THE EFFECTS OF ETHANOL EXTRACT PROPOLIS ABSORBED WITH NANOPARTICLES C60 FULLEREN ON ASTROGLIAL REACTIVITY AND CYTOSKELETON STATE IN PRIMARY RAT ASTROCYTE CELL CULTURE

Master Thesis

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Biology Department

Prof. Dr. Nedzvetskyi Viktor

2017

REPUBLIC OF TURKEY
BINGOL UNIVERSITY
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This master thesis on 17.01.2017 by the following jury memebers (The appropriate one will be stay, the other will be deleted) **Unanimously has been accepted.**

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AKNOWLEDGMENT

Praise be to God for giving us potency, primary astrocyte cell culture. I would like to express my deep appreciation and sincere graduate to my supervisor Prof. Dr. Nedzvetskyi Viktor for suggesting the project and for his generous help, guidance and research expertise. I would like to thank Prof. Dr. Giyasettin BAYDAŞ and Dr. Mudhir Sabir. They continuous support and help during my study. I wish to thank my brother Aso for his help and advices. I want to extend my thanks and appreciation to mother and father.

I would like to thank my colleagues Mr. Aso Nadhim, and all the staff members and postgraduate students in Molecular Biology Departments for their encouragement and support. Lastly,my deep thanks and appreciation to my manger Safin, and encouragement during my postgraduate study.

Dedication

I would like to dedicate this thesis to my wife Nazeera, who has been a constant source of support throughout the years of my postgraduate study. In addition, I would like to thank my family, especially my mother and father for constant support and encouragement.

To my brothers ,sisters, and my daughter Rona ,Rwany and Lanya

Raber Qader MAHMOOD

January 2017

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LIST OF ABBREVATIONS

EEP	:	Ethanol extract propolis
MCA	:	Mammary carcinoma
HeLa	:	Human epithelial carcinoma
HL	:	Human leukemia
SK-OV-3	:	Human ovarian carcinoma
NCI-H358	:	Human lung carcinoma
HepG2	:	Human hepatocellular carcinoma
ME180	:	Human cervical cancer
MDA	:	Malondialdehyde
CAT	:	Antioxidative enzyme catalase
ROS	:	Reactive oxygen species
GFAP	:	Clial fibrillary acidic protein
CNS	:	Central nervous system
IF	:	Intermediate filament
GLT-1	:	Glutamate transporters
SGZ	:	Sub granular zone
SVZ	:	Sub ventricular zone
bFGF	:	Basic fibroblast growth factor
FcR	:	Fc receptor
GDNF	:	Glial-cell line-derived neurotrophic factor
HSP	:	Heat-shock protein
ICAM	:	Intercellular cell-adhesion molecule
INOS	:	Inducible nitric oxide synthase
SCI	:	Cerebrospinal injury

OECs	:	Olfactory ensheathing cells
PGE	:	Prostaglandins
NO	:	Nitric oxide
BV	:	Blood vessel
AQP4	:	Aquaporin 4
ATPase	:	Adenosine tri phosphatase
TNFa	:	Tumor necrosis factor a
BBB	:	Blood brain barrier
EETs	:	Epoxyeicosatrienoic acid
CSF	:	Cerebrospinal fluid
GS	:	Glutamine synthase
MCP	:	Monocyte chemoattractant protein
MHC	:	Majorhistocompatibility complex
MRF	:	Microglial response factor
NGF	:	Nerve growth factor
TNF	:	Tumour necrosis factor
LPS	:	Iipopolysaccharide
hGFAP	:	Human GFAP gene
AD	:	Alzheimer's disease
AxD	:	Alexander disease
GFAPd	:	GFAP decrease
WHO	:	World health organization
ICH	:	Intracerebral hemorrhage
IS	:	Ischemic stroke
NF-kB	:	Nuclear factor-kappa-B
IκB-α	:	Inhibitor κB -α
COX-2	:	Cyclooxygenase-2
IKK	:	IκB-kinase

IL-1β	:	Interleukin-1β
NO	:	Nitric oxide
TNF-α	:	Tumor necrosis factor-α
DMEM	:	Dulbecco's modified Eagle's medium
MPO	:	Myeloperoxidase
NAD	:	Nicotinamide adenine dinucleotide
FBS	:	Fetal bovine serum
PBS	:	Phosphate buffered saline
MTT	:	Methylthiazolyl diphenyltetrazolium bromide
DMSO	:	Solution, dimethyl sulfoxide
PDL	:	Poly-D-lysine
SDS	:	Sodium dodecyl sulfate
TEMED	:	Ttetramethylethylenediamine

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İLKÖĞRETİM RAT ASTROSIT HÜCRE KÜLTÜRÜNDE ASTROGLIALREAKTİVİTE VE İSKELETİ DEVLET ÜZERİNE NANOPARTİKÜLLERİNİN C60 FULLEREN İLE EMİLEN ETANOL EKSTRAKTI PROPOLISIN ETKİLERİ

ÖZET

Astrositler insan beyninde en bol bulunan hücre tipidir ve normal şartlarda beyinde homeostasis ve sinaptik olgunlaşmada önemli rol oynar. Ayrıca, astrositler beyin hasarına yanıt olarak reaktif glioz gibi patolojik değişikliklerle karşılık verir. Propolis, birçok farklı kültürde kullanılan doğal bir üründür ve sağlığa faydalı birçok biyolojik aktivitesi vardır. Propolisle yapılmış birçok çalışma propolisin kimyasal yapısında bulunan birçok bileşiği ortaya çıkarmıştır ve bu çalışmalar göstermiştir ki propolisin kimyasal içeriği iklim, bitki örtüsü, bal arısı türleri gibi etkenlere bağlı olarak değişmektedir. Bu çalışmada propolisin etanol ekstraktının astroglial reaktivite ve astroglial hücre iskeleti üzerindeki etkilerinin moleküler açıdan gözlemlenmesi amaçlanmıştır. Sonuç olarak, propolisin etanol ekstraktı astrosit aktivasyonunu arttırmıştır. GFAP, Nf-kB ve anjiostatin ekspresyonunun artması da bu aktivasyonun bir göstergesidir. Normal doku ve hücreleri doksorubisin tarafından indüklenen kimyasal hasara karşı korumanın bir yolu da propolisin etanol ekstraktını başka doğal antioksidanlarla kullanmak olabilir. Son dönemde propolisin etanol ekstraktının antikanser çalışmalarında olumlu sonuç veren bir ajan olarak kullanıldığı bilinmektedir

Anahtar kelimeler: EEP, GFAP, Nf-kB, Angiostatines, Fullerene C60, astroglial reactivity.

THE EFFECTS OF ETHANOL EXTRACT PROPOLIS ABSORBED WITH NANOPARTICLES C60 FULLEREN ON ASTROGLIAL REACTIVITY AND CYTOSKELETON STATE IN PRIMARY RAT ASTROCYTE CELL CULTURE

ABSTRACT

Astrocytes are the most abundant cell-type of the human brain and play a variety of roles in brain homeostasis and synaptic maturation, under normal conditions. In addition, astrocytes undergo dramatic pathological changes in response to brain injury, such as reactive gliosis and glial scar formation. The implementation of new public healthcare models that stimulate the use of natural products from traditional medicine, as a so-called integrated medicine, refers to an approach that uses best of both conventional medicine and traditional medicine. Propolis is a widely used natural product by different ancient cultures and known to exhibit biological activities beneficial for health. A large number of studies conducted with propolis had identified that its chemical composition varies as a function of the climate, plant difference, and bee species and plays an important role on its therapeutic characteristics. The aim of the study is the molecular disclosure mechanisms of EEP effects on astroglial reactivity and cytoskeleton state in primary rat astrocyte cell culture. In Our study observed Ethanol Extract Propolis significantly induced astrocytes activation can lead to expression GFAP, Nf-kB, and angiostatin. One approach to protecting against Dox-induced chemical insult to normal tissues is a combined use of the EEP with antioxidants of a different nature. EEP has recently been recognized as a promising agent for use in anticancer therapy.

Key words: EEP, GFAP, Nf-kB, Angiostatines, Fullerene C60, astroglial reactivity.

1. INTRODUCTION

Propolis, or bee glue, is a sticky and resinous material produced by honey bees (Apis mellifera) to seal up their hives. This material is enhanced with salivary and enzymatic secretions. A constituent of propolis is directly derived from bud exudates collected by worker bees from wide variety of plants including birch, poplar, pine and alder. The composition also varies depending on the climatic and geographic area where it was collected; therefore, propolis from Europe, North and South America, Asia and Africa are different in their chemical composition. So far, more than 200 constituents have been identified indifferent kinds of propolis, with at least 300 ineachone (e.g. unsaturated fatty acids, esters, flavonoids, terpenes , β-steroids, aromatic aldehydes and alcohols) (Hayakari et al. 2013). The composition of propolis is directly related to bud exudates collected by honey bees from various trees. Flavonoids are also responsible for antioxidant activity. The antioxidant activity of these components is principally based on their radical scavenging effect. Propolis is also reported to inhibit the generation of superoxide anion. Besides, propolis has been determined to reverse the consumption of glutathione, which is synthesized in the liver and has radical scavenging activity (Yonar, Yonar, Coban, and Eroglu. 2014). Propolis has been used in folk medicine since ancient times, and even today it is one of the most famous remedies (Hayakari et al. 2013). In recent years, green propolis has been widely used because of its characteristic chemical composition and biological activities. In Japan in particular, Brazilian propolis is utilized extensively in foods and beverages with the aim of maintaining or improving human health (Shimazawa et al. 2005) . Although the composition of propolis varies as a function of its botanical and geographical. Propolis is viewed as biotherapy product because it contains organic secretions of the bees that produce it. The most known pharmacologically active chemical components in propolis are flavonoids phenolic acid, xanthones and guttiferones , which account for its antimicrobial, anti-inflammatory, antioxidant, antiviral, antifungal

and anticancer actions, among other. Climate variations might affect changes in the concentration of bioactive compounds of plants, with consequent in the biological activity of the various types of propolis. Although, therapeutic standardization of propolis is testing, and the relationship between definite types of propolis and specific biological activities is difficult to establish, the presence of a significant amount of one specific compound might lead to the expectance that the extract has the potential to show bioactivities connected to this potential. The chemical composition of Brazilian propolis is quite variant from that of propolis from European countries as a function of the tropical climate, plant diversity and bee species, the latter resulting from the crossing of European and African species. Those factors play a relevant role in the physical, chemical and biological properties of propolis (de Mendonca et al. 2015). The organic solvents were utilized to extract the chemical composition from propolis such as water, ethanol, methanol, hexane and acetone (Sun and Ho, 2005). The organic solvent can dissolve the different chemical compounds according to a polarity of substance. Ethanol was utilized to extract propolis to generate the fatty acid and flavonoids, while acetone extraction generated monosaccharide, glycerol and caffeic acid. Alkane, alcohol and bee wax were found in hexane fraction of propolis . Besides, the extraction time, light and temperature influenced propolis extraction (Khacha-ananda, Tragoolpua, Chantawannakul and Tragoolpua. 2013). The information about influence EEP as a potential therapeutic substance on such physiologically normal cells is extremely important. At the same time, EEP can leads to oxidative stress and mitochondrial misbalance into non cancer cells. These changes can directly cause hypoxia in the neurons and glial cells and not necessarily via ROS. In addition to the damages induced by ischemia and/or infarction, free radicals released may contribute to brain injuries by head trauma and hemorrhage. Toward the goal of reducing the oxidative stress in brain damage, a number of studies have focused on developing new neuroprotective agents. Pristine C_{60} fullerene, along with some water-soluble derivate, has recently gained considerable attention as a promising candidate for many biomedical applications. Recent data show high antioxidant potential of fullerenes C60 in vivo. Fullerene C60 can be used as free-radical eliminating agent for treating diseases, which are associated with abnormally high levels of membrane lipid per oxidation. It is well known that astrocytes are directly involved in the process of neuronal injury and regeneration. Astroglia also produce a host of atrophic factors, which are crucial for the survival of neurons. However, activated astroglia

become hypertrophic, exhibit increased production of glial fibrillary acidic protein (GFAP). Thus the characteristics astroglial reactivation could be beneficial for knowledge the effects of natural honeybee products combined with fullerene nanoparticles on the neural tissue cells. The aim of study is the disclosure molecular mechanisms of EEP effects on astroglial reactivity and cytoskeleton state in primary rat astrocyte cell culture (Markiewicz and Zukowska et al. 2012).

1. LITERATURE REVIEW

2.1. Propolis Beneficial Effects

Propolis has large amount of biology effects including antibacterial, antifungial, antiviral, hepatoprotective and immunomodulatory. Flavonoids, cinamic acid derivatives, steroids, amino acids and vitamins such as B1, B2, E, C belong to important components of propolis. Antiproliferative/cytotoxic in vivo activity of propolis in the cells of neural tissue remain unknown. The understanding cytotoxicity of honeybee products can provide its advancing for modulation of cell vitality and clearing of synergistic interaction of compounds in the propolis extract (Markiewicz and Zukowska et al. 2012). Flavonoids, cinamic acid derivatives, steroids, amino acids and vitamins such as B1, B2, E, and C belong to important components of propolis. The anti-cancer effects of ethanol extract propolis (EEP) have been presented in various cell lines such as mammary carcinoma (MCA), human epithelial carcinoma (HeLa), human leukemia (HL-60, CI41, U937), human ovarian carcinoma (SK-OV-3), human lung carcinoma (NCI-H358), human hepatocellular carcinoma (HepG2), human cervical cancer (ME180), human pancreatic cancer (PANC-1) and BxPC-3 cells . Although many studies presented the proapoptotic effect induced by propolis. Some researchers have demonstrated their different effect on cancer and normal cells. The Chinese hamster lung fibroblast cells V79 after treatment of propolis or human lung fibroblast WI-38 in the presence of CAPE demonstrated no changes that occurred in cancer cells. There was shown that the mixture of different flavonoids (baicalein, galangin, genistein, quercetin and naringenin) added to culture of human breast cell line were more cytotoxic for tumor cells than individual flavonoid alone. Recent research on cytotoxicity of propolis on HeLa cells demonstrated also synergistic antiproliferative activity of phenolic acid and flavonoids contained in propolis presented antyproliferative/cytotoxic in vitro activity of propolis in cancer cell lines and in human foreskin fibroblast cell line and suggested that such effects are due to existence or synergistic interaction of compounds in the propolis extract. These

observations suggest that different mechanism of cells growth inhibition may be involved in case of products containing the mixture of flavonoids compared to their individual compounds. Recent results suggest that not only main compounds as chrysin and CAPE but also other bioactive chemicals of EEP determine activity of natural honeybee products. Propolis is used by the patients with various diseases, especially by patients with glioma tumors. It was reported the anti-tumoral activity of propolis in C6 glioma cells. Moreover, the effect of this natural product and its active ingredients on the viability of nontumor cells as astrocytes is unknown. Astrocytes are the most abundant cells in the Central Nervous System that constitute no less 50% of all glial cells and modulate synaptic transduction through astrocyte-neuron interaction (Markiewicz and Zukowska et al. 2012). The examination of new antioxidants as potential therapeutic agents is an active field of biochemistry. A variety of organic forms of antioxidant molecules have been studied as natural therapeutic and preventive agents. Propolis is a phenolic compound which may be capable of preventing apoptosis by decreasing oxidative stress. The most widely utilized assay for lipid peroxidation is malondialdehyde (MDA) formation which represents the secondary lipid peroxidation product with the thiobarbituric acid reactive substances test. MDA is the final product of lipid peroxidation. The concentration of MDA is a measure of free radical damage to lipids. Lipid peroxides could change the properties of biological membranes, resulting in eventual cell damage.In order to minimize such damage, cells have evolved defense systems containing both enzymatic and nonenzymatic processes. An example of an enzymatic defense system is the antioxidative enzyme catalase (CAT). Flavonoids comprise nonenzymatic antioxidant molecule. Flavonoids are potent antioxidants, free radical scavengers and metal chelators. The different antioxidant properties of these compounds help to prevent the irreversible lipid peroxidation which occurs with oxidative stress (Kakoolaki Talas Cakir Ciftci and Ozdemir 2013).

2.2. Astrocytes

The term neuroglia or Nervenkitt (i.e. nerve-putty) was initially introduced by Rudolf Virchow, acelebrated pathologist in the 1850's. Virchow pictured neuroglia as small round-shaped cells that filled up the extracellular space and were part of the connective tissue. Although the term neuroglia survives, our knowledge on the diversity and

properties of neuroglial cells, and in particular astrocytes, has dramatically changed. Astrocytes have been viewed as a homogeneous cell population that have a star-shaped morphology [Figure 2.1]. Extend numerous processes surrounding neighboring neurons and blood vessels, and contain intermediate filaments (glial fibrils). While astrocytes are classically defined by their morphology and expression of glial fibrils, defining a cell as an astrocyte is not a simple task as discussed in a recent review. With the development of electrophysiological, molecular, and genetic tools, it is now well accepted that astrocytes represent a various population of cells with numerous functions. In addition, the finding that a subpopulation of GFAP-expressing cells displays neural progenitor or stem cell features and that astrocytes possess neuronal properties (Wang and Bordey 2008).

