verma IM. IkBγ, a 70kd protein identical to the C-terminal half of p110 NF-κB: a new member of the IkB family. Cell (1992) 68, 1109–1120.

Israël A. The IKK complex: an integrator of all signals that activate NF-κB? Trends in Cell Biology (2000) 10, 129-133.

Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y, Nagata S. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell (1991) 66, 233-243.

Jaffe EA, Nachman RL, Becker CG, Minick RC. Synthesis of antihemophilic factor antigen by cultured human endothlial cells. J. Chin. Invest. (1973) 52, 2745-2756.

Karsan A, Yee E, Harlan JM. Endothelial cell death induced by tumor necrosis factor-α is inhibited by the Bcl-2 family member, A1. J. Biol. Chem. (1996) 271(44), 27201-27204.

Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wideranging implication in tissue kinetics. Br. J. Cnacer (1972) 26, 239-257.

Kieran M, Blank V, Logeat F, Vandekerckhove J, Lottspeich F, Le-Bail O, Urban MB, Kourilsky P, Baeuerle PA, Israël A. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell (1990) 62, 1007–1018.

Kikuzaki H, Nakatani N. Antioxidant effects of some ginger constituents. J. Food Sci. (1993) 58, 1407–1410.

Kleijnen J, Knipschild P, Riet GT. Garlic, onions and cardiovascular risk factors. A review of the evidence from human experiments with emphasis on commercially available preparations. Br. J. Clin. Pharmacol. (1989) 28, 535–544.

Klosterhalfen B, Hauptmann S, Offner FA, Amo-Takyi B, Tons C, Winkeltau G, Affify M, Kupper W, Kirkpatrick CJ, Mittermayer C. Induction of heat shock protein 70 by zinc-bis-(DL-hydrogenaspartate) reduces cytokine liberation, apoptosis, and mortality rate in a rat model of LD100 endotoxemia. Shock (1997) 7(4), 254-262.

Kuang P, Li Z, Zhang F, Tao Y, Liu J, Wu W. Protective effect of radix Salviae Miltiorrhizae Composita on cerebral ischemia. J. Tradit. Chin. Med. (Chinese) (1995) 15(2), 135-140.

Kuang PG. The effect of Radix Salviae Miltiorrhizae (RSM) on substance P in cerebral ischemia: animal experiment. J. Tradit. Chin. Med. (1991) 11, 123.

Lam LKT, Zhang J, Hasegawa S, Schut HAJ. Inhibition of chemically induced carcinogenesis by citrus liminoids. In: Huang MT, Osawa T, Ho CT, Rosen RT, eds. Food phytochemicals for cancer prevention I. Fruits and vegetables. Washington, DC: American Chemical Society (1994) 209–219.

Larkin T. Herbs are often more toxic than magical. FDA Consum (1983) 17, 4–11.

LeBail O, Schmidt-Ullrich R, Israël A. Promoter analysis of the gene encoding the IκB-α/MAD-3 inhibitor of NF-κB: positive regulation by members of the rel/ NF-κB family. EMBO J. (1993) 12, 5043-5049.

Leist M, Gantner F, Bohlinger I, Germann PG, Tiegs G, Wendel A. Murine hepatocyte apoptosis induced in vitro and in vivo by TNF-alpha requires transcriptional arrest. J. Immunol. (1994) 153(4), 1778-1788.

Li CZ, Yang SC, Zhao FD. Effect of tanshinone II-A sulfonate on thrombus formation, platelet and conjunction in rats and mice. Acta. Pharmacol. Sin. (1984) 5, 39-42.

Li DY, Xu LN, Liu XG. Effects of water-soluble components isolated from Salvia miltiorrhiza on oxygen free radical generation and lipid peroxidation. Journal of Chinese Pharmaceutical Sciences (1995) 4(2), 107-112.

Li NL, Tan R, Chen WM. Salvianolic acid A, a new depside from roots of salvia miltiorrhiza. Planta. Med. (1984) 50, 227-228.

Li YJ, Wu JX, Yu XJ, Yan YF. Protective effect of tetramethyl-pyrazine against damages of aortic endothelial cells elicited by low-density lipoproteins. Acta. Pharmacol. Sin. (1994) 15, 407-410.

Lin YC, Brown K, Siebenlist U. Activation of NF-kappa B requires proteolysis of the inhibitor I kappa B-alpha: signal-induced phosphorylation of I kappa B-alpha alone does not release active NF-kappa B. Proc. Natl. Acad. Sci. U S A. (1995) 92(2), 552-556.

Lin YZ, Yao SY, Veach RA, Torgerson TR, Hawiger J. Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. J. Biol. Chem. (1995) 270(24), 14255-14258.