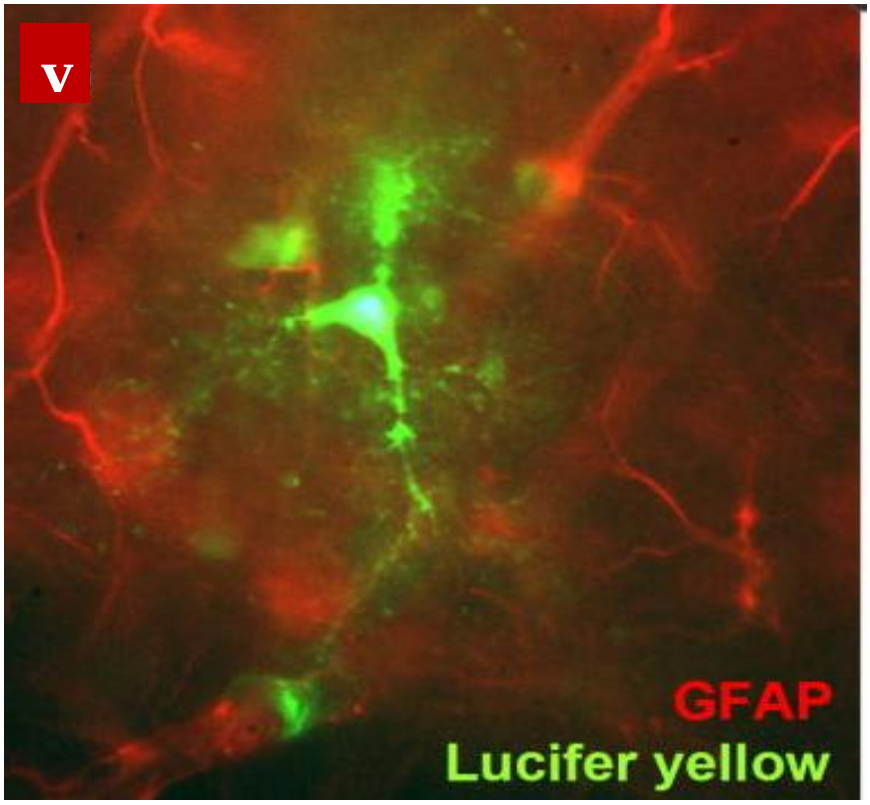

Figure 2.1. Morphology of astrocytes

Photographs of astrocytes recorded in the hippocampus and filled with lucifer yellow during patch clamp recording. Immunostaining for GFAP was overlaid with the lucifer yellow fill. The cell displays dye coupling to other cells and send a process ensheathing a blood vessel typical of an astrocyte, but does not stain for GFAP (Wang and Bordey 2008)

Glial cells are the main non neuronal component in the nervous system, and they have been suggested as playing a role in early pattern formation and in the establishment of neural pathways. In culture, glial cells are preferred substrates for axon elongation. Nonneural “primitive glial” cells also appear to provide a favorable substrate for early axon development in vivo. A scaffold of primitive glia may be involved in the initial patterning of axonal pathways in the insect nervous system and glial cell disturbances are sufficient to cause axon tract malformations. In vertebrates, the earliest tracts form superficially, and development cones extend in contact with the endfeet of radial neuroepithelial cells. Optic axons course along radial cell endfeet en route to their targets in the brain, and the hamster cerebral cortex. Other evidence suggests that several glial tissues form boundaries inhibitory to axon growth. Examples of structures that function as barriers include the roof plate of the spinal cord and the glial “knot” located in the rostral midline of the optic chiasm. Thus non neural cells may contribute to the patterning and formation of the nervous system by acting as guides or barriers at critical stages of improvement. Although embryonic non neural cells have often been called “primitive glia,” their glial identity is uncertain because it has often been based on morphological criteria alone. Electron microscopic studies of early axon out growth identified the neuroepithelial endfeet contacted by growth cones as belonging to “primitive radial glia, but in the loosing of any independent evidence, this nomenclature must be considered speculative. The recent development of cell-specific markers provides a useful method to study identified populations of cells, and in this study we use the glial-specific intermediate filament, glial fibrillary acidic protein, to identify presumptive glia during early stages of development. Antisera to GFAP have been utilized extensively to visualize mature and developing glial cells in many species. To investigate possible roles of glia in axon outgrowth, it is important to investigate the appearance and distribution of these cells in a system in which the development of the earliest neurons and their axonal tracts is well established (Marcus and Easter 1995). Although damage to adult mammalian central nervous system (CNS) leads to persistent functional deficits for the absence of axonal regeneration and reconnection with correct synaptic targets. The failure of spontaneous anatomical and functional repair is due not merely to the intrinsic incapacity of the neuron to regenerate but rather to the presence of a hostile environment in the lesion site. As the major cell type in CNS, astrocytes provide a variety of critical supportive functions that maintain neuronal homeostasis. When the CNS is damaged,

astrocytes undergo an injury response and become reactive, characterized by hyperplasia, hypertrophy and an massive up-regulation of intermediate filament (IF) proteins, and leads eventually to the formation of a dense glial scar network at the lesion site. The glial scar which composed primarily of reactive astrocytes has long been implicated as a major impediment to axon regeneration and functional outcome after SCI and other forms of CNS injury. It constitutes a mechanical obstacle and a biochemical barrier to preventing successful regeneration, as several classes of growth inhibitory molecules are up regulated and have been appeared to contribute to the failure of axon regeneration. On the other hand, increasing evidence indicates that glial scar might also possess several important beneficial functions for example stabilizing fragile CNS tissue after injury. After injury, reactive astrogliosis form a dense scar tissue that has been suggested to seclude inflammatory cells, demarcate the lesion area, and separate the injured tissue from its surroundings. Astrocytes have an important scavenging activity, which is crucial for regulating the top levels of glutamate, K^+ and other ions. However, the glial scar is reported to fill the gaps in the lesion area, creating a scaffold for the vascularization network. Olfactory ensheathing cells (OECs) are the glial cells that get from the olfactory placode and envelop olfactory axons in the course of migration from the olfactory epithelium to the bulb. Owing to the axonal growth-promoting properties and the superior capacity to interact with astrocytes, OECs transplantation has emerged as a promising experimental therapy to treat axonal injuries and been shown to induce anatomical and functional repair of lesions of spinal cord. After SCI, there foundation of neural connections depends not only on the ability of nerve fibers to regeneration but also on the provision of a pathway along which they can elongate to reach appropriate destinations. Transplanted OECs have been shown to migrate with regenerating axons through an unfavorable CNS environment and to blend well with astrocytes in adult brain. Interaction with astrocytes at the lesion site results in the creation of an OEC channel between the host astrocytic pathways on either side of the lesion, being devoid of inhibitory molecules and providing a pathway for the severed axons to regenerate successfully across the lesion and reach tissue targets. Moreover, the mechanism underlying OECs migration into the lesion region remains elusive. Here, we provided the first evidence that reactive astrocytes attract OECs migration by secreted TNF-a not only in vitro but also in damaged spinal cord, suggesting a mechanism for guiding OECs

migration into glial scar, which is crucial for OECs mediated axons regeneration beyond the lesion site (Su et al. 2009).

2.3. Astrocytes Development

The developmental generation of astrocytes tends to occur after the initial production of neurons in many CNS regions. Nevertheless, astrocytes exert a number of important functions during development of both gray and white matter. Molecular boundaries formed by astrocytes take part in guiding the migration of developing axons and certain neuroblasts. In addition, substantive occur is accumulating that astrocytes are essential for the formation and function of developing synapses by releasing molecular signals such as thrombospondin. Astrocytes appear also to influence developmental synaptic pruning via releasing signals that induce expression of complement C1q in synapses and thereby tag them for elimination by microglia. As regards the development of white matter, the loss of astrocyte connexins and gap junctions leads to dysmelination (Sofroniew and Vinters 2010).

2.4. Astrocytes Function

2.4.1. Regulation Of Blood Flow

Astrocytes make extensive contacts with and have multiple bidirectional interactions with blood vessels, including regulation of local CNS blood flow. Recent findings show that astrocytes produce and release various molecular mediators, such prostaglandins (PGE), nitric oxide (NO), and arachidonic acid (AA), that can increase or decrease CNS blood vessel diameter and blood stream in a coordinated manner. However, astrocytes may be primary mediators of changes in local CNS blood flow in response to changes in neuronal activity. Astrocytes have processes in contact with both blood vessels and synapses [Figure 2.2. A, B]. By these contacts, astrocytes titrate blood flow in relation to levels of synaptic activity, as demonstrated recently in the visual cortex where fMRI detected changes in blood flow in response to visual stimuli were shown to be dependent on astrocyte function (Sofroniew and Vinters 2010).

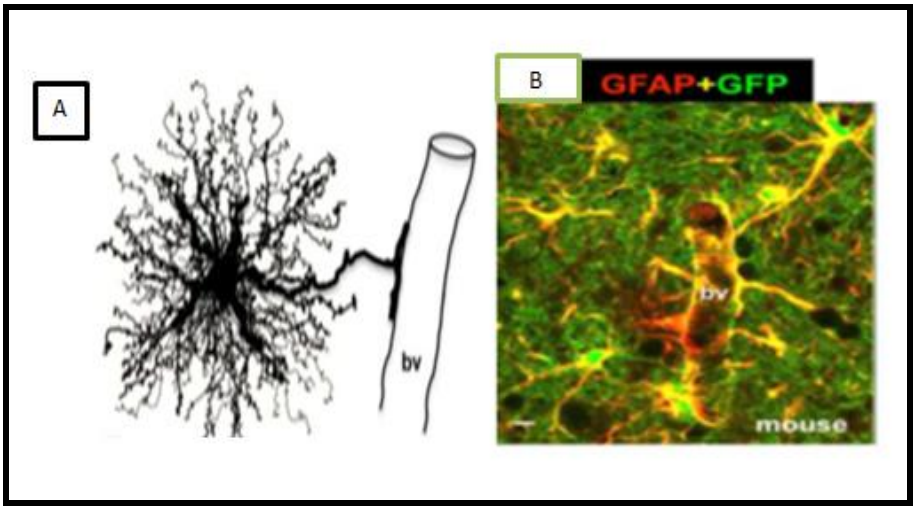

Figure 2.2. Astrocyte morphology and interactions with blood vessels in healthy and diseased tissue . A. Protoplasmic astrocyte giving rise to a dense network of finely branching processes throughout its local gray matter neuropil, as well as to a large stem branch that extends foot processes along a blood vessel (BV). B. Two color fluorescence showing astrocytes in healthy mouse gray matter stained immunohistochemically for GFAP (red) as well as the transgene-derived reporter molecule GFP (green) (Sofroniew and Vinters 2010)

2.4.2 Fluid, Ion, PH, And Transmitter Homeostasis

Astrocyte processes envelop fundamental all synapses and exert essential functions in preserving the fluid, ion, pH, and transmitter homeostasis of the synaptic interstitial fluid in a manner that is critical for healthy synaptic transmission [Figure 2.3.A]. Astrocyte processes are rich in the aquaporin4 (AQP4) water channel and in transporters for the uptake of K. Astrocyte membranes have variant means of proton shuttling, including the Na^{+2}/H exchanger, bicarbonate transporters, monocarboxylic acid transporters, and the vacuolar-type proton ATPase. AQP4 water channels are densely clustered along astrocyte processes that linked blood vessels and play a critical role in regulating fluid homeostasis in healthy CNS and play roles in both vasogenic and cytotoxic edema as discussed below. Astrocyte processes at synapses also play essential roles in transmitter homeostasis by expressing high levels of transporters for neurotransmitters such as glutamate, GABA, and glycine that serve to clear the neurotransmitters from the synaptic space. After uptake into astrocytes, the transmitters are changed by enzymes such as glutamine synthetase into precursors such as glutamine and recycled back to synapses for reconversion into active transmitters [Figure 2.3]. Networks of astrocytes linked together by gap junctions

are able to rapidly dissipate small molecules such as potassium and glutamate and prevent their potentially detrimental accumulation (Sofroniew and Vinters 2010).

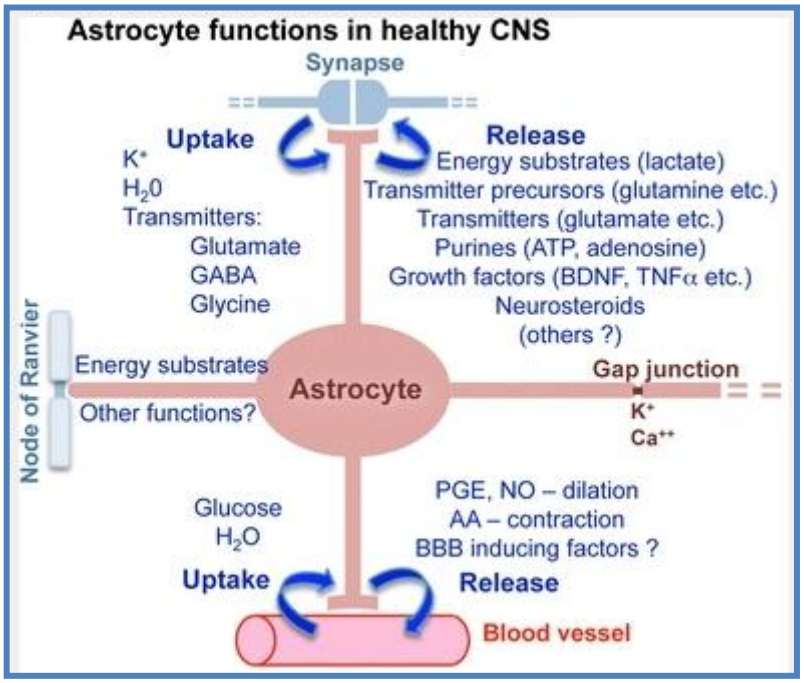

Figure 2.3. Schematic representations that summarize astrocyte functions in healthy CNS (Sofroniew and Vinters. 2010)

2.4.3. Roles İn Synapse Function

There is now steadily accumulating evidence that astrocytes play direct roles in synaptic transmission through the regulated release of synaptically active molecules such as glutamate, purines (ATP and adenosine), GABA, and D-serine. The release of such gliotransmitters occurs in response to changes in neuronal synaptic activity, involves astrocyte excitability as reflected by increases in astrocyte [Ca^{+2}], and can alter neuronal excitability. such has given rise to the 'tripartite synapse' hypothesis, which posits that astrocytes play direct and interactive roles with neurons during synaptic activity in a manner that is fundamental for information processing by neural circuits. Besides, to having direct effects on synaptic activity via the release of gliotransmitters, astrocytes have the potential to exert powerful and long-term influences on synaptic function through the release of growth factors and related molecules [Figure 2.3]. Molecular mechanisms have been identified through which astrocytes play a role in the formation,

maintenance, and pruning of synapses during development. Such mechanisms may also provide astrocytes with the means of exerting powerful effects on synaptic remodeling and pruning in the healthy adult CNS or in response to CNS disorders. Cytokines including tumor necrosis alpha (TNFa) have been shown to influence homeostatic synaptic scaling by inducing the insertion of AMPA receptors at post-synaptic membranes. Although it is not certain whether astrocytes or microglia are primary sources of TNFa in the CNS in vivo, the effects on synaptic function of astrocyte derived growth factors and cytokines warrant further study. Astrocytes are also sources of neuroactive steroids (neurosteroids), such as, estradiol, progesterone and various intermediaries and metabolites that can have synaptic effects, particularly at GABAA receptors (Sofroniew and Vinters 2010).

2.4.4. Roles In Energy

A growing body of evidence also now indicates that astrocytes make important contributions to CNS metabolism. Astrocytes, which have processes that on the one hand contact blood vessels and on the other hand linked with neuronal perikarya, axons (at nodes of Ranvier), and synapses, are well positioned to take up glucose from blood vessels and furnish energy metabolites to different neural elements in gray and white matter [Figure 2.4.]. Although it was known for many years that astrocytes are the principal storage sites of glycogen granules in the CNS and that the greatest accumulation of astrocytic glycogen occurs in areas of high synaptic density, the functional contribution of these stores was originally discounted. Compelling evidence now demonstrates that astrocytic glycogen usage can sustain neuronal activity during hypoglycemia and during periods of high neuronal activity. In this regard, it is noteworthy that astrocyte glycogen content can be modulated by transmitters including glutamate and that glucose metabolites can be passed across gap junctions in a manner that is regulated via glutamate and neuronal activity. Other lines of evidence indicate that during hypoglycemia, astrocyte glycogen breaks down to lactate that is transferred to adjacent neural elements (both synapses in gray matter and axons in white matter) where it is used aerobically as fuel. Moreover, computer-based modeling studies suggest that during periods of high neuronal activity, inhibition of phosphofructokinase leads to impairment of neuronal glycolysis, with the consequence that lactate effluxes from

astrocytes becomes the preferred energy substrate for neuron (Sofroniew and Vinters 2010).

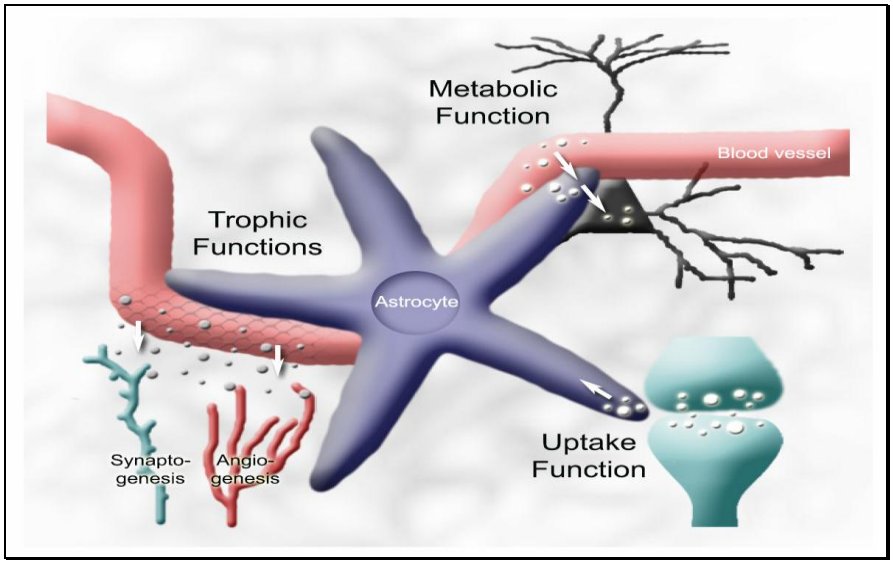

Figure 2.4.The well-established functions astrocytes have several homeostatic functions maintaining a viable nervous system environment for neurons. These functions include: (1) providing metabolic support for neurons, (2) taking up K^+ and neurotransmitters, (3) Synaptogenesis, angiogenesis, and BBB maintenance (Wang and Bordey 2008)

2.4.5. Blood Brain Barrier

The blood brain barrier (BBB) is a diffusion barrier that impedes the influx into brain parenchyma of numerous molecules on the basis of polarity and size. The principal cellular constituents of the BBB are cerebral capillary endothelial cells that form tight junctions and are surrounded by a basal lamina, perivascular pericytes, and astrocyte end feet. The role of pericytes in BBB function is not well studied and the role of astrocytes is controversial. The main functional components of the BBB are the endothelial tight junctions. Various lines of in vitro evidence indicate that astrocytes can induce barrier properties in cerebral and other endothelial cells as well as in related epithelial, arguing in favor of a role for astrocytes in BBB induction. In contrast, numerous aspects of the BBB become functional in vivo before the appearance of astrocytes during development. In such manner, it is interesting to note that embryonic neural progenitor cells are able to induce BBB properties in cerebral endothelial cells (Sofroniew and Vinters 2010).

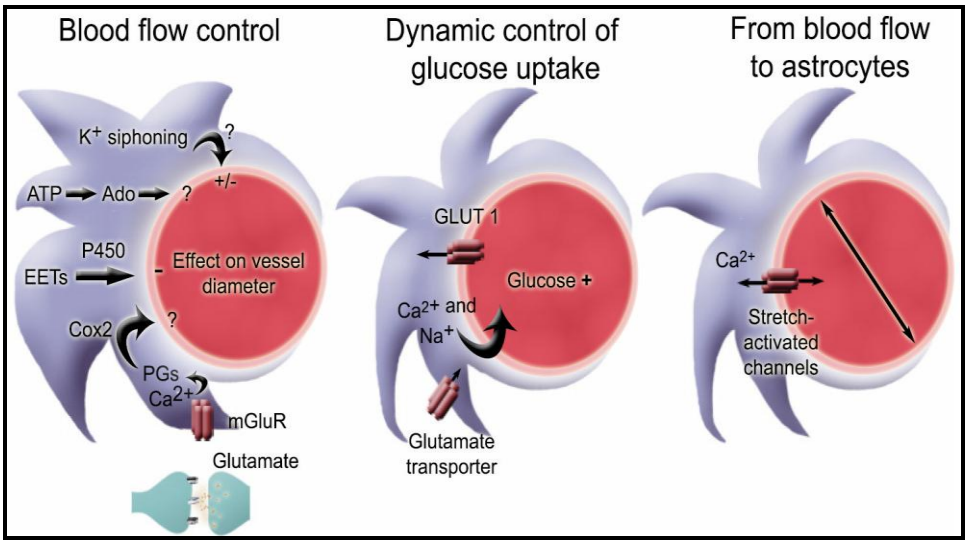

Figure 2.5. Astrocyte interactions with the vasculature (Sofroniew and Vinters 2010)

Astrocytic processes ensheath blood vessels, including arterioles, which are composed of endothelial cells and smooth muscles cells. The smooth muscle cells allow the vessels to contract or dilate. Neuronal activity induces mGluR [Figure 2.5]. Activation in astrocytes leading to the synthesis of arachidonic acid and the formation of downstream messengers, including prostaglandins (PGs) and epoxyeicosatrienoic acid (EETs) via Cox^2 and P450, respectively. PGs (in particular PGE2) and EETs induce vessel dilation. AA can also pass from astrocytes to smooth muscle cells where its downstream product 20-hydroxyeicosatetraenoic acid induces vasoconstriction. In addition the activation of Ca^{2+} activated K^+ channels in astrocyte end feet and the efflux of K^+ from astrocytes and subsequently from smooth muscle cells has been suggested to modify vascular tone by hyper polarization and relaxation of smooth muscle cells, but this does not occur in the retina (Metea et al. 2007). Besides, Glutamate uptake into astrocytes is accompanied by Na^+ entry leading to $Ca2^+$ elevation as a result of Na^+/Ca^{2+} exchange. Together Na^+ and Ca^{2+} can stimulate glucose uptake from the blood into astrocytes via GLUT1. One newer hypothesis is whether changes in blood vessel diameter can affect the biology of astrocytes via activation of stretch-activated channels that can be permeable to Ca^{2+} or other ions (Wang and Bordey 2008).

2.4.6. Spine Formation

Astrocytes promote synaptogenesis during development by releasing many diffusible molecules. In 1992, it was shown that astrocyte-conditioned medium promotes the proliferation of spines prior to the appearance of axons, an event that introduce synapse formation. Protrusive dendritic activity is prominent in developing neurons. It is thought that a subset of these locomotive protrusions are stabilized and transformed into stable spines with synaptic contacts. A recent study using two-photon time-lapse imaging in cultured slices observed that astrocytic motility was essential for stabilization of individual dendritic protrusions and their subsequent maturation into spines [Figure 2.6]. In this study, manipulating Rac1-dependent signaling in astrocytes resulted in institution of longer, filopodia like dendritic protrusions. Rac1 belongs to the family of small GTPases of the Rho family that are important regulators of the actin cytoskeleton. Furthermore, in the same study, manipulation of ephrin/Eph-dependent neuron-astrocyte signaling suggested the involvement of this signaling pathway in astrocyte-dependent stabilization of newly generated dendritic protrusions. An earlier study showed that the membrane-bound ligand, ephrin-A3, on astrocytes dynamically regulates spine morphology in the hippocampus via local activation of EphA receptors on spines in acute slices. Improvements in imaging resolution and molecular tools to label variant cell types and manipulate their properties would improve our understanding of the function of astrocytes on spine formation and stability (Wang and Bordey 2008). Astrocytes encapsulate synapses including spines. This ensheathment allows spines to remain stable. At the molecular level, the ephrin-A3/EphA4 receptor signaling between astrocytic processes and spines has been shown to regulate the morphology of dendritic spines. In the absence of astrocytic processes, motile filopodia extend from dendrites. The astrocytic contact allows the filopodia to transform into a mature spine. Besides, Astrocytes are an active synaptic partner. Not only they take up glutamate via high affinity transporters, but they sense glutamate escaping synaptic clefts and release neuroactive substances upon glutamate receptor activation and intracellular Ca^{2+} elevation. Neuroactive substances include glutamate that is released via either vesicles or via Ca^{2+} dependent chloride channels, or both. Glial glutamate activates extrasynaptic receptors, including NR2B, on neurons leading to changes in synaptic integration (Wang and Bordey 2008).

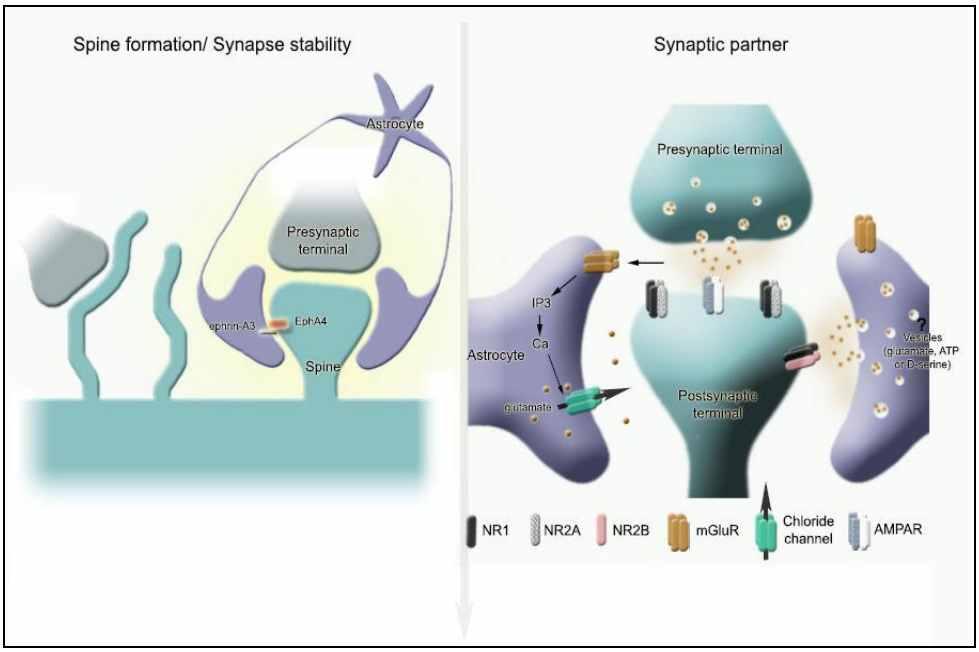

Figure 2.6. Astrocyte interactions with synapses (Wang and Bordey 2008)

2.4.7. Detoxification And Immune Functions

One of the most important roles of astrocytes is to protect neurons against excitotoxicity via capturing excess ammonia and glutamate and converting them into glutamine. In addition, astrocytes may also participate in the uptake of some heavy metals, such as lead. Astrocytes contain metal binding proteins including metallothioneins that endow astrocytes with both neuroprotective and neuroregenerative properties following injury or exposure to toxic metals. Astrocytes can protect a bridge between the CNS and immune system. In particular, astrocytes can phagocytose cells and act as antigen-presenting cells. Such as, cultured astrocytes were shown to present antigens to T lymphocytes in a specific manner which is restricted by the major histocompatibility complex, and in particular they could activate myelin basic protein specific encephalitogenic T-cell lines. Astrocytes capable to express class II major histocompatibility complex antigens and costimulatory molecules (B7 and CD40) that are critical for antigen presentation and T-cell activation. Moreover, astrocytes were found to express receptors involved in innate immunity, including Toll-like receptors, nucleotide-binding oligomerization domains, double-stranded RNA-dependent protein kinase, scavenger receptors, mannose receptor and components of the complement system. Finally, astrocytes produce a wide array of

chemokines and cytokines that act as immune mediators in cooperation with those produced by microglia. One intriguing idea is that molecules involved in immune responses may protect additional roles as adhesion molecules between astrocytes and neuronal elements such as dendritic spines. However, this proposed function has not been explored and remains to be elucidated (Wang and Bordey 2008).

2.5. Astrocyte Heterogeneity

The increasing awareness of the complexity, importance, and variety of astrocyte functions is giving rise to a growing interest in the potential for specialization and heterogeneity among astrocytes. The notion of astrocyte heterogeneity is not new. Various anatomical distinctions have long been recognized among gray (protoplasmic) and white (fibrous) matter astrocytes in different CNS regions, but these have long been over looked or discounted. Moreover, there is a long-standing recognition of an extended family of astroglial cells that share similarities with, but also exhibit differences to, protoplasmic and fibrous astrocytes, including Muller glia in the retina, Bergmann glia of the cerebellum, tanycytes at the base of the third ventricle, pituicytes in the neurohypophysis, cribrosocytes at the optic nerve head, and others. These different cell types express various astrocyte-related molecules such as GFAP, S100b, glutamine synthetase and others, and exert functions similar to astrocytes in manners specialized to their locations. In addition, these different types of astroglial cells share with astrocytes the ability to become reactive in response to CNS insults and these cells have the potential to play important roles in pathological changes in their specific locations as discussed below. As regards protoplasmic and fibrous astrocytes, various lines of evidence suggest that there is considerable molecular, structural, and potentially functional diversity of astrocytes at both the regional and local levels, but such investigations are at an early stage. In this context it is interesting to note that the number, complexity, and diversity of astroglial cells related with neurons has increased considerably with evolution, such that the ratio of astrocytes to neurons is 1:6 in worms, 1:3 inrodent cortex, and 1.4:1 in human cortex, implying that astrocyte roles increase in importance with sophistication of neural tissue. The human cerebral neocortex contains multiple subtypes of astrocytes, such as types that do not appear to exist in rodent cortex. As molecular markers become more sophisticated, it seems likely that astrocytes will be

revealed to be considerably more heterogeneous than thus far imagined. Such knowledge is likely to impact on concepts about astrocyte roles in both health and disease (Sofroniew and Vinters 2010).

2.6. Astrocytic Biomarkers

Astrocytes are commonly distinguished by the presence of intermediate filaments (glial fibrils), which are more prominent in white matter than gray matter astrocytes. The major component of glial fibrils, glial fibrillary acidic protein (GFAP), is thought to be specific for astrocytes in the CNS. Moreover, the low expression of GFAP may not be readily detected by immunohistochemistry, leading to confusing results regarding the identity of astrocytes. As an example, the cell filled with the fluorescent dye lucifer yellow in Figure 2B was GFAP-immunonegative, but had endfeet covering a blood vessel, suggesting that it may be an astrocyte. It is possible that whole cell patch clamp recordings render the GFAP antigen inaccessible for the anti-GFAP antibody. Other defining characteristics or ways to visualize filaments (such as by electron microscopy) are thus required to define a cell as an astrocyte. In addition, GFAP is expressed by other cell types in the CNS (i.e. ependymal cells, which are also derived from radial glia) that share similar properties with astrocytes, but are not typically part of the astrocyte family. Ependymal cells form an epithelial layer lining the walls of the cerebral ventricles and display morphological features different from astrocytes such as motile cilia. They function as a barrier between the brain parenchyma and cerebrospinal fluid (CSF) and play a role in cerebral fluid balance, toxin metabolism and secretion into the CSF. They thus have specific functions that distinguish them from astrocytes. Amazingly, GFAP has also been situated in rat kidney glomeruli and peritubular fibroblasts, leydig cells of the testis, skin keratinocyte, osteocytes of bones, chondrocytes of epiglottis, bronchus, as well as stellate-shaped cells of the pancreas and liver. Another commonly used astrocytic marker is S100B, which belongs to the S100 family of EF-band calcium binding proteins. Although, S100B is only expressed by a subtype of mature astrocytes that ensheath blood vessels and by NG2-expressing cells. NG2-expressing cells are found in both the progressing and adult mammalian CNS, and until recently, were referred to as smooth protoplasmic astrocytes because of their astrocytic appearance with less branched processes and a paucity of intracellular filaments. However, they are GFAP immunonegative and have been located

in the oligodendrocyte lineage, and now they are commonly referred to as oligodendrocyte precursor cells (OPCs) or NG2 cells. Other markers thought to be exclusive to astrocytes include, but are not fixed to, the glutamate transporters GLT-1, glycogen granules, and glutamine synthase (GS), which is an enzyme that catalyzes the alteration of ammonia and glutamate to glutamine. However, GS is also observed in white and gray matter oligodendrocytes (Wang and Bordey 2008).

2.7. Implications For Parenchymal Astrocytes

The presence of these neurogenic astrocytes challenges the traditional definition of astrocytes as described in section, which are viewed as mature, fully-differentiated cells. In addition, this raises some interesting questions regarding astrocytes: Can all astrocytes act as stem cells if placed in the right environment? Or do mature astrocytes undergo an irreversible genetic mutation which seals their fate as postmitotic cells? Can astrocytes in the adult brain be reactivated to regain stem cell features and help repair the damaged brain. These questions will likely lead to mixed answers. First, transplantation experiments have underscored the importance of the niche in regulating cell potential. For instance, cultured SGZ progenitors produce granule neurons when transplanted into another SGZ, but not to non-neurogenic brain regions. The instructive role of the niche is also exhibit when SGZ progenitors grafted into the rostral migratory stream can adopt the fate of SVZ neural precursors to produce olfactory bulb interneurons. Based on this evidence, we can speculate that the radial glial-derived astrocytic stem cells need the unique components of the niche in order to retain their neurogenic potential. Second, it was recently demonstrated that postnatal astrocytes from P5-7 mice can be reverted into a stem cell when reactivating the proper genetic program in vitro. In particular, the proneural genes neurogenin-2 and Mash1 possess the ability to reprogram these mature astrocytes to stem cells that can generate neurons. These astrocyte-derived neurons display neuronal excitability and also receive synaptic inputs when co-cultured with embryonic cortical neurons, although they fail to generate a functional presynaptic output within the culturing period. These findings are very exciting and raise hopes that reactive astrocytes that have been known to revert to an immature phenotype could be reprogrammed into neural stem cells and provide newborn neurons for repairing the damaged brain (Wang and Bordey 2008).

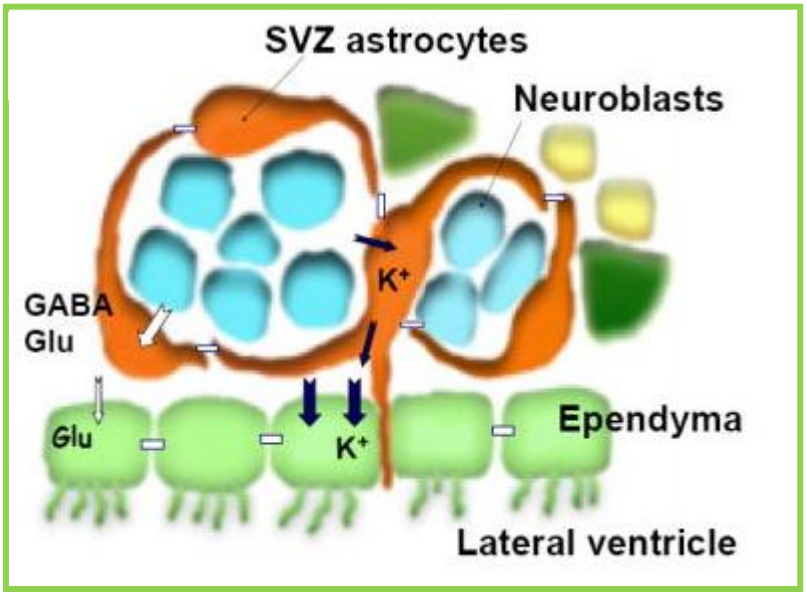

Figure 2.7. The SVZ astrocyte (Wang and Bordey 2008)

2.8. Reactive Astrogliosis And Glial Scar Formation Descriptive Features Of Reactive Astrogliosis And Glial Scars

Although reactive astrogliosis is used widely as a pathological hallmark of diseased CNS tissue, definitions of reactive astrogliosis can vary considerably among authors and there are no widely accepted categories of intensity or severity. Based on a large body of observations in experimental animals, a definition of reactive astrogliosis has recently been proposed that encompasses four key features: (1) reactive astrogliosis is a spectrum of potential molecular, cellular and functional changes in astrocytes that event in response to all forms and severities of CNS injury and disease including subtle perturbations, (2) the converts undergone by reactive astrocytes vary with severity of the insult along a gradated continuum of progressive alterations in molecular expression, progressive cellular hypertrophy, and in severe cases, proliferation and scar formation, (3) the changes of reactive astrogliosis are regulated in a context-specific manner by inter, and intracellular signaling molecules, (4) the changes undergone during reactive astrogliosis have the potential to alter astrocyte activities both through gain and loss of functions that can impact both beneficially and detrimentally onsurrounding neural and non-neural cells (Sofroniew and Vinters 2010). For at least a century, the neuropathology literature has documented that damage to the CNS results in conversion of microglia and astrocytes

into their ‘reactive’ or ‘activated’ form. Microglia and astrocytes constitute subtypes of glial cells, glia and neurons being the main cellular constituents of the nervous system. Injury-induced conversion of microglia and astrocytes into their ‘activated’ phenotype often is referred to as ‘reactive’ gliosis or simply, gliosis. Trauma, ischaemia, infectious and neurological diseases and, more recently, chemical exposures, are all known to have the capacity to induce gliosis. Despite the century-old recognition of gliosis as a response to nervous system damage, only recently have biochemical features of this cellular response been documented. Thus, evaluation of gliosis is hardly a novel approach for assessing brain damage but it is one for which a ‘biomarker’ component is just now being explored. What is now known, is that early in the time course of a nervous system cell type response to injury, microglia and astroglia become activated, elaborating their cellular processes and increasing their expression of glial genes [Figure 2.8]. Moreover, these deliberations, in turn, led us to embark on an investigation of gliosis as a potentially common feature to unify all neurotoxic responses (O'Callaghan and Sriram 2005).

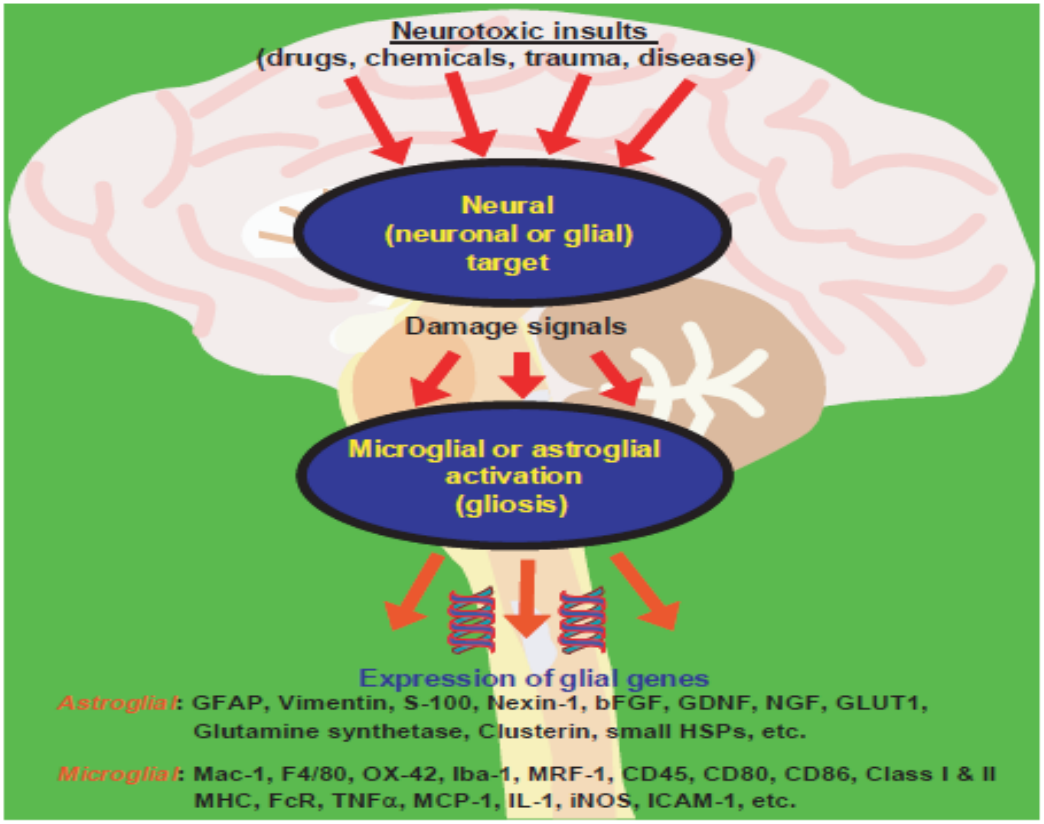

Figure 2.8.Cellular events associated with disease, injury or toxicant-induced gliosis (O'Callaghan and Sriram 2005)

2.8.1. Mild To Moderate Reactive Astrogliosis

In moderate reactive astrogliosis there is variable up regulation of expression of GFAP and other genes, as well as hypertrophy of cell body and processes that can differ in degree but that occurs within the domains of individual astrocytes. Without substantive intermingling or overlap of method of neighboring astrocytes or loss of individual domains [Figure 2.9.B]. There is little or no astrocyte proliferation in mild or moderate reactive astrogliosis, moreover, the up regulation of GFAP expression in astrocytes that do not express detectable levels of GFAP in healthy tissue [Figure 2.9.A].Can lead to the staining of more cells, sometimes giving the false impression of proliferation. Mild or moderate reactive astrogliosis is generally associated with mild non penetrating and non-contusive trauma, diffuse innate immune activation (viral infections, system bacterial infections), and in areas that are some distance to focal CNS lesions. Due to there is little or no reorganization of tissue architecture, if the triggering mechanism is able to resolve, then mild or moderate reactive astrogliosis exhibits the potential for resolution in which the astrocytes return to an appearance similar to that in healthy tissue (Sofroniew and Vinters 2010). Mild to moderate reactive astrogliosis consists of changes (up- or down-regulation) in gene expression and hypertrophy of cell body and processes without substantive loss of individual astrocyte domains and little or no astrocyte proliferation, upregulation of GFAP expression is prominent. This type of response is generally associated with mild non-penetrating and non-contusive trauma, diffuse innate immune activation (viral infections, system bacterial infections) and areas that are some distance to focal CNS lesions. Due to there is little or no reorganization of tissue architecture, if the triggering mechanism is able to resolve, then mild or moderate reactive astrogliosis exhibits the potential for resolution in which the astrocytes return to an appearance similar to that in healthy tissue (Sofroniew and Vinters 2010).