Liou HC, Baltimore D. Regulation of the NF-κB/rel transcription factor and IκB inhibitor system. Curr. Opin. Cell Biol. (1993) 5, 477–487.

Liou HC, Nolan GP, Ghosh S, Fujita T, Baltimore D. The NF-κB p50 precursor, p105, contains an internal IkB-like inhibitor that preferentially inhibits p50. EMBO J. (1992) 11, 3003–3009.

Liu GT, Zhang TM, Wang BE, Wang YW. Protective action of seven natural phenolic compounds against peroxidative damage to biomembranes. Biochemical Pharmacology (1992) 43(2), 147-152.

Liu SM, Tang TQ. Clinical and experimental studies of feiyaning in treating pulmonary arterial hypertension in corpulmonale [Chinese]. Chung-Kuo. Chung. Hsi. i. Chieh. Ho. Tsa. Chih. (1994) 14, 469-473.

Liu SY, Sylvester DM. Antiplatelet activity of tetramethylpyrazine. Thromb. Res. (1994) 75, 51-62.

Lux SE, John KM, Bennett V. Analysis of cDNA for human erythrocyte ankyrin indicates a repeated structure with homology to tissue-differentiation and cell-cycle control proteins. Nature (London) (1990) 344, 36–42.

Manach C, Regerat F, Texier O. Bioavailability, metabolism and physiological impact of 4-oxo-flavonoids. Nutr. Res. (1996) 16, 517–544.

Martinou JC, Dubois-Dauphin M, Staple JK, Rodriguez I, Frankowski H, Missotten M, Albertini P, Talabot D, Catsicas S, Pietra C. Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron. (1994) 13(4), 1017-1030.

Marui N, Offermann MK, Swerlick R, Kunsch C, Rosen CA, Ahmad M, Alexander RW, Medford RM. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. J. Clin. Invest. (1993) 92(4), 1866-1874.

McNutt K. Medicinals in foods. Nutr. Today (1995) 30, 218–222.

Mellits KH, Hay RT, Goodbourn S. Proteolytic degradation of MAD-3 (IkBα) and enhanced processing of the NF-κB precursor p105 are obligatory steps in the activation of NF-κB. Nucleic. Acids Res. (1993) 21, 5059–5066.

Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A, Rao A. IKK-1 and IKK-2: Cytokine-Activated IB Kinases Essential for NF-κB Activation. Science (1997) 278, 860-866.

Messmer UK, Briner VA, Pfeilschifter J. Tumor necrosis factor-alpha and lipopolysaccharide induce apoptotic cell death in bovine glomerular endothelial cells. Kidney Int. (1999) 55 (6), 2322-2337.

Mostefa-Kara N, Pauwels A, Pines E, Biour M, Levy VG. Fatal hepatitis after herbal tea. Lancet (1992) 340, 674 (letter).

Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. Cell (1996) 85(6), 817-827.

Nagata S. Apoptosis by death factor (review). Cell (1997) 88, 355-365.

Natoli G[1], Costanzo A, Guido F, Moretti F, Bernardo A, Burgio VL, Agresti C, and Levrero M. Nuclear Factor kB-independent Cytoprotective Pathways Originating at Tumor Necrosis Factor Receptor-associated Factor 2. J. Biol. Chem. (1998) 273, 31262-31272.

Natoli G[2], Costanzo A, Guido F, Moretti F, Levrero M. Apoptotic, non-apoptotic, and anti-apoptotic pathways of tumor necrosis factor signalling. Biochem. Pharmacol. (1998) 56(8), 915-920.

Neri AC, Lombardi L, Salina M, Corradini P, Maiolo AT, Dalla-Favera RSK. B cell lymphoma associated chromosomal translocation involves candidate oncogene lyt-10, homologous to NF-κB p50. Cell (1992) 67, 1075–1087.

Nicolson GL, Painter RG. Anionic sites of human erythrocyte membranes. II. Antispectrin-induced transmembrane aggregation of the binding sites for positively charged colloidal particles. J. Cell. Biol. (1973) 59, 395-406.

Nielsen I, Pederson RS. Life-threatening hypokalaemia caused by liquorice ingestion. Lancet (1984) 1, 1305 (letter).

Niwa M, Hara A, Kanamori Y, Matsuno H, Kozawa O, Yoshimi N, Mori H, Uematsu T. Inhibition of tumor necrosis factor-alpha induced neutrophil apoptosis by cyclic AMP: involvement of caspase cascade. Eur. J. Pharmacol. (1999) 371(1), 59-67.