2.8.2. Severe Diffuse Reactive Astrogliosis

In severe diffuse reactive astrogliosis there is pronounced up regulation of expression of GFAP and other genes,together with pronounced hypertrophy of cell body and processes. Moreover, astrocyte proliferation, resulting in considerable extension of processes beyond the previous domains of individual astrocytes. As a result, there is substantive

intermingling and overlapping of neighboring astrocyte processes with blurring and disruption of individual astrocyte domains [Figure 2.9.C]. These changes can result in long-lasting reorganization of tissue architecture that can extend diffusely over substantive areas without the formation of dense, compact barriers as found in glial scars along borders to necrotic tissue. Severe diffuse reactive astrogliosis is generally found in areas surrounding severe focal lesions, infections or areas responding to chronic neurodegenerative triggers (Sofroniew and Vinters 2010).

2.8.3. Severe Reactive Astrogliosis With Compact Glial Scar Formation

Severe reactive astrogliosis with compact glial scar formation includes changes associated with milder forms, such as pronounced up regulation of GFAP and other genes, and pronounced hypertrophy of cell bodies and processes. In addition, glial scar formation progresses beyond these changes with groups of reactive astrocytes that exhibit pronounced overlapping of reactive astrocyte processes, obliteration of individual astrocyte domains, evidence of substantive astrocyte proliferation, and the clear formation of dense, narrow, and compact glial scars [Figure 2.9.C]. Recent experimental evidence indicates that these astrocyte scars act as neuroprotective barriers to inflammatory cells and infectious agents, and that they form in particular along borders to severe tissue damage, necrosis, infection or autoimmune-triggered inflammatory infiltration. An important feature of these glial scars is the interaction of reactive astrocytes with other cell types, in particular fibromeningeal and other glial cells, and the deposition of a dense collagenous extracellular matrix that include many molecular cues that inhibit axonal and cellular migration. Triggering insults include penetrating trauma, severe contusive trauma, invasive infections or abscess formation, neoplasm, chronic neurodegeneration, systemically triggered inflammatory challenges. It is noteworthy that the glial scar formation is associated with substantive tissue reorganization and structural changes Schematic representations that summarize different gradations of reactive astrogliosis. A. Astrocytes in healthy CNS tissue. B. Mild to moderate reactive astrogliosis comprises variable changes in molecular expression and functional activity together with variable degrees of cellular hypertrophy. Such changes event after mild trauma or at sites distant from a more severe injury, or after moderate metabolic or molecular insults or milder infections or inflammatory activation. These changes vary

with insult severity, involve little anatomical overlap of the processes of neighboring astrocytes and exhibit the potential for structural resolution if the triggering insult is removed or resolves. C. Severe diffuse reactive astrogliosis such changes in molecular expression, functional activity and cellular hypertrophy, as well newly proliferated astrocytes (with red nuclei in figure), disrupting astrocyte domains and causing long-lasting reorganization of tissue architecture. Such changes are found in areas surrounding severe focal lesions, infections or areas responding to chronic neurodegenerative triggers. D. Severe reactive astrogliosis with compact glial scar formation occurs along borders to areas of overt tissue damage and inflammation, and includes newly proliferated astrocytes (with red nuclei in figure) and other cell types (gray in figure) such as fibromeningeal cells and other glia, as well as deposition of dense collagenous extracellular matrix. In the compact glial scar, astrocytes have densely overlapping processes. Mature glial scars tend to persist for long periods and act as barriers not only to axon regeneration but also to inflammatory cells, infectious agents, and non CNS cells in a manner that maintain healthy tissue from nearby areas of intense inflammation that are long lasting and persist long after the triggering insult may have resolved. The findings summarized here show that there are pronounced differences along the continuum of potential responses of reactive astrocyte to insults of different kinds and severities. These differences are likely to be of consequence when considering the functions and impact of reactive astrogliosis on CNS disorders and pathologies (Sofroniew and Vinters 2010).

2.8.4. The Role Of Astrocyte Proliferation In Reactive Astrogliosis And Glial Scar Formation

The contribution of astrocyte proliferation to reactive astrogliosis warrants specific consideration. The wide spread and often exclusive use of GFAP as a marker for astrocytes has led to a tendency to overestimate the contribution of astrocyte proliferation to reactive astrogliosis. As discussed above, many astrocytes in healthy CNS do not express GFAP at immunohistochemically investigate levels, or express only very low levels [Figure 2.9.A]. During mild or moderate astrogliosis, there is a marked up regulation of GFAP expression as well as cellular hyper trophy in essentially all astrocytes, which can lead to the false impression of an increase in astrocyte number

because more astrocytes are immunohistochemically stained and the larger astrocytes seem more densely packed [Figure 2.9.B]. In healthy CNS tissue, astrocyte turnover is low and there are few proliferating or newly generated astrocytes and it appears that the majority of astrocytes are post-mitotic and long-lived. Experimental analysis of cell proliferation indicates that GFAP up regulation and hypertrophy can occur in mild or moderate astrogliosis in the loss of proliferation and increase in cell number. The appearance of newly proliferated astrocytes occurs in severe diffuse reactive astrogliosis and severe reactive astrogliosis with compact glial scar formation as discussed above [Figure 2.9.C]. In human specimens, actively dividing reactive astrocytes have been particularly reported in association with infection and acute demyelinating lesion. The source of newly divided scar-forming astrocytes is not well established, and may include mature astrocytes that re-enter the cell cycle as well as progenitor cells in the local parenchyma or in the periventricular regions (Sofroniew and Vinters 2010).

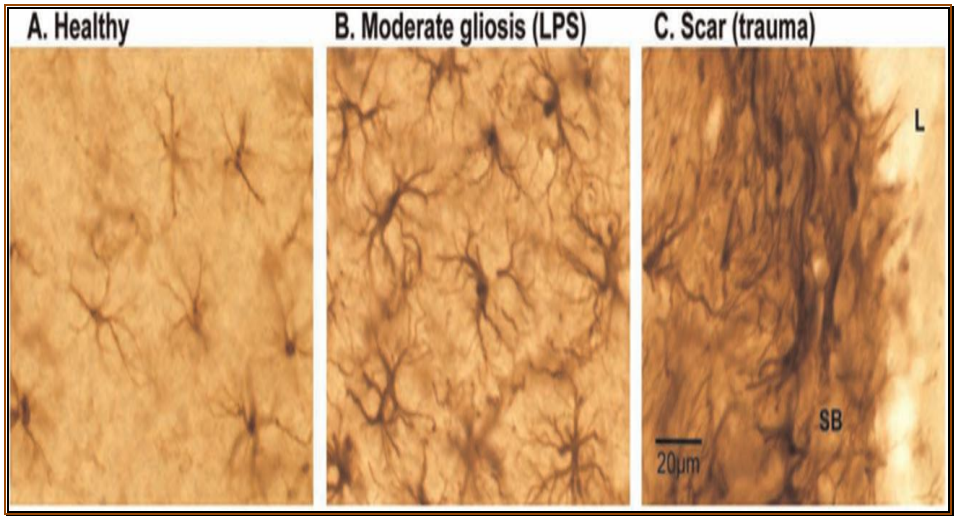

Figure 2.9. Appearance of astrocytes and different types of reactive astrocytes in mouse cerebral cortex Images show immunohistochemistry for the intermediate filament protein, GFAP, which visualizes the cell cytoskeleton (Sofroniew and Vinters 2010)

2.8.5. Mechanisms For Contributions Of Reactive Astrogliosis To Cns Disorders And Pathologies

Astrocytes and reactive astrocytes have the potential to impact on essentially all aspects of neural function through regulation of blood flow, provision of energy substrates, or by

influencing synaptic function and plasticity. Increasing evidence indicates that dysfunctions of the mechanisms of reactive astrogliosis and scar formation have the potential to contribute to, or to be primary causes of, CNS disease mechanisms either through loss of normal functions or through gain of detrimental effects (Sofroniew and Vinters 2010). Potential for loss of essential functions by reactive astrocytes As discussed earlier, in healthy neural tissue, astrocytes play important roles in many functions, including regulation of blood flow, homeostasis of extracellular fluid, ions and transmitters, energy provision, and regulation of synapse function, and synaptic remodeling. In addition, different types of transgenic deletion studies show that reactive astrocytes exert a number of essential beneficial functions in response to CNS insults, including BBB repair, neural protection and restricting the spread of inflammatory cells, and infection. The disturbance or loss of these functions during the process of reactive astrogliosis has the potential to underlie neural dysfunction and pathology in various conditions including trauma, stroke, multiple sclerosis, and others. Genetic animal models are providing evidence for ways in which loss or attenuation of reactive astrocyte functions might worsen outcome after various kinds of CNS insults, for example, through excitotoxic neurodegeneration due to failure or attenuation of glutamate uptake or promote inflammation or infection due to loss or failure of astrocyte barrier functions (Sofroniew and Vinters 2010).

2.8.6. Functions Of Reactive Astrogliosis And Glial Scar Formation

Concepts about reactive astrogliosis have long been dominated by the recognition over 100 years ago that scars formed by reactive astrocytes inhibit axon regeneration and by the interpretation that this scar formation was the main impediment to functional repair after CNS injury or disease. The ensuing over 100-year-long emphasis on glial scar formation as an inhibitor of axon regeneration has often led to a generalized negative view of reactive astrogliosis, and there has been a tendency among certain authors to typecast the entire process of reactive astrogliosis as a uniformly negative and maladaptive phenomenon that unavoidably causes neurotoxicity, inflammation, or chronic pain. This stereotyped viewpoint has sometimes led to the simplistic notion that total inhibition of reactive astrogliosis can be regarded as a therapeutic strategy. This absolutely negative viewpoint of reactive astrogliosis is no longer tenable and it is now

clear from many different lines of experimental occur that there is a normal process of reactive astrogliosis that exerts essential beneficial functions and does not do harm. As reviewed in detail elsewhere, many application using transgenic and experimental animal models provide compelling evidence that reactive astrocytes protect CNS cells and tissue by (1) uptake of potentially excitotoxic glutamate, (2) protection from oxidative stress by glutathione production, (3) neuroprotection by adenosine release, (4) preservation from $NH4^{+2}$ toxicity,(5) neuroprotection by damaged of amyloid-beta peptides, (6) facilitating blood brain barrier recover, (7) decrease vasogenic edema after trauma, stroke or obstructive hydrocephalus, (8) maintaining extracellular fluid and ion balance and reducing seizure threshold, and (9) limiting the spread of inflammatory cells or infectious agents from areas of destroyed or disease into healthy CNS parenchyma (Sofroniew and Vinters 2010).

2.8.7. Reactive Astrocytes Restrict Inflammation After SCI

Inflammation is a variable and inadequately understood component of the tissue response to SCI. Different studies report that inflammatory cells may either progress or exacerbate outcome after SCI. Cellular interactions that regulate the entry and subsequent exit of variable types of inflammatory cells after SCI are uncertain. In tissue culture, astrocytes produce both pro and anti-inflammatory molecules. The discovering here and in the fore brain. View that ablation of reactive astrocytes leads to a pronounced increase in the number of activated inflammatory cells in CNS tissue 14 days after injury. It is noteworthy that tissue from which astrocytes were intentionally ablated in our transgenic mice was similar in appearance to the central necrotic lesion observed in non-transgenic mice after crush SCI here and as reported by others after crush or contusion SCI. In both transgenic and non-transgenic mice, tissue lacking reactive astrocytes contains activated macrophages, whereas areas that contain viable reactive astrocytes have few phagocytic inflammatory cells. These findings agree with studies observing that astrocytes suppress the phagocytic capacity of microglia in vitro. Taken together, these findings suggest that after SCI, scar-forming reactive astrocytes surround and wall off regions that loss astrocytes but contain activated inflammatory cells and act to restrict infiltration of activated inflammatory cells into surrounding tissue. Nevertheless, our findings need not be interpreted that reactive astrocytes are exclusively anti-inflammatory, particularly

immediately after SCI, when they may produce pro-inflammatory cytokines. Available evidence suggests that astrocyte roles after SCI are likely to be dynamic and context-dependent, such that astrocytes may exert either pro- or anti-inflammatory functions at different situation or at different times in the response to injury and during repair (Faulkner et al. 2004).

2.9. Glial Fibrillary Acidic Protein

A key component of the astrocyte's cytoskeleton, that warrants cell integrity and resilience, is the intermediate filament (IF) network. In addition, the pivotal role in the cell's structural properties, novel IF network functions associated with transduction of biomechanical and molecular signals have emerged. Glial fibrillary acidic protein (GFAP) is the major IF protein in astrocytes, in addition to vimentin, nestin and synemin. A noteworthy asset of GFAP is that about eight different isoforms of this IF proteins have been identified. Recent data show that these isoforms are expressed in specific subsets of astrocytes and that they can change the properties of the IF network of a cell. Classically GFAP is a marker for astrocytes, known to be induced upon brain damage or during CNS degeneration, and to be more highly expressed in the aged brain. Due to the discovery of the different isoforms, which may execute distinct functions, it is important to reevaluate the published literature and to reveal how this earlier work may relate to a specific isoform or to GFAP in general. GFAP belongs to the family of IFs that, along with microtubules and microfilaments, make up the cytoskeleton of most eukaryotic cells. IFs diameter (8–12 nm) between thin actin microfilaments (7nm) and thick microtubules (25nm). IF proteins are subdivided into 6 classes based upon sequence homology. GFAP, together with vimentin, desmin and peripherin is classified as a type III IF protein (Middeldorp and Hol 2011). Glial fibrillary acidic protein (GFAP) expression initiated already during embryonic development in radial glia and is highly sensitive to any type of pathology such as acute brain injury (stroke, trauma), chronic neurodegeneration (Alzheimer's and Parkinson's disease) and aging. In the past two decades, 2.2 kb fragment 59 upstream of the open reading frame of the human GFAP gene (hGFAP promoter) (Bai et al. 2013).

2.9.1. The GFAP Protein Isoforms

GFAP is the principal 8–12 nm IF type III protein in mature astrocytes of the CNS. All kind of IF proteins are arranged into the following three main domains amino-terminal 'head' ,central helical 'rod', and carboxy-terminal 'tail' domains. The rod domains (310–350 aa residues) are highly conserved among IF proteins, while size and amino acid sequences of head and tail domains vary (Middeldorp and Hol 2011).

2.9.2. GFAP Expression In The Developing, Adult And Aging Nervous System

While vimentin is the main IF protein in the neonatal brain, GFAP is the major IF protein in the adult brain and is a characteristic protein in mature astrocytes. Initial expression of GFAP in the developing human brain starts in radial glia, which are bipolar cells in the ventricular zone (VZ) expressing vimentin and nestin, and that can act as neural stem cells [Figure 2.10.]. Moreover the exact age at which GFAP protein expression starts is not clear. Several studies have reported that GFAP immunostaining appeared in radial glia of the human brain at gestational week (gw) 9–12. One study observed a small population of GFAP expressing cells in the premordium plexiform layer already at gw6, at the onset of corticogenesis. Moreover, other studies have reported little or no GFAP staining until 14–25 weeks. The differences found between studies may be explained by a difference in the brain areas investigated, antibodies used and the staining methods applied, which have significantly progressed over the years. In most reports the number of GFAP expressing cells increases with gestational age and is much higher during the second half of gestation. Around this time, a second proliferative zone becomes discernable between the VZ and the cell sparse intermediate zone, called the subventricular zone (SVZ). Neural precursor cells in this area also express GFAP, which persists into adulthood, in contrast to the VZ that will disappear before birth. During the transform of precursor cells into astrocytes, there is a shift in the prevalence of IF proteins from vimentin to GFAP. Vimentin expression decreases in some astrocytes to undetectable levels, although several astrocytes also co-express GFAP and vimentin in the adult animal, such as the Bergmann glia and the astrocytes in the corpus callosum and the hippocampus. GFAP expression increases progressively during aging in humans and inbred laboratory rodents, evaluated by GFAP mRNA and protein. Although the cell

volume of astrocytes increases during aging, the number of astrocytes expressing GFAP appearance much more modest changes. The increased GFAP expression during aging is due to an increased transcription of GFAP, as shown by in situ hybridization at a cellular level with intronic cRNA probes. It has been suggested that increased GFAP transcription during aging is caused by the increased load of oxidatively damaged proteins, which appear in tissues throughout the body during aging, such as the brain. The enlargement of astrocytes and the increased expression of GFAP is an indication of reactive gliosis, a process which has shown to be highly associated to brain damage and aging. At the age of the average human life-span 77 years, humans show an increase in GFAP mRNA in the hippocampus, frontal cortex and temporal cortex. The hippocampus is always the site most affected by reactive gliosis and presumably also the initial region affected during aging, since this phenomenon is never observed in other regions alone. Reactive gliosis in the adjacent entorhinal cortex, where neuropathological changes are initially found in the early stages of AD, is systematically accompanied by an increased glial reaction in the hippocampus. According to dramatic GFAP production in the hippocampal region during aging mainly concerns soluble GFAP and not the filamentous form. Demonstrated that the more soluble and acidic GFAP forms are more susceptible to degradation. By using 2D electrophoresis and Western blots, they revealed a complex GFAP pattern with variant modification and degradation forms in aging and AD. Another explanation might be that some of these spots represent the different GFAP isoforms. besides ,to an increased GFAP expression in the brain, demonstrated an age-dependent increase of GFAP levels in cerebrospinal fluid (CSF) from neurological healthy individuals (age range 16–77years). On the contrary, in blood of both children (0.2–20 years) and adults (age 64 years) an age-dependency could not be appeared for GFAP. Differences in GFAP expression throughout development and aging of the brain are indicative of different functions of astrocytes and also of changes in astrocyte functions over time. Moreover, changes in GFAP expression might alter the morphology of astrocytes, which could indirectly affect other cell types and the structure of the brain (Middeldorp and Hol 2011).

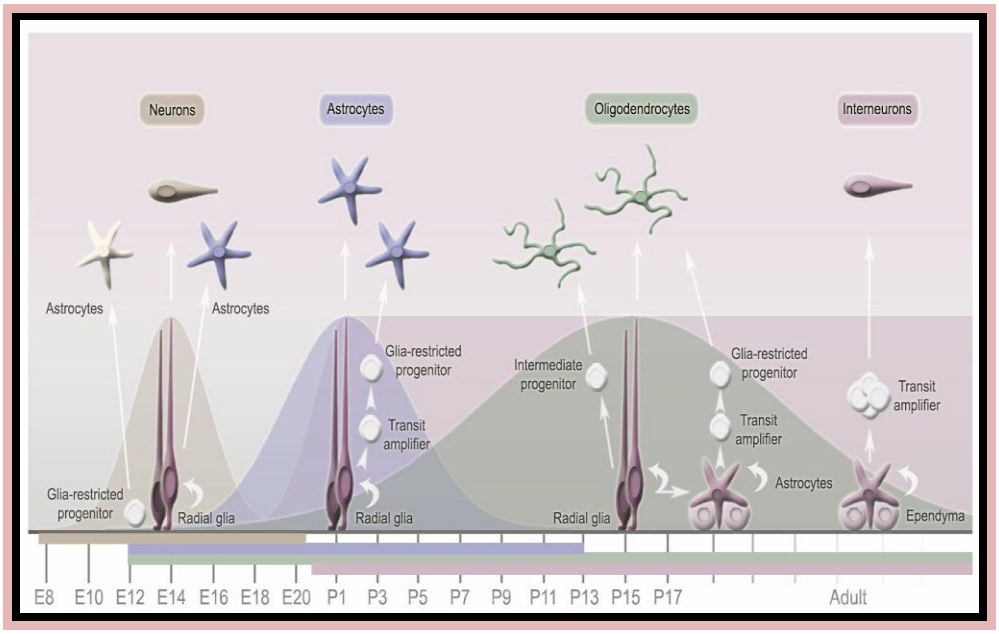

Figure 2.10. Summarizing the sequence of neuron and glia development (Wang and Bordey 2008)

2.9.3. GFAP Function

All type III IF proteins are formed in the structure and function of the cell's cytoskeleton. Perhaps the most well-known function of IFs is to provide mechanical support for the plasma membrane where it comes into contact with other cells or with the extracellular matrix. GFAP is expressed in astrocytes, where it is thought to help maintain mechanical strength, as well as the shape of cells. In the last twenty years there is a growing realization that astrocytes have a much broader function than only supporting the neurons in the brain, as they have specialized functions in inducing and regulating the blood brain barrier (BBB), protect neurons against neurotransmitter excesses, promote synaptic plasticity, coordinate neuronal activity via direct communication with neurons and are the neural stem cells in the adult brain. In the last 15 years several mouse models have been generated that either lack or overexpression GFAP (Middeldorp and Hol 2011).

2.9.4. Glial Fibrillary Acidic Protein As A Key Biomarker

If gliosis is a dominant and universal response to nervous system damage, what are the key biomarkers of this cellular reaction to injury? Of the many glial genes now known to be expressed during gliosis the oldest and most well documented is glial fibrillary acidic

protein (GFAP). The astroglial component of gliosis is characterized at the electron microscopic level via the accumulation of glial filaments, of which GFAP is the major constituent. By definition, therefore, astrocytic gliosis is accompanied by an increase in GFAP. Indeed, the use of antibodies to GFAP in histological studies has firmly established the existence of reactive gliosis as a dominant response to many different types of brain injuries. Although GFAP immunohistochemistry has not been applied in the context of first level neurotoxicity screens, where tissue sections from neurotoxicant exposed animals have been applied, enhanced expression of this protein has been seen in target-appropriate brain regions. Thus, based on the accumulated morphological data, the ideal 'biomarker' of all types of nervous system injuries is enhanced expression of GFAP (O'Callaghan and Sriram 2005).

2.9.5. GFAP In Disease

Astrocytes are involved in a wide range of CNS pathologies, including trauma, ischemia, and neurodegeneration. In response to essentially any CNS pathology, astrocytes undergo a characteristic change in observed, i.e. the hypertrophy of their cellular processes, a phenomenon referred to as reactive gliosis. A well-known feature of reactive astrocytes is increased production of IFs, a result of the increased expression of GFAP, but also vimentin and nestin. Diseases which show increased GFAP mRNA and protein include Alzheimer's disease, scrapies and Creutzfeldt-Jacob disease. Other types of injuries in the CNS which view increased GFAP are for instance cerebrovascular accidents, stab wounds and other lesions and experimental allergic encephalomyelitis, an animal model for multiple sclerosis. Howeover, many different diseases of the CNS show an increased GFAP expression, which is usually regarded as secondary to neurodegeneration. IF encoding genes are selectively expressed in certain cell types and during differentiation. This feature is relevant to the association of mutations in these genes with a broad range of tissue-specific diseases. Most IF associated diseases are keratin or lamin-related, like the skin disease epidermolysis bullosa simplex and CharcotMarie-Tooth disease. The first example of neurologic diseases by mutations in the GFAP gene is AxD, which is the first known primary genetic disorder of astrocytes (Middeldorp and Hol 2011).

2.9.6. Diseases Which Show Increased GFAP Mrna And Protein Or Absent GFAP

Including

- Alexander disease
- Alzheimer's disease
- Parkinson's disease
- GFAP in neurodegenerative diseases
- Amyotrophic lateral sclerosis
- GFAP isoforms in disease
- Other diseases

Besides, to these neurodegenerative diseases and others like Pick's and Huntington's disease, GFAP expression has also been reported to be altered in different neurological conditions including developmental, infectious and inflammatory, vascular, and mood disorders. For example a decrease in GFAP expression in different brain areas has been correlated to depression, whereas increased GFAP was discover in relation to autism, such as auto-antibodies to GFAP in plasma and elevated GFAP levels in the CSF. High GFAP levels in the CSF in general have been shown in the context of acute central nervous system injury, such as brain infarction and traumatic brain injury. Furthermore, decreased GFAP expression has been relation with the growth of gliomas, more appearing in high-grade than in low-grade gliomas, and serum GFAP has proven a valuable analytic marker for glioblastoma multiforme. Studied GFAP decrease immunostaining in different types of reactive gliosis, i.e. Chaslin's gliosis, Ammonhorn sclerosis and gliosis induced by several types of cancer. In Chaslin's gliosis, which is characterized by a dense fibrillary meshwork in for instance the glia limitans, robust GFAP decrease immunostaining was observed only in the cell bodies and proximal processes. Individual astrocytes could be distinguished with GFAPd in contrast to the normal GFAP immunostaining pattern in which split cell bodies were hardly distinguishable. Comparable to control tissues, GFAPd expression was very similar to vimentin expression in gliotic tissues. Both GFAPd and vimentin were observed in the perikaryon and proximal processes of activated astrocytes, but not in their distal part. GFAPd expression in tumor cells was not systematically related with vimentin

expression. Various types of tumors, e.g. pilocytic astrocytomas, WHO grade II ependymomas, gemistocytic astrocytomas and oligodendrogliomas showed GFAP decreased (Middeldorp and Hol 2011).

2.9.7. Nuclear Factor-Kappa-B (NF-kB)

NF-κB is a protein complex that controls the transcription of DNA and is a central regulator of cellular stress in all cell types in humans. NF-κB plays a key role in regulating the immune response to infection and in acute and chronic inflammation. The activation of NF-κB in rats can stimulate the expression of IL-1β, which increases the expression of proinflammatory molecules. The transcription factor NF-κB has a pivotal role in a different of physiological processes throughout the body, including immune responses, cell proliferation and inflammation. NF-κB elicits its effects by promoting the transcription of a range of cytokines, enzymes, chemokines and antiapoptotic and cell growth factors. Many in vitro and in vivo studies have described diverse biological activities of CAPE (at micromolar concentrations), such as a specific inhibition of NF-κB and a suppression of the lipoxygenase pathway of arachidonic acid metabolism during inflammation. It has also been shown that CAPE acts to suppress the NF-κB activation stimulated by ROS-generating agents in human histiocytic and coronary artery endothelial cells. It is believed that, rather than preventing the degradation of κB inhibitor-α (IκB-α), CAPE suppresses NF-κB activation via inhibiting the interaction between NF-κB proteins and DNA [Figure 2.11]. Anti‑inflammatory effect of CAPE is most likely due to the inhibition of ROS. The activation of inactive NF-κB proteins existing in the cytoplasm is induced by numerous factors, including inflammatory cytokines (IL‑1 and TNF‑α), bacterial products and protein synthesis inhibitors. However, agents that can down regulate the activation of NF-κB have potential for therapeutic interventions, whereas the activation of NF-κB promotes inflammation in animals. The binding of TNF-α to cell surface receptors engages multiple signal transduction pathways, including three groups of MAPKs. Extracellular signal regulated kinases, c‑Jun N‑terminal kinases and p38 MAPKs. These MAPK signaling pathways induce a secondary response via increasing the expression of several inflammatory cytokines that contribute to the biological activity of TNF-α. MAPKs, therefore, function both upstream and downstream of signaling by TNF-α receptors. In almost all cell types,

the exposure of the cells to TNF-α induces the activation of NF-κB and leads to the expression of a range of genes related with inflammation.

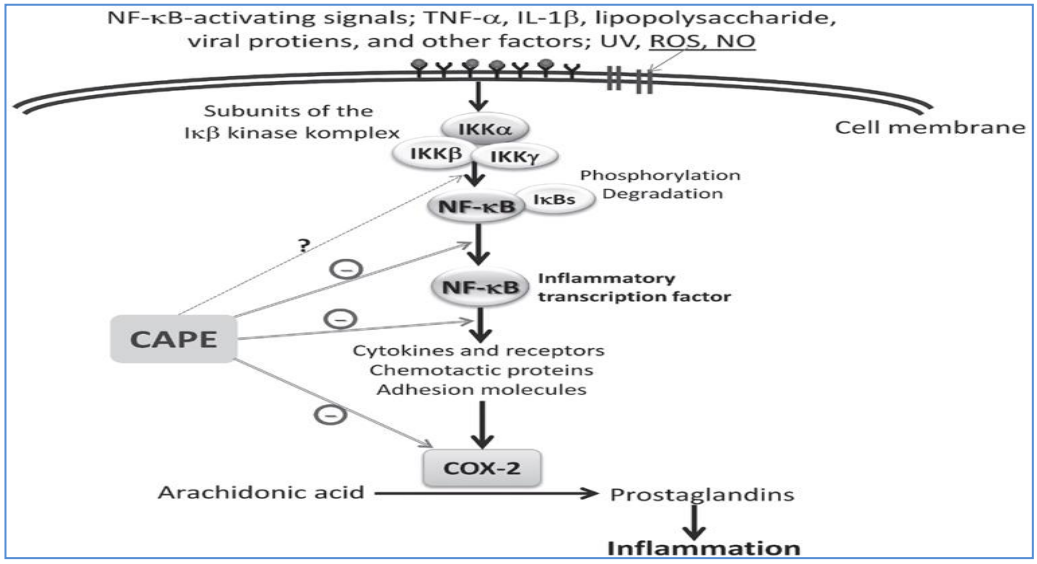

Figure 2.11. Schematic presentation of the anti-inflammatory effects of propolis in the inhibition of inflammation (Armutcu Akyol Ustunsoyand Turan 2015)

CAPE possesses strong antioxidant, anti-inflammatory and healing properties, and its effects on the wound repairing have been attributed to the inhibition of NF-κB. Consistent with these two cited studies .Reported that treatment with CAPE increase wound healing, particularly wound healing following burns; decreased inflammatory parameters and oxidative damage and inhibited the activity of cyclooxygenase and lipooxygenase. Under most inflammatory conditions, such as in thermal injury, NO production is enhanced. In addition to performing histological and biochemical analyses, Santos et al increase the anti-cluster of differentiation 68 (CD68) and NO levels, as well as myeloperoxidase (MPO) activity. CAPE exhibited an anti‑inflammatory action on rat burn healing by reducing MPO activity, NO levels and the number of CD68-positive cells .Showed that CAPE reduced neurovascular inflammation and protected the rat brain following transient focal cerebral ischemia by down regulating NF-κB and certain mediators, such as cytokines and iNOS (Armutcu et al. 2015). Although neurotrophic factors, such as glial-derived neurotrophic factor (GDNF), basic fibroblast growth factor, and brain-derived neurotrophic factor (BDNF), have been identified in these activated astrocytes, a line of growing evidence has demonstrated that LPS or inflammatory factors

also trigger astrocytes to release the pro-inflammatory cytokines NO and ROS, which are detrimental to the neighboring neurons. Genomic analysis of an LPS mouse model revealed that the activated astrocytes exhibit a detrimental phenotype. Consistently, we observed significant increases in the pro-inflammatory factors iNOS and ROS in the activated astrocytes following stimulation with LPS in combination with other cytokines. Thus, the identification of new regulators that control inflammatory astrogliosis and the prevention of the progression of Parkinson's disease (PD) by the elimination of these activated astrocytes with detrimental properties may be essential (Sun et al. 2016).

2.9.8. Angiostatines

Angiostatines is a proteolytic fragment of plasminogen [Figure 2.12]. Including the first four-kringle subunits (K1–4). It was first found in a mouse Lewis Lung carcinoma model of concomitant resistance. Besides to being generated by cancer and inflammatory cells, angiostatin is further present in healthy humans. It is found in abundance in human plasma, and it is constitutively generated by platelets and released in active form upon aggregation. Angiostatin suppress angiogenesis by inhibiting endothelial cell proliferation, migration, and can even enhance endothelial apoptosis (Radziwon Balicka Moncada Rosa Zielnik Doroszko and Jurasz 2013).

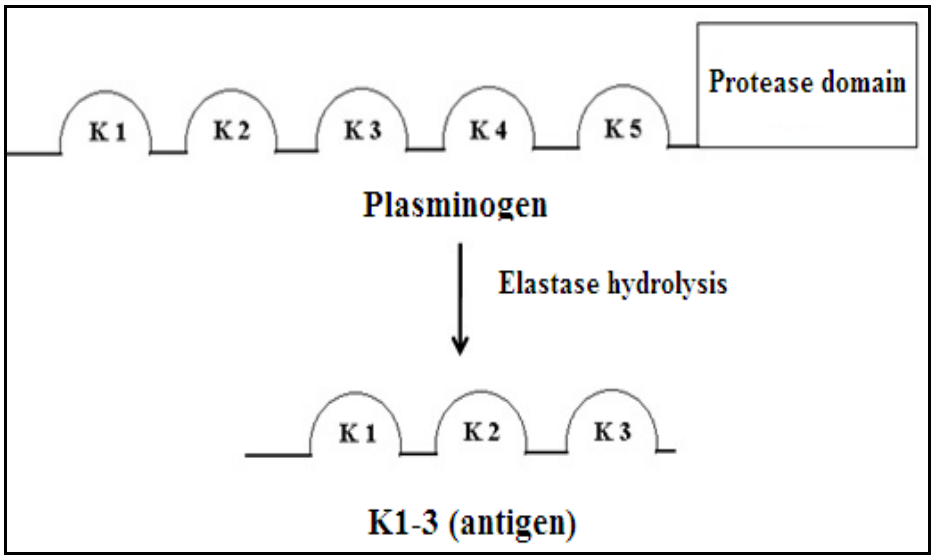

Figure 2.12. Structure of plasmogen and K1-3 kringle

Moreover, inflammatory diseases of the central nervous system (CNS) receive some adaptive responses that include angiogenesis, the process via which new blood vessels are produced from pre-existing vasculature. While angiogenesis is part of the natural response to injury if it becomes excessive or persistent then it can preserve inflammation and contribute to disease severity. Many factors regulate angiogenesis and include a main role for the 165 amino acid isoform of Vascular Endothelial Growth Factor-A in humans (164 amino acids in mice). VEGF plays in mediators to increase new blood vessel production. These mediators include angiopoietin (Ang)-1, Ang-2, Tumor necrosis factor and other growth factors. Despite the multiple factors implicated in support of angiogenesis, the 164/165 amino acid isoform of VEGF-A is observed as a key orchestrator of angiogenesis in pathologic or inflammatory settings. Here, we propose strategies to inhibit VEGF that hold promise for the treatment of inflammatory disorders. Kringle(1-3), the first three kringle domains of angiostatin, an angiogenesis inhibitor that directly targets vascular endothelial cells. Angiostatin is a proteolytic splitting product of plasminogen composed of its first four kringle domains (a triple loop structure including about 80 amino acids;) that inhibits angiogenesis in a different of contexts. The first three kringle domains are used instead of the complete protein due to the inhibit angiogenesis with higher potency (MacMillan et al. 2014). Plasminogen kringle 5, a proteolytic fragment of plasminogen, is a potent angiogenic inhibitor. Intravitreal injection of K5 inhibited retinal NV and reduced retinal vascular leakage in diabetic retinopathy (DR) models. Moreover, K5 and its deletion fragment also displayed inhibitory influences on tumor growth by inhibiting angiogenesis. Moreover, the molecular mechanism for K5′s antiangiogenic influences has not been performed. Decline K5 receptor expression in the retina, a potential pathogenic mechanism for diabetic retinopathy. Plasma plasminogen is the zymogen form of the serine protease, plasmin. Plasminogen is a ~90 kDa protein of the extracellular matrix. Plasma plasmin is the precursor of the angiogenesis blocking. Angiostatin is an N-terminal fragment of plasmin consisting of kringle domains 1-4 and part of kringle 5. The generation of angiostatin involves splitting of both disulfide and peptide bonds in plasmin. By definition, allosteric disulfides are bonds that control the function of the mature protein in which they reside by triggering a change when they are cleaved. These bonds are cleaved via oxidoreductases or thiol/disulfide exchange and functional changes in ligand binding, substrate hydrolysis, proteolysis, or oligomer formation have been identified. The functional change upon cleavage of the plasmin

allosteric disulfides is proteolysis of the protein. Reduction of the kringle five allosteric disulfides involves the interaction of plasmin with other proteins. A plasmin-binding protein that is secreted by tumor cells is the glycolytic enzyme, phosphoglycerate kinase (PGK). In glycolysis, PGK catalyzes phosphoryl transfer from 1, 3-bisphosphoglycerate to ADP to form 3-phosphoglycerate and ATP. PGK secretion via cultured tumor cells positively interacts with oxygen stress, which is by hypoxia being a driver of tumor angiogenesis. Furthermore, plasma levels of PGK negatively correlate with tumor angiogenesis and tumor growth in mice. Plasmin activity has been implicated in, for example, tissue remodeling, injury healing, angiogenesis, and cancer invasion. But, also in cell injury. This can probably be attributed to the ability of plasmin to degrade components of the extracellular matrix and basal membrane and to activate matrix metalloproteases. Plasmin is probably best known for its critical role in fibrinolysis. With its capability to digest fibrin fibers, plasmin controls the extent of coagulation and is of central importance for solubilization of blood clots. Hence it is somewhat surprising that plasminogen deficiency as a sole abnormality is mainly related to ligneous conjunctivitis, but does not seem to increase the risk of thrombosis in human subjects. Interestingly, plasminogen deficiency was recently associated with the disease atypical hemolytic uremic syndrome (aHUS) (Hyvarinen and Jokiranta 2015). The mechanism of reduction of the disulfides has been investigated in this study. That disulfide bond is already cleaved in a fraction of plasma plasminogen and show that this reduced Plasminogen is the precursor for PGK-mediated angiostatin formation. Moreover, the plasmin(ogen) allosteric disulfides have configurations that are shared by allosteric disulfides in other proteins. Angiostatin, which is potent angiogenesis inhibitors, specifically inhibit the secretion of vascular endothelial cell and fibroblast growth factors. This process inhibits the proliferation of vascular endothelial cells but spares non-endothelial cells. Experiments have shown that angiostatin can be used to treat Lewis lung carcinoma, breast cancer, and bladder cancer (MacMillan et al. 2014).

2.9.9. C60

Since their founded in 1985 by Kroto, fullerenes have attracted great interest in research due to their single properties, and their production on a preparative scale was underway already in 1990. Besides, engineered C60 fullerenes, combustion-derived C60, as identified in

particulate matter emitted from coal-fired power plants (Spohn etal. 2009). Oncological diseases, particularly breast cancer, lung cancer, and leucosis, are common and carry a great mortality rate. C60 fullerene has recently been identified as a promising agent for use in anticancer therapy. C60 fullerene and its derivatives are biocompatible, show no toxic effects on normal tissues at low concentrations, possess strong free radical- scavenging and antioxidant potential, and demonstrate a protective effect against Dox-induced chronic cardio- and hepatotoxicity. The tumor inhibitory activity of nanoparticles containing C60 fullerene has been studied in models of murine hepatocarcinoma. And rat colorectal cancer and mammary carcinoma. The biological effects of pristine C60 fullerene are limited by its hydrophobicity, difficulties involved with reaching sufficient concentration, and aggregation in water. For this purpose, hydrophilic derivatives obtained by chemical alteration of the C60 fullerene outer surface were done in most studies of C60 fullerene antioxidant and antineoplastic effects. Though, derivatization does not completely prevent cluster generation. Besides, substantial modification of the C60 fullerene core appears to cause a disorder of the conjugated electronic system of cyclohexatriene assemblies on the molecular surface and so may affect C60 fullerene reactivity. The use of a constant water colloid dispersion of pristine C60 fullerene has shown for the general analysis of C60 fullerene biological effects and antitumor efficiency (Prylutska et al. 2014). To check this hypothesis, we investigated the effects of C60 against the radical-related toxicity of carbon tetrachloride in rats, which provides an important model for elucidation of the mechanism of action of hepatotoxic effects including fatty degeneration, hepatocellular death, and carcinogenicity. This work allowed us as well to check the harmlessness of C60 to another rodent species (Gharbi et al. 2005).