Nolan GP, Ghosh S, Liou HC, Tempst P, Baltimore D. DNA binding and IkB inhibition of the cloned p65 subunit of NF-κB, a *re*l-related polypeptide. Cell (1991) 64, 961–969.

Oroszlan G, Lakatos L, Balazs M, Karmazsin L. D-penicillamine decreases the H2O2 and phenylhydrazine induced lipid peroxidation in the erythrocyte membrane. Acta. Paediatr. Hung. (1986) 27(1), 43-46.

Palombella VJ, Rando OJ, Goldberg AL, Maniatis T. The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell (1994) 78(5), 773-785.

Peng W, Duan SF. Effects of ligustrazine controlled release capsule in chronic pulmonary heart disease. J. Tongji. Med. Univer. (1991) 11, 101-105.

Pennica D, Nedwin GE, Hayflick JS. Human tumor necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature (1984) 312, 724-729.

Pincemail J, Dupuis M, Nasr C, Hans P, Haans-berrurier M, Anton R, Deby C. Superoxide anion scavenging effect and superoxide dismutase activity of Ginkgo Biloba extract. Experientia. (1989) 45, 708-712.

Pincemail J, Dupuis M, Nasr C, Hans P, Haans-berrurier M, Anton R, Deby C, Hebbel RP, Leung A, Mohandas N. Oxidation-induced changes in microrheologic properties of the red blood cell membrane. Blood (1990) 76, 1015-1020.

Raff MC. Social controls on cell survival and cell death. Nature (1992) 356(6368), 397-400.

Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y, Jacobson MD. Programmed cell death and the control of cell survival: lessons from the nervous system. Scienc. (1993) 262 (5134), 695-700.

Rath PC, Aggarwal BB. TNF-induced signaling in apoptosis. J. Clin. Innunol. (1999) 19, 350.

Regnier CH, Song HY, Gao X, Goeddel DV, Cao Z, Rothe M. Identification and characterization of an IkappaB kinase. Cell (1997) 90, 373-383.

Rice-Evans C. Free radical, cell damage and disease, Richelieu Press, London, (1986) 179.

Robbers JE, Speedie MK, Tyler VE. Pharmacognosy and pharmacobiotechnology. Baltimore: Williams & Wilkins, (1994).

Rosenbaum DM, Kalberg J, Kessler JA. Superoxide dismutase ameliorates neuronal death from hypoxia in culture. Stroke (1994) 25(4), 857-862.

Rosette C, Karin M. Cytoskeletal control of gene expression: depolymerization of microtubules activates NF-κB. J. Cell Biochem. (1995) 128,1111-1119.

Ruben SM, Dillon PJ, Schreck R, Henkel T, Chen CH, Maher M, Baeuerle PA, Rosen CA. Isolation of a *rel*-related human cDNA that potentially encodes the 65 kD subunit of NF-κB. Science (1991) 251, 1490–1493.

Ryseck RP, Bull P, Takamiya M, Bours V, Siebenlist U, Dobrzanski P, Bravo R. RelB, a new Rel family transcription activator that can interact with p50-NF-κB. Mol. Cell. Biol. (1992) 12, 674–684.

Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual. 2nd ed, Cold Spring Harbor, New York (1989).

Saxe TG. Toxicity of medicinal herbal preparations. Am. Fam. Physician (1987) 35, 135–142.

Scheidereit C. Signal transduction. Docking IkappaB kinases. Nature (1998) 395(6699), 225-226.

Scherer DC, Brockman JA, Chen Z, Maniatis T, Ballard DW. Signal-Induced Degradation of IB Requires Site-Specific Ubiquitination. Proc. Natl. Acad. Sci. USA (1995) 92, 11259-11263.

Schmid RM, Perkins ND, Duckett CS, Andrews PC, Nabel GJ. Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. Nature (London) (1991) 352, 733–736.

Schmitz ML, Baeuerle PA. The p65 subunit is responsible for the strong transcription activating potential of NF-κB. EMBO J. (1991) 10, 3805– 3817.

Schrier SL. Red cell membrane biology: introduction. Clin. Haematol. (1985) 14, 1-12.

Schrier SL, Mohandas N. Globin-chain specifity of oxidation-induced changes in red blood cell membrane properties. Blood (1992) 79, 1586-1592.

Sharma RD, Raghuram TC. Hypoglycaemic effect of fenugreek seeds in non-insulin dependant diabetic subjects. Nutr. Res. (1990) 10, 731–739.

Sharma RD, Raghuram TC, Rao VD. Hypolipidaemic effect of fenugreek seeds. A clinical study. Phytotherapy Res. (1991) 5, 145–147.