2.9.9.1. Doxorubicin

Many cytostatic drugs, including doxorubicin (Dox), are applied in the therapy of malignant tumors. Dox is an antibiotic of the anthracycline group, with a broad spectrum of clinical activity against tumors and hemoblastoses, acute leukemia, lung carcinoma and. Dox non-covalently binds to DNA, inhibition of the synthesis of nucleic acids, and shows high antimitotic activity and pronounced mutagenic effect, but exercises toxic effects in normal tissues and cells. One path to protecting against Dox-induced chemical insult to normal tissues is a mixed use of the cytostatic drug with antioxidants of a diverse

nature. Recent results indicate that C60 fullerene complexation with Dox may be a key process leading to alteration of the antitumor effect of doxorubicin (Prylutska et al. 2014).

2.9.9.2. Hydroxynonenal

Reactive oxygen species including, hydrogen peroxide, superoxide anion hydroxyl radicals, singlet oxygen, and lipid peroxyl radicals, are ubiquitous and regarded as byproducts of aerobic life. Most of these chemically reactive molecules are short-lived and react with surrounding molecules at the site of the formation while some of the most stable molecules diffuse and cause damages far away from their sites of production. Excess of these ROS, named oxidative stress, may make oxidation of polyunsaturated fatty acids in cellular membranes through free radical chain reactions and produce lipid hydroperoxides as primary products. Some of these primary oxidation products may decompose and lead to the formation of reactive lipid electrophiles. Among this lipid peroxidation (LPO) products, 4-hydroxy-2-nonenals (4-HNE) represents one of the most bioactive and well-studied lipid alkenes. 4-HNE can modulate some signaling processes essentially through forming covalent adducts with nucleophilic functional groups in proteins, nucleic acids, and membrane lipids. 4-hydroxynonenal (4-HNE), are biomarkers for oxidative stress and important players for mediating some signaling pathways. The biological effects of 4-HNE are primarily caused covalent alteration of important biomolecules including proteins, DNA, and phospholipids containing the amino group. Mitochondria are important for cellular bioenergetics and regarded as the main cellular site for ROS production and showed that mitochondria are also an important site for 4-HNE production. Besides, it has been abundantly texted that oxidative stress and ROS generation are intimately associated with cancer. Mitochondrial macromolecular adducts from 4-HNE are included in the beginning and improvement of cancer. The role of 4-HNE in cancer and focus on the involvement of mitochondria: formation of 4-HNE from the oxidation of cardiolipin, covalent modification of mitochondrial biomolecules including proteins, DNA, and lipids, and therapeutic targeting the mitochondrial pathways caused by 4-HNE in the context of cancer pathogenesis (Zhong and Yin 2015). Moreover, Oxidative stress increases lipid peroxidation and lipid peroxides induce a variety of cellular damage directly or indirectly by covalently modifying membrane-associated or intracellular proteins. 4-HNE, derived from peroxidation of n-6

polyunsaturated fatty acids such as linoleic acids. At high levels, 4-HNE is cytotoxic to many cell types, while micromolar and submicromolar concentrations of 4-HNE have been exhibited to induce several nontoxic, cell-specific effects (Zhang et al. 2013).

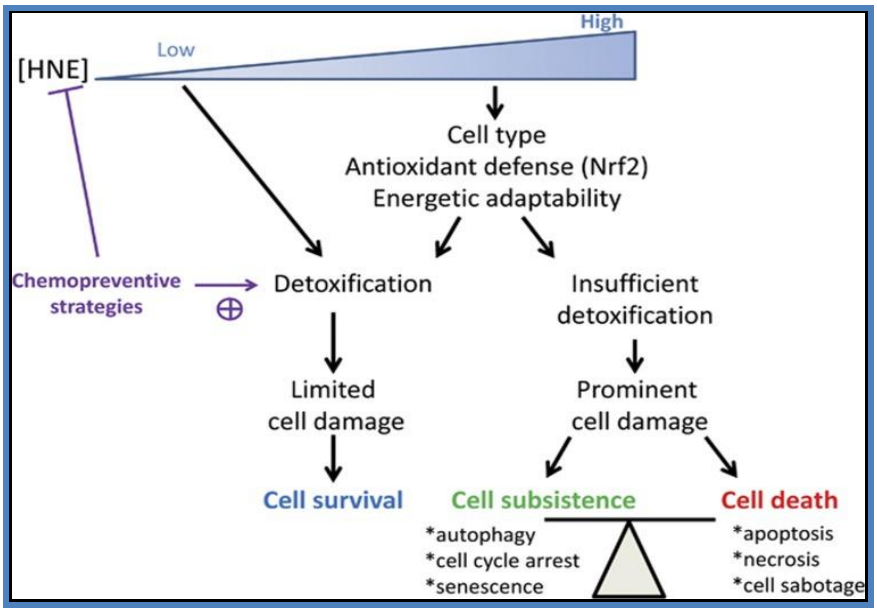

Figure 2.13. Summary of cell fate upon HNE appearance (Dalleau Baradat Gueraud and Huc 2013)

2. MATERIALS AND METHODS

3.1. Reagents

10% heat-inactivated fetal bovine serum (FBS), 2.5% trypsin-EDTA, penicillin, streptomycin were purchased calcium-free phosphate buffered saline (PBS) was from Biomed, methylthiazolyl diphenyltetrazolium bromide (MTT), Puck's-D1 solution, dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich). Acrylamide/ bis-acrylamide solution, poly-D-lysine (PDL) at a concentration of 50 μg/ml, ammonium persulfate, bovine serum albumin, lysis buffer, bromophenol blue, calcium chloride, glycine, glycerol, TMZ, sodium dodecyl sulfate (SDS), N,N,N'N' tetramethylethylenediamine (TEMED), trichloroacetic acid, Tris-Triton buffer, triton X-100, trizma base were obtained from Sigma-Aldrich. Hank's solution, Novus Biological (Littleton, CO), antibodies against glial fibrillary acidic protein (GFAP; rabbit polyclonal, ABCAM).

3.2. Characterization Of Raw Propolis Samples

Propolis is a complex mixture, containing resins, balsamic products, wax, essential oils, pollen, and microelements, besides other components. The samples presented a characteristic aroma, balsamic and/or resinous, malleable (red) to rigid (brown and green) consistencies at room temperature, with a very heterogeneous granulometry. It is observed that the analysis of the physical chemical composition is of great importance to determine the quality of the studied material, considering the incorporation of this matrix in food products (Machado et al. 2016). The results of the physical-chemical characterization of the different samples of propolis are shown. The results for the content of certain minerals present in the samples. The value of humidity and total solids varied among the specimen. In relation to the contents of ash, protein, lipids and fibers, a significant variation was observed among the samples the determination of the total ash

content is particularly important in samples of propolis commercialized in powder form, as this analysis can identify a possible adulteration of the material through the presence of impurities, or even residues from previously extracted propolis. The samples were within the limit established via the Brazilian legislation (maximum 5%). Among the microelements analysed and identified in the samples, we can highlight the high contents of potassium on the three samples of green propoli. Some studies show aluminium, vanadium, iron, calcium, silicon, manganese, strontium and potassium, as the major microelements present in propolis samples (Machado et al. 2016).

3.3. Collection And Extraction Of Propolis

Method constructing propolis extraction may influence its activity, because different solvents solubilize and extract different compounds. There are three methods commonly used for extraction with ethanol, methanol, water and its composition or propolis is directly dependent with the composition of the vegetation of the region (Kakoolaki et al. 2013). The propolis used in the present study was poplar propolis collected manually from honeybee. when accumulated green propolis from the bee hive in February to March from north west of Iraq during winter 2016. The propolis should be clean and protect the light. Ethanol extraction was prepared by extraction of 30g of bee glue with 450m of ethanol 98% for 12h of a darkened plaice in the shaker machine, then it was filtered by qualitative filter paper (125mm and 110mm), vacuum dried and evaporated in rotary evaporation the yield of prepared extracted (% w/w) as that original product. The extracts were dissolved in DMSO and prepared 1mg/mL stock solution (calculate on the dry mg of extracts) Afterwards, the extracts were stored in tubes covered in aluminium foil in inert atmospheric conditions (N^2) in order to avoid degradation of the material. The material was kept at 5°C until analysis (Machado et al. 2016).

3.4. Cell Isolation

Fresh rat pups normal brain tissue samples from a 24h old were obtained at the time of dissection from the Department of Bingol University in Turkey, The tissue was accumulated immediately after a surgical resection. It was cut, homogenized, and seeded into tissue culture plates with a medium containing Dulbecco’s modified Eagle’s medium

(DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. The medium was changed 2–3 times a week. Under these conditions, the cells got attached and grew for a few weeks and were used after VI passages. Sub confluent cells were detached with a trypsin-EDTA solution in PBS and counted in a Neubauer hemocytometer.

3.4.1. Cell Culture

Mixed cell isolation for astrocyte cultures can be performed using P1 to P4 rat pups. In order to achieve proper astrocyte density it is necessary to use 4 rat pup cortices per T75 tissue culture flask (Schildge Bohrer Beck and Schachtrup 2013). All experimental procedures conformed to the "Guidelines for Proper Control of Animal Experiments" approved by the site ethics regulations. Astrocytes are seed in T75 tissue culture flask at a starting density $6x10^4$ cells/ml (20 ml/flask) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin and incubated at 37°C in atmosphere containing 10% CO_2. After 9–10 days, these cell cultures should wash with Hank's solution (Ca_2, Mg_2-free; in mM: 50 NaCl, 5 KCl, 0.2 KH_2PO_4, 0.17 Na_2HPO_4, 5.0 glucose, 58.4 sucrose, pH 7.4), detached from the plastic with trypsin-EGTA (0.05%/0.02% wt/vol) solution. Then DMEM containing 10% (vol/vol) (FBS) is added to stop the action of trypsin. After the cells were centrifuged at 500 g for 5 min. The cells were plated for the experiments in DMEM with 10% (vol/vol) FBS. For experiments cells were used between day 11 and 13 in culture. These cultures are characterized to contain more than 90% astrocytes. Then discarding the supernatant, the pellet of cells was again suspended in a complete RPMI medium and then the inoculum of cells corresponding to the final concentration of $1x10^5$cells/ml was calculated. After distributing the culture medium in plaques of 24 wells, the compounds were added to two concentrations of 50μg/ml and 100μg/ml, and the DMSO was applied as a diluting control and statistic parameter (Machado et al. 2016).

3.4.2. Preparation Of Samples

Cells should be rapidly frozen to avoid protease degradation of proteins or accumulated and lysed as quickly as possible. Solid tissue is mechanically broken down, usually using a homogenizer or by sonication in a lysis buffer. Tissue preparation can be performed at cold temperatures to avoid denaturation and degradation of protein. Lysis buffer should be used to enable lysis of cells and to solubilize proteins. Lysis buffer contains 50 mM triz pH 6,8, 5% glycerol, 3% SDS, 2 mM dithiotreitol, 0,01% bromphenol blue. Lysis buffers used in sample preparation for western blotting should enable efficient protein extraction and maintain antisera recognition of the protein (MacPhee 2010). For cytoskeleton proteins the buffer should contains Tris-Triton buffer: 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate. As soon as lysis occurs, proteolysis, dephosphorylation and denaturation begin. These events can be slowed down tremendously if samples are kept on ice or at 4°C at all times and appropriate inhibitors are added fresh to the lysis buffer.

3.4.3. Immunoblot Method

PAAG electrophoresis. For optimal separation, it is important to evaluate the ideal bisacrylamide acrylamide ratio prior to electrophoresis. The samples are loaded onto the gel. One lane should include a molecular weight marker that is used to determine the molecular weight of the target protein. Another lane should include an internal control, ideally with a known concentration and molecular weight to determine. Polyacrylamide gels are formed from the polymerization of two compounds, acrylamide and N, N-methylenebisacrylamide (Bis, for short). Bis is a cross-linking agent for the gels. The polymerization is initiated by the addition of ammonium persulfate along with either DMAP or TEMED. The gels are neutral, hydrophilic, three-dimensional networks of long hydrocarbons crosslinked by methylene groups. The separation of molecules within a gel is determined by the relative size of the pores formed within the gel. The pore size of a gel is determined by two factors the total amount of acrylamide present (designated as %T) and the amount of cross linker (%C). As the total amount of acrylamide increases, the pore size decreases. With cross- linking, 5%C gives the smallest pore size. Any increase or decrease in %C increases the pore size. Gels are designated as percent

solutions and will have two necessary parameters. The total acrylamide is given as a percentage (w/v) of the acrylamide plus the bis-acrylamide. A standard migration buffer (also called running buffer) for PAGE is 1X Tris-glycine: 25 mM Tris base, 190 mM glycine, 0.1% SDS, pH 8.3. Transferring of proteins. Once electrophoresis is complete, the separated proteins should be transferred from within the gel onto a membrane (a western blot) made of nitrocellulose, polyvinylidene difluoride (Kurien and Scofield. 2006). Transferring proteins from a gel to a membrane should realize 90 – 120 min with constant current 150 – 200 mA. Its main advantages are speed and completeness of transfer. This process applied an electric current to pull proteins from the gel onto the membrane. The effectiveness of protein transfer is dependent on the type of gel applied, the molecular mass of the protein, and the type of membrane. Visualization of protein sorbtion onto membranes. To check for success of transfer, wash the membrane in TBS. Staining with Ponceau Red. The stock is made of 2% Ponceau S in 30% trichloroacetic acid and 30% sulfosalicylic acid. Incubate on an agitator for 5 min. Wash extensively in water until the water is clear and the protein bands are well-defined. The membrane may be destained completely by repeated washing in TBST or water. When using a PVDF membrane, reactivate the membrane with methanol then wash again in TBST. Blocking and Antibodies. To block nonspecific binding, the membrane is placed in a dilute solution of protein such as bovine serum albumin and nonfat dry milk Blocking helps mask any potential nonspecific binding sites on the membrane. After blocking, the most popular method is to incubate the membrane with primary antibody, wash, reblock, and then incubate with secondary antibody and wash again. It is important to determine the optimal concentration of antibodies before running all the samples as optimization is a prime determinant of the sensitivity of the assay (Burnette 1981). The antibody concentration should be optimized to provide the best signal to noise ratio. Both monoclonal and polyclonal antibodies can be used for western blot analyses, with advantages and disadvantages in using either type (MacPhee 2010).

Detection the protein bands. The probes that are labeled and bound to the protein of interest need to be detected on the western blot. Chemiluminescent detection is used most often and therefore, will be briefly described. Enhanced chemiluminescence (ECL) is a sensitive method and can be applied for relative quantitation of the protein of interest (Kurien Scofield 2006 and MacPhee 2010). The primary antibody binds to the protein of

interest and the secondary antibody linked to horseradish peroxidase, is applied to cleave a chemiluminescent agent. Once exposures have been captured, blots can be washed in a buffer and then ‘‘stripped,’’ which involves removing bound antisera to enable reuse of the blot. Blots can then be stored for future reprobing many more times. However, subsequent reprobing can interfere with protein antigens, resulting in a decreased signal (Alegria Schaffer Lodge and Vattem 2009). Relative quantification and analysis. The blot results can be analyzed by densitometry to measure the relative amount of a specific protein on the blot by comparing it with a control or specific time point. This quantification is necessary to compare samples. There are commercial software programs for image analysis of bands on film or membrane. Relative levels of protein expression can then be obtained via comparing ratios of intensities of a reference band (e.g. b-actin or glyceraldehyde-3-hosphate dehydrogenase [GAPDH]) or a band of known protein concentration. Relative optical density units can be plotted in a graph, and the appropriate statistical diagnosis can be performed on the samples that have been converted to optical density units (Jensen Wood and Keller Wood 2007).

3.5. Determination Of Protein Concentration

The protein concentrations of the samples to be loaded on a gel need to be evaluated. Quantification for total protein can be achieved by measuring samples at 280 nm on a spectrophotometer, but the buffer must not contain absorbing materials. When the buffer contains absorbing materials, the Bradford assay (Bradford 1976). Can be applied where a standard curve is created to determine unknown sample concentrations.

3.6. Preparation Of Pristine C60 Fullerene Water Colloid Solution

The C60 fullerene powder with a purity of (99.8 % was prepared according to a published protocol. The highly stable and reproducible pristine C60 fullerene aqueous colloid solution (C60 1.0 mg/ml, purity (99.5 %) was prepared, as published previously, by the transfer of C60 fullerene from toluene to an aqueous phase with the aid of ultrasonic.

3.7. Statistical Analysis

Data are presented as the mean SEM. Statistical comparisons were made by means of a one way analysis of variance (ANOVA) followed by a Bonferroni test using GraphPad Prism software (GraphPad Prism, v.6.01). A $P<0.05$ was considered statistically significant, data sets that are significant at different levels: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3. RESULTS

Events the results of western blot the astrocytes samples of treated with EEP in a range of concentration 10 μg/ml - 100 μg/ml shown total increased of GFAP 49kDa content in primary astrocyte cell culture. Though, there was not direct depending on effect/dose for 24 hours later treatment with EEP. The small dose of 10μg/ml EEP affected increased of cleaved GFAP contents about 22% to compare with control. On the opposite, the treatment with 25μg/ml induced the increase of GFAP contents about 89%. At the same time, a treatment primary astrocyte cell culture with dose 100 μg/ml during 24 hours lead to rising of the GFAP content on 67% to compare with control group [Figure 3.1]. Maximum rising of GFAP expression content was observed in growth of astrocyte samples treated with EEP 25 μg/ml a proximal to 1.9 times more compared with control. Moreover, increasing GFAP expression in astrocyte samples treated with 25μg/ml EEP and EEP+LPS indicated 2.43 times compared with control. LPS lead to increasing GFAP expression in 243/1.89 times. EEP with LPS can induce primary astrocyte reactivation with distinct mechanisms that targeted to cell proliferation and differentiation. The treated with 10μg/ml EEP primary astrocyte cell culture induce small increasing GFAP expression with constant cytoskeleton of primary astrocyte cells. The rising of GFAP expression was about 22% compared with control. Moreover, the treatment with 25 μg/ml concentration EEP induce more intensive rising GFAP expression about 89%. In contrast, treatment primary astrocyte with 100μg/ml EEP lead to increasing expression GFAP content on 67% [Figure.4.1].

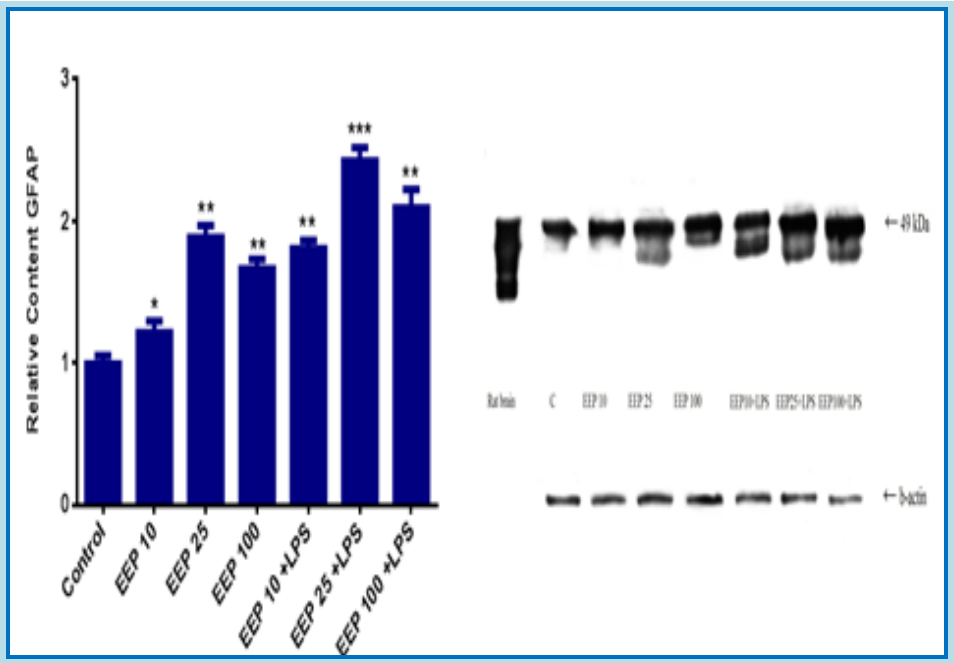

Figure 4.1. Relative content of GFAP in control and primary astrocytes cells culture treated with Ethanol Extraction Propolis (EEP) and (EEP+LPS), data sets that are significant at different levels: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Result analysed by a Bonferroni test using GraphPad Prism software (GraphPad Prism, v.6.01)

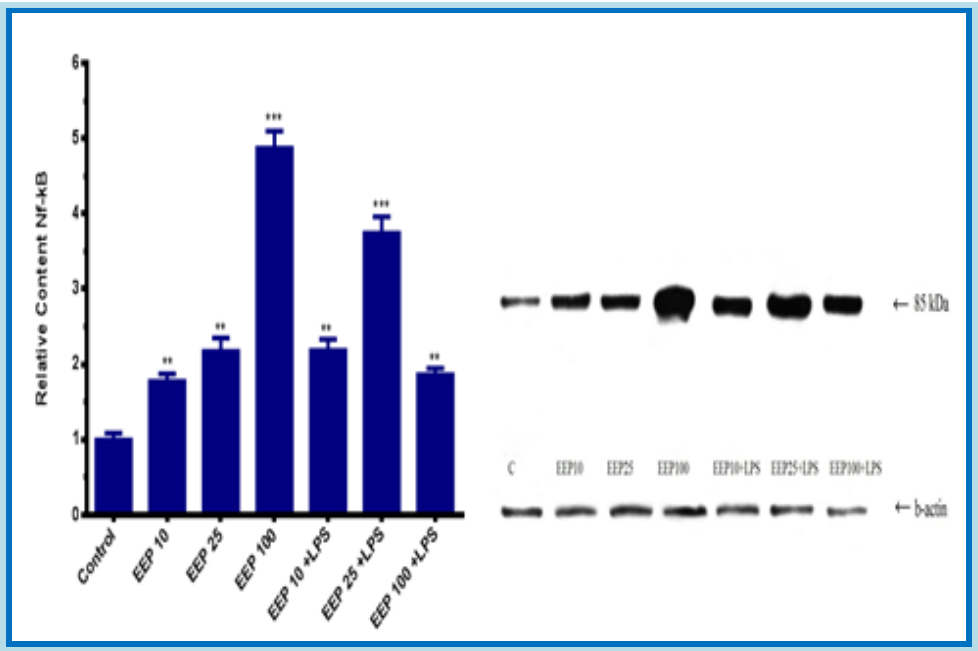

Figure 4.2. Relative content of Nf-kB in control and primary astrocytes cells culture treated with Ethanol Extraction Propolis (EEP) and (EEP+LPS), data sets that are significant at different levels: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Result analysed by a Bonferroni test using GraphPad Prism software (GraphPad Prism, v.6.01)

Results of western blot analyses the samples of primary astrocytes treated with EEP in a range of concentration 10µg/ml, 25µg/ml shown increasing of expression Nf-kB 60 kDa content in primary astrocyte culture. Moreover, there was not linear depend effect/dose for 24 hours later treatment with EEP. The small dose of EEP induced increasing of expression Nf-kB contents about 78% to compare with control. On the contrary, the treatment with 25µg/ml induced the rising of expression Nf-kB contents about 200%. The same time a treatment primary astrocyte culture with dose 100 µg/ml during 24 hours lead to dramatic enhancing of expression Nf-kB content on 488% to compared with control group. The combined treatment with EEP and LPS induced different results in the groups 25µg/ml, 100µg/ml studied compared EEP group treatment. The effects of EEP+LPS treatment on expression Nf-kB content for 25 and 100 µg/ml treatments were 157%, 300% accordingly[Figure 4.2]. In this results observed may be related with indirect effects of bioactive EEP compounds on multifactorial pathways that modulate of activation of the expression Nf-kB.

Our observed results are according to this data shown that treated with 100µg/ml EEP high expression Nf-kB than the treated with 100µg/ml EEP+LPS, its evaluated that LPS 0.01 µg/ml activated primary astrocytes cell, it can support some mechanisms to lower effect of the high level of expression Nf-kB.

Recently, new biologically active substances have appeared that can be useful in plasminogen of the astrocytes treated with EEP in a range of concentration 10 µg/ml - 100 µg/ml shown total enhanced except 100 µg/ml EEP+LPS deep decrease angiostatines 38kDa content in primary astrocyte culture. However, there was not linear depend on effect/dose for 24 hours later treatment with EEP. The small dose of EEP induced increasing of angiostatines contents about 45% to compare with control. On the contrary, the treatment with 25µg/ml induced the high expression angiostatines 38 kDa contents about 114% [Figure 4.3]. The same time a treatment primary astrocyte culture with dose 100 µg/ml during 24 hours lead to an increase of the angiostatines 38 kDa content on 63% to compare with control group.

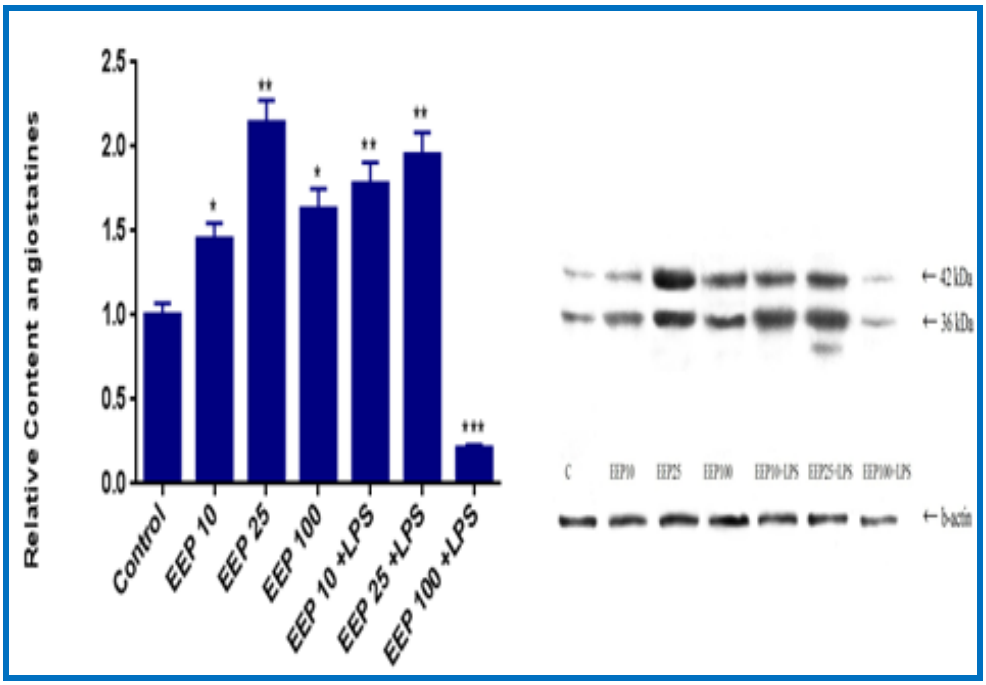

Figure 4.3. Relative content of angiostatins in control and primary astrocytes cells culture treated with-Ethanol Extraction Propolis (EEP) and (EEP+LPS), data sets that are significant at different levels:* p<0.05, ** p<0.01, *** p<0.001. Result analysed by a Bonferroni test using GraphPad Prism software (GraphPad Prism, v.6.01)

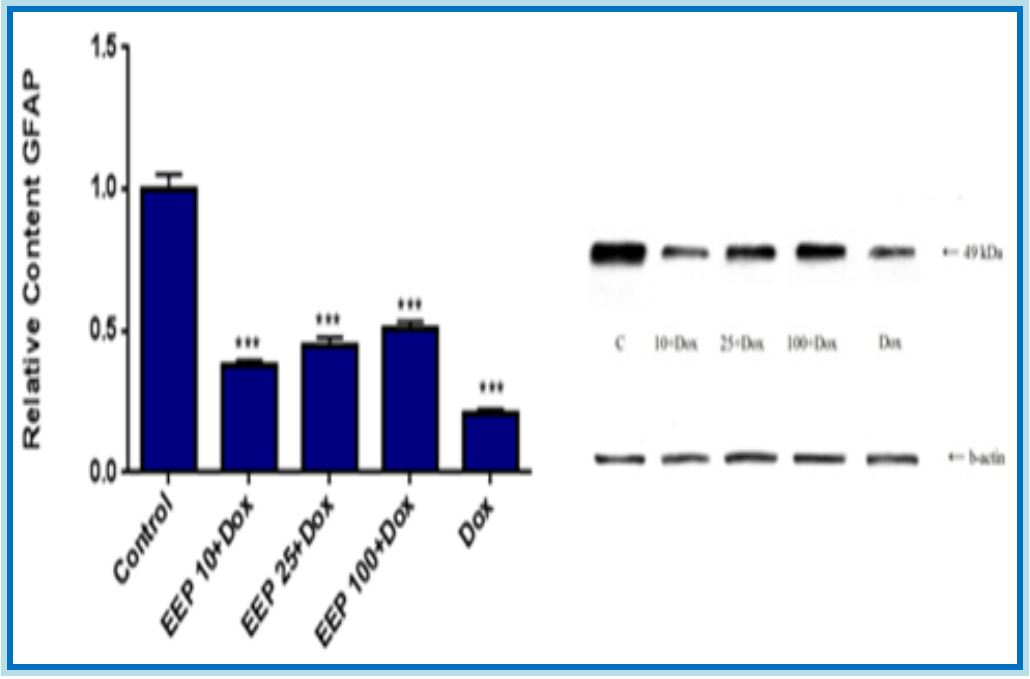

Figure 4.4. Effect of Dox on the expression of GFAP in primary astrocytes cell culture with or with out EEP, data sets that are significant at different levels: * p<0.05, ** p<0.01, *** p<0.001. Result analysed by a Bonferroni test using GraphPad Prism software (GraphPad Prism, v.6.01)

Western blot showed that primary astrocytes treated with EEP in a different concentration 10µg/ml -100µg/ml total decreasing of cleaved GFAP content in primary astrocyte culture. However, the small dose of 10µg/ml EEP+Dox-induced decreasing of cleaved GFAP contents about 83% compares with control. On the contrary, the treatment with 25µg/ml induced the decline of cleaved GFAP contents about 73%. The same time a treatment primary astrocyte culture with dose 100 µg/ml during 24 hours lead to the low decline of the GFAP content on 77% to compare with the control group, while Dox without EEP dramatically decline of the GFAP on 85% compared with the control group[Figure 4.4]. The result in western blot astrocytes treated with EEP, a range of concentration 10µg/ml - 100µg/ml shown total decreasing of expression angiostatines content in primary astrocyte culture. The small dose of Dox + EEP 10 µg/ml induced decreasing of kringle angiostatin contents about 85% to compare with control [Figure 4.5]. On the contrary, the treatment with Dox+EEP µg/ml 25 µg/ml caused the decline of kringle angiostatin contents about 65%. While treatment primary astrocyte culture with dose 100 µg/ml during 24 hours lead to the low decline of the angiostatin content on 8% to compare with the control.

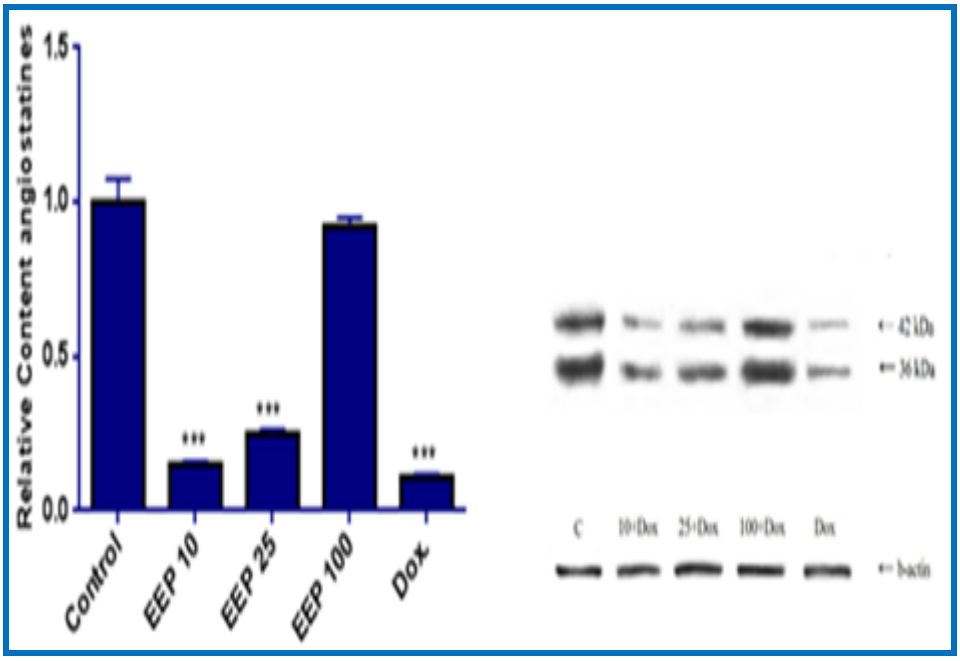

Figure 4.5. Effect of Dox on the expression of angiostatines in primary astrocytes cell culture with or without EEP, data sets that are significant at different levels: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Result analysed by a Bonferroni test using GraphPad Prism software (GraphPad Prism, v.6.01)

The results of western blot the shown that astrocytes treated with EEP in a range of concentration 10µg/ml -100µg/ml shown total decreasing of expression Hydroxinonenal (64 kDa) content in primary astrocyte culture. However, there was not linear depend on effect/dose for 24 hours later treatment with EEP. The small dose of EEP induced decreasing of Hydroxinonenal contents about 27% to compare with control. On the contrary, the treatment with 25µg/ml induced the decline of Hydroxinonenal contents about 14%. The same time a treatment primary astrocyte culture with dose 100 µg/ml during 24 hours lead to the dramatic decline of the Hydroxinonenal content of 77% to compare with control group [Figure 4.6]. The combined treatment with EEP and LPS induced various results. The effects of EEP+LPS treatment on Hydroxinonenal content for 100µg/ml treatments more decreased compare with control group contents about 89%, accordingly. In our experiment results may be related to indirect effects of bioactive EEP compounds on multi antioxidative mechanisms that modulated during the ROS content in primary astrocytes.

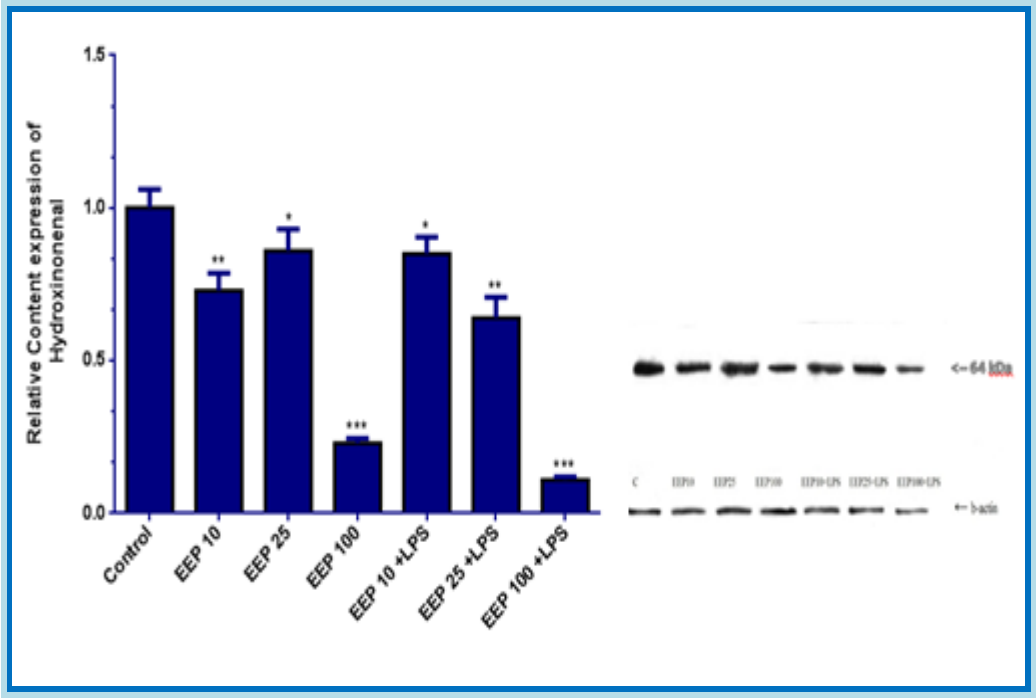

Figure 4.6. Hydroxinonenal effect. on the expression primary astrocytes cell culture with or with out EEP,EEP+LPS, data sets that are significant at different levels: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Result analysed by a Bonferroni test using GraphPad Prism software (GraphPad Prism, v.6.01)

Also, treated with 100µg/ml EEP+LPS compared with 10µg/ml EEP+LPS vary about 74% [Figure 4.7], in our results observed the treatment with EEP in the low concentration 10,25 µg/ml EEP and EEP+LPS limited effect on Hydroxynonenal, it can be produced ROS and activation antioxidative enzymes in primary astrocyte cells against ROS. The results of Immunoblot shown that samples of astrocytes activation by LPS (0.01 µg/ml) in primary astrocyte culture increases activation GFAP, Nf-Kb, and angiostatin stimulation compared to controls. But the Hydroxinonenal range was limit decreased in LPS-treated cells compared to controls. However, at same dose of LPS induced increased of GFAP contents about 44% to compare with control. On the contrary, the treatment with LPS 0.1µg/ml induced the expression of Nf-kB contents about 72%, Also the treatment with LPS 0.1µg/ml stimulate the rising of angiostatin to contain about 57%. The same time a treatment primary astrocyte culture with dose LPS 0.1µg/ml during 24 hours lead to the decline of the cleaved hydroxinonenal content on 5% to compared with control group [Figure 4.7].