Shibata S. Antitumor-promoting and anti-inflammatory activities of licorice principles and their modified compounds. In: Huang MT, Osawa T, Ho CT, Rosen RT, eds. Food phytochemicals for cancer prevention II. Teas, spices and herbs. Washington, DC: American Chemical Society, (1994) 308–321.

Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of NF-kappa B. Annu. Rev. Cell Biol. (1994) 10, 405-455.

Smith TJ, Yang CS. Effects of food phytochemicals or xenobiotic metabolism. In: Huang MT, Osawa T, Ho CT, Rosen RT, eds. Food phytochemicals for cancer prevention I. Fruits and vegetables. Washington, DC: American Chemical Society, (1994) 17–48.

Snyder LM, Fortier NL, Trainor J, Leb J. Effect of hydrogen peroxide exposure on normal human erythrocyte deformability, morphology, surface characteristics, and spectrin-hemoglobin cross-linking. Journal of Clinical Investigation (1985) 76, 1971-1977.

Steinmetz KA, Potter JD. Vegetables, fruit, and cancer. II Mechanisms. Cancer Causes Control (1991) 2, 427–442.

Steller H, Grether ME. Programmed cell death in Drosophila. Neuron. (1994) 13(6), 1269-1274.

Stern A. Red cell oxidative damage. Academic Press, London, (1985) 331-349.

Sun SC, Ganchi PA, Ballard DW, Greene WC. NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. Science (1993) 259, 1912-1915.

Sutter MC, Wang YX. Recent cardiovascular drugs from Chinese medicinal plant [Review]. Cardiovasc. Res. (1993) 27, 1891-1901.

Tanaka M, Ito H, Adachi S, Akimoto H, Nishikawa T, Kasajima T, Marumo F, Hiroe M. Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. Circ. Res. (1994) 75(3), 426-433.

Traenckner EBM, Wilk S, Baeuerle PA. A proteasome inhibitor prevents activation of NF-κB and stabilizes a newly phosphorylated form of IκB-α that is still bound to NF-κB. EMBO J. (1994) 13, 5433-5441.

Traenckner EB, Pahl HL, Henkel T, Schmidt KN, Wilk S, Baeuerle PA. Phosphorylation of human I kappa B-alpha on serines 32 and 36 controls I kappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli. EMBO J. (1995) 14(12), 2876-2883.

Truman JW, Schwartz LM. Steroid regulation of neuronal death in the moth nervous system. J. Neurosci. (1984) 4(1), 274-280.

Tyler V. Herbs of choice. The therapeutic use of phytomedicinals. New York: Haworth Press, (1994).

Ursini F. The multilevel system against lipid peroxidation in living tissues. Richelieu Press, London, (1985) 1-32.

Uyama O, Matsuyama T, Michishita H, Nakamura H, Sugita M. Protective effects of human recombinant superoxide dismutase on transient ischemic injury of CA1 neurons in gerbils. Stroke (1992) 23(1), 75-81.

Van-Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNF--Induced Apoptosis by NF-κB. Science (1996) 274, 787-789.

Vaux DL, Haecker G, Strasser A. An evolutionary perspective on apoptosis. Cell. (1994) 76(5), 777-779.

Virchow R. Cellular pathology as Based upon Physiological and Pathological Histology. (transl. from 2nd ed. by F. Chance, 1860). Churchill, London (1858).

Wang CY, Mayo MW, Baldwin ASJ. TNF- and Cancer Therapy-Induced Apoptosis: Potentiation by Inhibition of NF-κB. Science (1996) 274, 784-787.

Wang L, Huang X, Ding Z, Chen H, Peng R, Yuan G, Zhou D. Adhesion of erythrocytes of patients with cerebral thrombosis to injured endothelial cells. Hua. Hsi. i. Ko. Ta. Hsueh. Hsueh. Pao. (1995) 26(3), 266-270.

Warshafsky S, Kramer RS, Sivak SL. Effect of garlic on total serum cholesterol: a meta-analysis. Ann. Intern. Med. (1993) 119, 599–605.

Wilhelmsen KC, Eggleton K, Temin HM. Nucleic acid sequences of the oncogene v-*rel* in reticuloendotheliosis virus strain T and ist cellular homolog, the proto-oncogene c-*re*l. J. Virol. (1984) 52, 172–182.

Williams GT. Programmed cell death: apoptosis and oncogenesis. Cell (1991) 65(7), 1097-1098.

Wong GH, Elwell JH, Oberley LW, Goeddel DV. Manganous superoxide dismutase is essential for cellular resistance to cytotoxicity of tumor necrosis factor. Cell (1989) 58, 923.