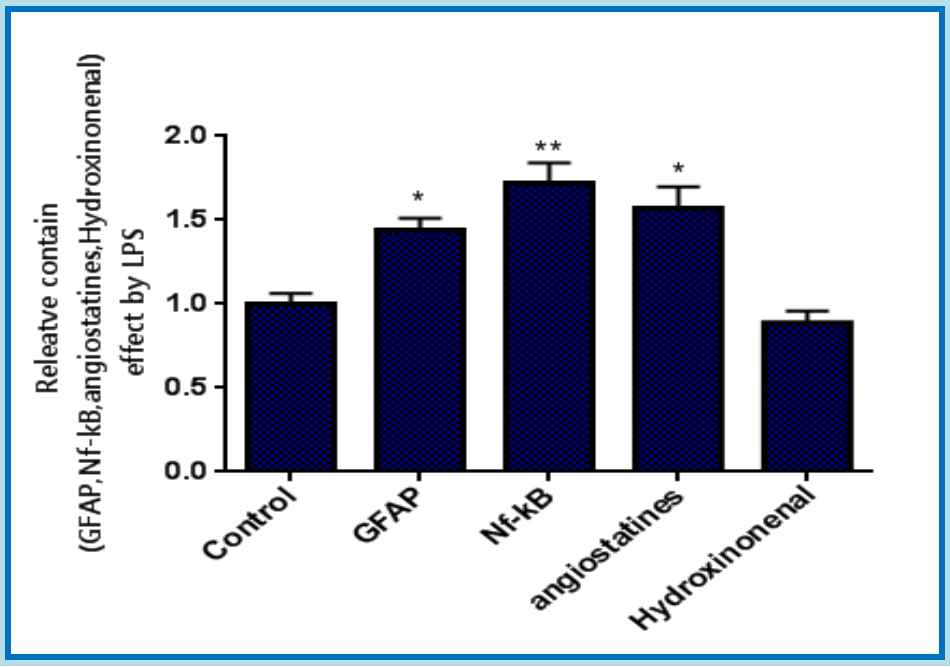

Figure 4.7. Comparative analyses of the changes induced in primary astrocytes cell culture treated with EEP, data sets that are significant at different levels: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Result analysed by a Bonferroni test using GraphPad Prism software (GraphPad Prism, v.6.01)

Observed our results may be related with indirect effects of LPS on expression factors that induced expression of GFAP, Nf-kB and angiostatines, activated primary astrocytes cell.

Events the results of western blot the astrocytes samples of treated with EEP in a range of concentration 10µg/ml - 100µg/ml shown total increased of GFAP 49kDa content in primary astrocyte cell culture. Though, there was not direct depending on effect/dose for 24 hours later treatment with EEP. Treated with C60 alone it was decreasing GFAP contents about 3% compared with control. Also, the small dose of 10µg/ml EEP+C60 affected to increased expression GFAP contents about 15% compare with control. On the opposite, the treatment with 25µg/ml EEP+C60 induced the increase expression of GFAP contents about 51%. At the same time, a treatment primary astrocyte cell culture with 100µg/ml EEP+ C60 during 24 hours lead to increasing GFAP expression content on 42% to compare with control group [Figure 4.8]. Nano-C60 elicited certain features of autophagy at relatively low concentrations that did not cause significant cell death. Given the considerable toxicity of fullerenes, and particularly Nano-C60, towards normal cells, it would be highly desirable to explore whether the capacity of fullerenes to induce autophagy at low and non-cytotoxic. Concentrations could be exploited to improve cancer chemotherapy (Q. Zhang et al. 2009). The cultivation of primary astrocytes cell culture treated with EEP+C60 fullerene during one day leads to significant increase of cell proliferation, especially 25µg/ml EEP+C60.

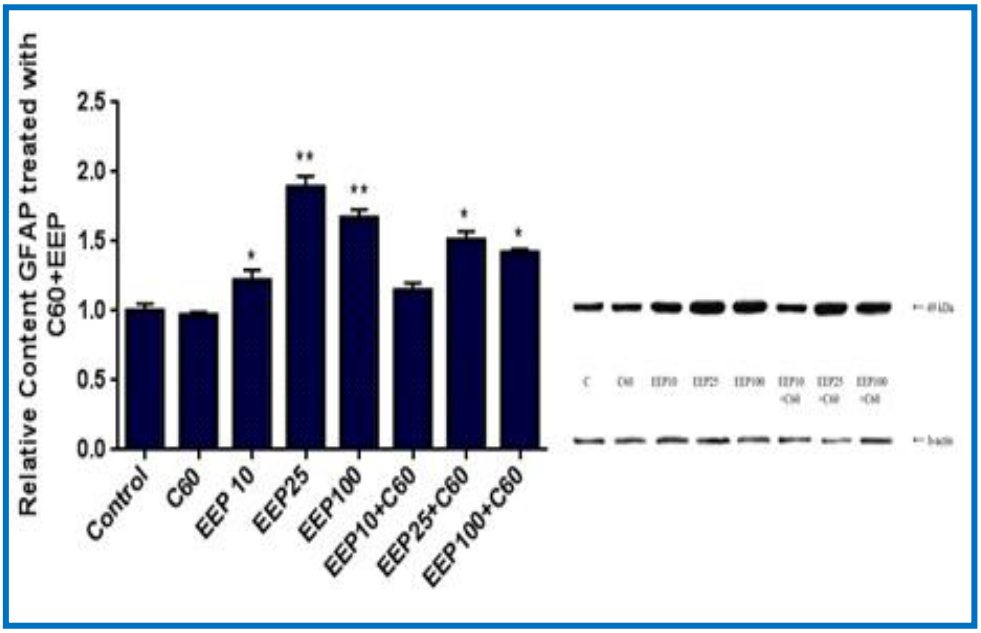

Figure 4.8. GFAP expression in primary astrocytes cell culture and treated with C60 alone and C60+ EEP, data sets that are significant at different levels: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Result analysed by a Bonferroni test using GraphPad Prism software (GraphPad Prism, v.6.01)

At the same time, C60 fullerene alone limited suppress the cell proliferation, shown that C60 fullerene alone affects it suppress level after one-day treatment of primary astrocytes cell versus control cells, only however, no significant changes were observed after 24 hr. In our results shown treated with 25µg/ml EEP+C60 highly significant to expressions GFAP from primary astrocytes cell culture than the treated with 10 µg/ml EEP+C60.

4. DISCUSSION

Astrocytes are one of the major cell types in the CNS and differ in their shapes. Protoplasmic astrocytes in the gray matter exhibit dense and complex branches, have contacts with the blood vessels for homeostasis and modulate synaptic functions. They express low levels of GFAP, which could be induced in injured conditions. Interestingly, human protoplasmic astrocytes are more complex, with more than 40 large processes and 27-fold greater volume than rodents. Thus, the diverse morphology of astrocytes does not simply reflect differences in their shape but is relevant to their functions in diseased states. The morphological changes of astrocytes in response to external factors have been mainly reported in primary astrocytes grown in vitro. Astrocytes respond to cocktails of growth factors, including FGF, and develop more branches with elevated GFAP expression (Kang Lee Han Choi and Song 2014). Hence, comprises variable changes in molecular expression and functional activity together with variable degrees of cellular hypertrophy. Although the high dose of propolis tended to over expression GFAP, statistical significance was not attained. Such changes occur after mild trauma or at sites distant from a more severe injury, or after moderate metabolic or molecular insults or milder infections or inflammatory activation. These changes vary with insult severity, include a little anatomical overlay of the processes of neighboring astrocytes and present the potential for structural resolution if the triggering insult is removed or resolves. Severe diffuse reactive astrogliosis includes changes in molecular expression, functional activity, and cellular hypertrophy (Sofroniew and Vinters 2010).

It is known that LPS activate primary astrocyte for producing proinflammative factors and reactivation. The combined treatment EEP with LPS induced similar directed changes in all examined groups. The effects of EEP+LPS treatment on GFAP expression content 10, 25 and 100 µg/ml treatments were 81%, 143% and 110% [Figure 4.1]. Respectively, in this result, the treatment with 25µg/ml EEP +LPS more intensive rising GFAP expression about 2.43% compared with control it may be cell over atrophy and death. Recognized

results may be related to indirect effects of bioactive EEP compounds on multifactorial pathways that modulate of generation of GFAP.

NF-κB is a protein complex that controls the transcription of DNA and is a central regulator of cellular stress in all cell types in humans. NF-κB plays a key role in regulating the immune response to infection and in acute and chronic inflammation (Armutcu Akyol Ustunsoy and Turan 2015). The activation of NF-κB in rats can stimulate the expression of IL-1β, which increases the expression of proinflammatory molecules. And regulation gene expression, especially proinflammatory element and antioxidative stress and growth factor, glial derived factor (GDF, DNF-2). During astrocyte activation. The transcription factor NF-κB has a pivotal role in a different of physiological processes throughout the body, including immune responses, cell proliferation, and inflammation. The activation of inactive NF-κB proteins existing in the cytoplasm is induced by numerous factors, including inflammatory cytokines (IL‑1 and TNF‑α). EEP stimulate some enzymes which are related to activation antioxidative mechanisms inside primary astrocytes cell culture and supporting neuron protection, treated with EEP+LPS 25µg/ml three times more three times than the control lead to activation expression Nf-kb. Hence, Nf-kB activated some oxidative enzymes also activated some repair enzyme in astrocyte cell [Figure 4.2]. However, treated with 10µg/ml EEP that defense the cell to survival but treated 100µg/ml EEP that causes cell proliferation cell death, due to expression Nf-kB rising 5 time than the control. Rising of Nf-kB content in astrocytes treated with 10, 25µg/ml EEP plays an important role in preventing of ROS-damaged. Nf-kB regulates expression antioxidative enzymes, and its activation can lead to repairing cell.This mechanism of regulation reactivity may be important for the neuroprotective effect of EEP. Nf-kB activation compares with GFAP expression from the concentration 10, 25µg/ml treated with EEP and10, 25µg/ml EEP+LPS are similar while treated with 100µg/ml EEP alone over activation Nf-kB lead to primary astrocyte cells death.

Plasmin (itself a fragment of plasminogen) enclosing three to five contiguous kringle domains. Each module contains two small beta sheets and three disulfide bonds (Cao et al. 1996). Angiostatin derived from cleaved plasminogen called Kringles. While differences of Kringles determinate its fore effectiveness inhibition of endothelial cells. More affective is Kringle 1-3. Presented results show that the dose 25µg/ml EEP induce maximum

production of the angiostatin in primarily astrocyte cell culture compared with 10µg/ml and 100µg/ml EEP. This result may be related to toxicity high dose of EEP fore mammalian astrocyte. The treatment concentration 100µg/ml EEP may have dual character. But, the dose EEP10 µg/ml and EEP 25 µg/ml are more appropriative for stimulation cells of neural tissue than EEP 100 µg/ml. The dose can induce molecularly and cell mechanism that limits cells reactivation. Producing of angiostatin of astrocytes treated with EEP has a character similar to changes in GFAP expression. Angiostatins inhibits the growth of vessels more active lead to an optimum balance between angiogenic and angiostatic factor. Our results of the angiostatin expression under the EEP treatment optimum concentration EEP 10µg/ml, lead to positive effect but from the concentration100µg/ml EEP+LPS lead to negative expression can lead cell damage especially primary astrocyte. The intensity of new blood vessel formation depends on the balance between proangiogenic factors, such as basic fibroblast growth factor-2, vascular endothelial growth factor (VEGF), and antiangiogenic factors, such as angiostatin, angiopoietin 2, and endostatin. Shifting of this equilibrium due to the increased expression of antiangiogenic or reduction of proangiogenic factors inhibits angiogenesis. Inhibition of tumor angiogenesis suppresses tumor growth and prevents metastasis (Butera et al. 2014). The combined treatment with EEP and LPS induced vary results in all studied groups. The effects of EEP+LPS treatment on cleaved 38 kDa content for 10, 25 and 100 µg/ml treatments were increased 78%, 95% and decreased 79% accordingly. Observed results may be related to indirect effects of bioactive EEP compounds on multifactorial mechanism that stimulate of generation angiostatin 38 kDa. In our results the treatment with 25µg/ml EEP with EEP+LPS have over expression there was not linear depend on effect/dose for 24 hours later treatment with EEP. Angiostatin is a 38 kDa fragment of a larger protein. Also, angiostatin stimulation compared with GFAP expression from the treatment 10µg/mlEEP+LPS, 25µg/ml EEP are similar while treatment with 100 µg/m EEP+LPS angiostatin dramatically decreased the lead to primary astrocyte cells death. Doxorubicin is one of the most potent anticancer drugs. One approach to protecting against Dox-dependent chemical insult has combined the use of the cytostatic drug with antioxidants (Prylutska et al. 2014). In our study was to estimate the antitumor effect and antioxidant enzyme activity of combined EEP and Dox in the primary astrocyte cell culture compared with Dox treatment alone. Doxorubicin anti-tumors lead to cell death (chemotherapeutic treatment) EEP can be effected against toxicity Dox On depletion

expression GFAP at the concentration 100 µg/ml Dox+EEP, Dox Generation of the oxidative stress. Hence, EEP antioxidant activity by this characteristic the main against Dox Towards normal cells, it would be highly desirable to explore whether the ability of EPP to induce autophagy at low and noncytotoxic concentrations could be exploited to improve cancer chemotherapy. Dox non-covalently binds to DNA, blocking the synthesis of nucleic acids, and exhibits high antimitotic activity and pronounced mutagenic impact, but exerts toxic effects in normal cells and tissues (Prylutska et al. 2014). One approach to protecting against Dox-induced chemical insult to normal tissues is a combined use of the EEP with antioxidants of a different nature. EEP has recently been recognized as a promising agent for use in anticancer therapy. While Dox without EEP dramatically decline of the angiostatines on 89% compared with the control group. The effects of Dox+EEP treatment on Kringle angiostatin content for 10, 25 and 100 µg/ml treatments were 85%, 65% and 8% accordingly [Figure 4.4]. Observed results may be related to indirect effects of bioactive EEP compounds on vary mechanisms that modulate of generation kringle angiostatin. Dox is an antibiotic of the anthracycline group, with a wide range of clinical activity against solid tumors and hemoblastoses lung carcinoma, and acute leukemia. Dox non-covalently binds to DNA, blocking the synthesis of nucleic acids, and demonstrates high antimitotic activity and pronounced mutagenic effect, but exerts toxic effects in normal tissues and cells (Prylutska et al. 2014). However, disorders in blood vessel development may foster diseases like chronic inflammatory disorders. Development of new vessels is also essential for the growth and metastasis of tumors, in which pro-angiogenic molecules like vascular endothelial growth factor (VEGF) and fibroblast growth factor play critical roles (Wierzbicki et al. 2013). EEP in this cell line did not lead to significant cell death. The combination treatment of EEP100 µg/ml with Dox Resulted in 8%death of drug-resistant primary astrocyte cell culture, while the same dose of Dox alone caused cell toxicity [Figure 4.5].

HNE is a ‘crossroads’ fragment regulating signal transduction, gene expression, cell proliferation, stress-mediated signaling and cell death. The specificity of HNE is highly dependent on its concentration, the continuance of exposure and the cell type. The inception of oxidative stress tolerance is intrinsic to each cell type in a particular environment. At low concentrations (less than five µM), HNE has been reported to increase proliferation, and a silent stimulation of antioxidant responses via low levels of HNE (hormesis) can be

preserve against more drastic assaults such as by carcinogens. At higher concentrations (20–100 μM), HNE causes cell cycle arrest, disturbs differentiation and triggers cell death. The modalities of apoptosis-induction pathways are governed by the inherent nature of the cell, prone or not to ROS production, the level of antioxidant defense and the induction of HNE metabolizing enzymes (Dalleau Baradat Gueraud and Huc 2013). Oxidative stress-induced lipid peroxidation has been related to human physiology and diseases including cancer. Overwhelming data suggest that reactive lipid mediators produced from this process, such as 4-hydroxynonenal (4-HNE), are important players for mediating some signaling pathways and biomarkers for oxidative stress. The biological effects of 4-HNE are primarily due to covalent modification of important biomolecules including DNA, proteins, and phospholipids containing an amino group. In this review, the role of 4-HNE in pathogenesis of cancer and concentrate on the intentness of mitochondria generation of 4-HNE from oxidation of mitochondria-specific phospholipid cardiolipin covalent modification of mitochondrial proteins, lipids, and DNA potential therapeutic strategies for targeting mitochondrial ROS production, lipid peroxidation, and 4-HNE (Zhong and Yin 2015). In our study the treated with 100 μg/ml EEP and EEP+LPS more suppression to activity primary astrocytes cell than the treated with10, 25μg/ml EEP and EEP+LPS While the treated with 25μg/ml EEP and EEP+LPS limited effect primary astrocytes cell compared with control. The comparison of treated with EEP+LPS100 μg/ml more decreased than treated with EEP100 μg/ml alone, it means Hydroxynonenal also cleaved by LPS but not more. LPS activity GFAP, Nf-kB, and angiostatin 44%,72%, and 57% it is one way for cell activity lead to activate proinflammation and response for astrocyte LPS activator for angiostatin it is not direct activate proinflammation way and does not control angiogenetic. So, angiostatin control angiogenetic by different kind of mechanism one mechanism it is regulation of gene expression but Nf-kB control another gene that controls with angiostatin. Angiostatin in the glial reactivity and astrocyte activate GFAP lead to increased angiostatin expression. LPS-induced systemic inflammation can increase generation iNOS and the activations of both astrocytes and microglial cells in the hippocampus and midbrain, and these activations are paralleled by the time-dependent apoptosis of astrocytes and microglia (Sun et al. 2016). Astrocytes express GFAP constitutive. Moreover, the expression of GFAP is increased during activation of astrocytes and astrogliosis. Due astroglial activation is further associated with the generation of NO we studied the role of nitric oxide in the expression of GFAP. Mouse primary astrocytes were

stimulated with various concentrations of LPS, the prototype inducer of various immune cells including CNS astrocytes. GFAP was expressed via control astrocytes; though, to monitor the increase by LPS, we used the semi quantitative approach. It is clear from that LPS dose-dependently enhanced the expression of GFAP in primary astrocytes with the maximum rise observed at the highest concentration (0.01μg/ml) of LPS tested. Assay of nitrite in culture supernatants also observed that the expression of GFAP was coordinate with the generation of NO. Viability assay revealed that LPS was unable to induce cell death at different concentrations tested. Subsequently, to correlate the expression of GFAP with NO production, we analyzed time-dependent expression of GFAP and production of NO in astrocytes. We observed that LPS was inefficient to increase the mRNA expression of GFAP within six h of stimulation. The increase in GFAP expression was apparent at 12 h, and a higher degree of increase was seen at a later hour of stimulation with the maximum increase observed at 24 h of stimulation. Interestingly, the induction of NO generation also began at 12 h and reached the maximum at 24 h. Therefore, the parallel induction of GFAP and NO suggests a possible involvement of NO in the enhanced expression of GFAP in astrocytes (Brahmachari Fung and Pahan 2006).

C60 fullerene has recently been identified as a promising agent for use in anticancer therapeutics. C60 fullerene and its derivatives are biocompatible, show no toxic effects on normal tissues at low concentrations, possess strong free radical- scavenging and antioxidant potential, and demonstrate a protective effect against Dox-induced chronic cardio- and hepatotoxicity. The tumor inhibitory activity of nanoparticles containing C60 fullerene has been studied in models of murine hepatocarcinoma, and rat colorectal cancer and mammary carcinoma. The study of the biological effects of pristine C60 fullerene is limited by its hydrophobicity, difficulties associated with reaching sufficient concentration, and aggregation in water. For this reason, hydrophilic derivatives taken by chemical alteration of the C60 fullerene outer surface were used in most studios of C60 fullerene antioxidant and antineoplastic effects (Prylutska et al. 2014).

CONCLUSIONS AND FUTURE ASPECTS

1. There were demonstrated that EEP and its major components induced induced overexpression of GFAP, Nf-KB, and generation of angiostatines.

2. Our findings indicated effect of EEP for the level of proliferation in primary astrocytes cell culture. Hence the propolis can be a possible potential candidate (protective agent) at low concentration (10µg/ml, 25µg/ml EEP).

3. Obtained results shown that EEP with higher dose 100µg/ml ameliorate cytotoxic effect of Doxorubicin (antitumor drug) on primary astrocytes cell cultures. This effect of EEP can applied for lowering toxic chemotherapy strategy.

4. Combining EEP with C60 fullerene treatment shown decreasing of astroglial reactivity induced only EEP treatment. However, C60 has not any effect on astrocytes while treated alone.

5. Treatment activated and nonactivated astrocytes with EEP induced decreasing hydroxinonenal content that reflects antioxidant effect of EEP.

6. Taking together obtained results, EEP combined with C60 fullerene may serve as a soft activator of primary astrocytes cell line to protect against oxidative stress.

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