Wu W, Kuang P, Zhu K. The effect of radix salviae miltiorrhizae on the changes of ultrastructure in rat brain after cerebral ischemia. J. Tradit. Chin. Med. (Chinese) (1992) 12(3), 183-186.

Wyllie AH, Kerr JF, Currie AR. Cell death: The significance of apoptosis: Int. Rev. Cytol. (1980) 68, 251-306.

Xi SC, Che DY, Zhang WR. The inhibitory effect of Radix Salviae miltiorrhizaeon hypoxic structural remodeling of intra-acinar pulmonary arteries. J. Tongji Med. Univ. (Chinese) (1994) 14(3), 149-152.

Xia P, Wang L, Gamble JR, Vadas MA. Activation of sphingosine kinase by tumor necrosis factor-alpha inhibits apoptosis in human endothelial cells. J. Biol. Chem. (1999) 274(48), 34499-34505.

Yonehara S, Ishii A, Yonehara M. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptorof tumor necrosis factor. J. Exp. Med. (1989) 169, 1747-1756.

Zhang X, Wu W, Kuang P, Takemichi K, Kogo O, Hirobumi M, Yasaburo O. The effect of radix salviae miltiorrhizae composita and ligustrazine on lipid peroxidation in low density lipoprotein due to copper dichloride. J. Tradit. Chin. Med. (Chinese) (1994) 14(4), 292-297.

Zhang ZH, Yu SZ, Wang ZT, Zhao BL, Hou JW, Yang FJ, Xin WJ. Scavenging effects of tetramethylpyrazine on active oxygen free radicals. Acta. Pharmacol. Sin. *(*1994) 15*,* 229-231.

Zheng GQ, Zhang J, Kenney PM, Lam LKT. Stimulation of glutathione S-transferase and inhibition of carcinogenesis in mice by celery seed oil constituents. In: Huang MT, Osawa T, Ho CT, Rosen RT, eds. Food

phytochemicals for cancer prevention. I. Fruits and vegetables. Washington, DC: American Chemical Society, (1994) 230–238.

Zink S, Rosen P, Lemoine H. Micro- and macrovascular endothelial cells in beta-adrenergic regulation of transendothelial permeability. Am. J. Physiol. (1995) 269(5 Pt 1), C1209-1218.

Zou ZW, Xu LN, Tian JY. Antithrombotic and antiplatelet effects of rosmarinic acid, a water- soluble component isolated from radix Salviae miltiorrhizae (danshen). Yao Hsueh Hsueh Pao (Chinese) (1993) 28(4), 241-245.

8. Abbreviations

ActD	Actinomycin D
Amp	Ampicillin
APS	Ammoniumpersulfat
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets
d H_2O	Distilled water
Dhpl	3,4-dihydroxyphenyl lactate
DTT	Dithiothreitol
EC	Endothelial cells
EDTA	Ethylendiamintetraacetat
EGTA	Ethylene glycol-O,O'-bis (2-aminoethyl)-N,N,N',N',-Tetraacetic acid
EMSA	Electrophoretic Mobility-Shift Assay
H_2O_2	Hydrogen peroxide
IκB	Inhibitor of NF-κB
NF-κB	Nuclear transcription factor kappa B
NP-40	Nonidet P40
PAA	Polyacrylamid
PAC	Protocatechuic Acid
PAL	Protocatechuic Aldehyde
PBS	Phosphate buffered saline
RBC	Red blood cells
RSM	Radix Salviae miltiorrhizae
TEMED	N,N,N',N',-Tetramethylethylendiamin
TMP	Tetramethylpyrazine

TNFα	Tumor necrosis factor-alpha

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Yang FK, Wu ZQ, Zhou JF, Artmann G, Shi YD. Effects of ligustiazine on RBC deformation by Elias-c cytomechanical parameter analyzer. Medical Biophysics (Chinese), 1999 (9-10), 149-154.

Yang FK, Wu ZQ, Zhou JF, Artmann G, Shi YD. Chuan Qiong Qin (ligustiazine) from Ligusticum wallichii Frangchat inhibits platelet aggregation induced by adrenaline. Medical Biophysics (Chinese), 1999 (9-10), 144-148.

Sun AY, Zheng P, Zhou JF, Artmann GM. Salvia Miltiorrhiza (Danshen) extract induces pharmacological preconditioning and ischemic tolerance in rat brain. Submitted to Journal of Cerebral Blood Flow Metabolism, 2001.

Zhou JF, Yang FK, Shi YD, Yedgar S, Artmann G M. Antioxidative drugs and compounds evaluated by flow channel techniques on cell properties. Submitted to Journal of Biorheology, 2001.

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