THE EFFECTS OF ETHANOL EXTRACT PROPOLIS ON EXPRESSION NF-KB, P53 AND PARP IN PRIMARY RAT ASTROCYTE CELL CULTURE

Honar Sulaiman KAREEM

Master Thesis

Biology Department
Supervisor: Prof. Dr. Viktor NEDZVETSKYI

2017

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REPUBLIC OF TURKEY
BINGOL UNIVERSITY
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This thesis on 15.02.2017 by the following jury members unanimously has been accepted.

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LIST OF ABBREVATIONS

GFAP	:	Glial Fibrillary Acidic Protein
PARP	:	Poly (ADP-Ribose) Polymerase
JNKs	:	C-Jun N-Terminal Kinases
ICAM	:	Intracellular Adhesion Molecule
FBS	:	Fetal Bovine Serum
EDTA	:	Ethylenediaminetetraacetic Acid
MTT	:	Methylthiazolyl Diphenyltetrazolium Bromide
DMSO	:	Dimethyl Sulfoxide
SDS	:	Sodium Dodecyl Sulfate
TEMED	:	N,N,N'N' Tetramethylethylenediamine
GBM	:	Glioblastoma
TMZ	:	Temozolomide
PVDF	:	Polyvinylidene Difluoride
ECL	:	Electrogenerated Chemiluminescence
GAPDH	:	Glyceraldehyde-3-Phosphate Dehydrogenase
NAD	:	Nicotinamide Adenine Dinucleotide
MKP-1	:	MAP Kinase Phosphosphatase-1
TNF	:	Tumor Necrosis Factor
Fas	:	Fatty Acid Synthase
CAPE	:	Caffeic Acid Phenyl Ester
LPS	:	Lipopolysacharide
FGF	:	Fibroblast Growth Factor
KA	:	Kainic Acid
NO	:	Nitric Oxide

NOS	:	Nitric Oxide Synthase
PTZ	:	Pentylenetetrazole
ROS	:	Reactive Oxygen Species
CC	:	Cerebral Cortex
TNFα	:	Tumor Necrosis Factor Alpha
BS	:	Brain Stem
CNS	:	Central Nervous System
Ca2+	:	Calcium
IL-6	:	Interleukin 6 Receptor
TGF-B1	:	Transforming Growth Factor-B1
COX-2	:	Cyclooxygenase-2
PAMPs	:	Pathogen-Associated Molecular Patterns
TBI	:	Traumatic Brain Injury
FGFRs	:	Fibroblast Growth Factor Receptors
CNTF	:	Ciliary Neurotrophic Factor
TGF-β	:	Transforming Growth Factor Beta
STAT3	:	Signal Transducer And Activator Of Transcription 3
SOCS3	:	Suppressor Of Cytokine Signaling 3
cAMP	:	Cyclic Adenosine Monophosphate
OM	:	Outer Membrane
TM	:	Transmembrane
MOMP	:	Mitochondrial Outer Membrane Permeabilization
PTP	:	Permeability Transition Pore
MTP	:	Mitochondrial Transmembrane Potential
VDAC	:	Voltage-Dependent Anion Channel
PCD	:	Programmed Cell Death
AIF	:	Apoptosis Induce Factor
DNA	:	Deoxyribonucleic Acid
FADD	:	Fas-Associated Protein With A Death Domain
DISC	:	Death-Inducing Signaling Complex

DMEM	:	Dulbecco's Modified Eagle's Medium
AMID	:	Apoptosis-Inducing Factor-Homologous Mitochondrion-Associated Inducer Of Death
HI	:	Hypoxic–Ischemic
PostC	:	Postconditioning
EEP	:	Ethanol Extract Propolis
ADP	:	Adenosine Diphosphate
Dox	:	Doxorubicin

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PROPOLİSİN ETANOL EKSTRAKTININ PRİMER RAT ASTROSİT HÜCRE KÜLTÜRÜNDE NF-KB, P53 VE PARP EKSPRESYONLARI ÜZERİNE ETKİLERİ

ÖZET

Deneysel çalışmamızda primer rat astrosit hücreleri, propolisin etanol ekstraktının (EEP) farklı konsantrasyonlarına 24 saat maruz bırakılmıştır**.** EEP, primer rat hücre kültüründe hücre reaktifliği üzerine etki göstermiştir. Propolis, alternatif tıpta doğal ürün olarak dünya genelinde yaygın olarak kullanılmaktadır. Bu çalışmada propolisin etanol ekstraktı kullanılmıştır. EEP, kullanılan doza bağlı olarak hücrelerde çalışılan proteinlerin ekspresyonlarını azaltan ya da arttıran ajan olarak etki göstermiştir. İmmunoblotlama sonuçlarına göre EEP eklenen hücrelerdeki GFAP ekspresyonu artmıştır. 25 µg/ml EEP, GFAP ekspresyonunu kontrol grubuna göre istatiksel olarak en fazla arttıran doz olarak bulunmuştur ve % 89 oranında arttırmıştır ($p<0,01$). Western blotlama sonuçlarına göre PARP aktivasyonu kontrol grubuna göre önemli oranda değişmiştir. EEP'nin 10-25 µg/ml değişen konsantrasyonlarda hücrelere uygulanması PARP aktivasyonunu kontrol grubuna göre % 150 ve % 170 oranında arttırmıştır. 25 µg/ml EEP PARP aktivasyonunu en çok arttırmıştır. Ayrıca, 100 µg/ml EEP ile birlikte 0,01 µg/ml LPS eklenmesi PARP aktivasyonunu kontrol grubuna göre önemli oranda düşürmüştür. Yaklaşık olarak % 94'lük bir düşüş gerçekleşmiştir. 100 µg/ml EEP eklenmesi Nf-kB ekspresyonunu kontrol grubuna göre 4,88 kat arttırmıştır. Normal astrosit hücrelerinde değişlik konsantrasyonlarda EEP eklenmesi, p53 ekspresyonu üzerinde önemli bir değişikliğe yol açmamıştır. Bu sebeble insan glioma hücreleri kullanılmıştır ve EEP eklenmesi p53 ekspresyonunu değiştirmiştir. Bu çalışmada, hücrelere EEP eklenmesinin Nf-kB ve GFAP ekspresyonunu arttırarak hücre reaktifliğine yol açtığı ve bu durumun astrosit hücre reaktifliğinin sebeblerinden birisi olduğu bulunmuştur. Ek olarak 100 µg/ml EEP'nin astrosit reaktifliği için yüksek doz olduğu gözlemlenmiştir. PARP'ın inaktif formdan aktif forma dönüşümünü azaltan Nf-kB'nin yüksek aktivasyonu, Nf-kB'nin PARP'a doğrudan bağlanması sebebiyledir ve PARP aktivasyonunun azalmasının önemli sebeblerinden biri olabilir.

Anahtar kelimeler: Propolis, astrosit, Nf-kB, GFAP, PARP, P53.

THE EFFECTS OF ETHANOL EXTRACT PROPOLIS ON EXPRESSION NF-KB, P53 AND PARP IN PRIMARY RAT ASTROCYTE CELL CULTURE

ABSTRACT

Our experimental model employed primary cultures of rat astrocytes were treated with different concentrations of ethanol extract propolis (EEP) in vitro for 24 hours. EEP that showed cell reactivity of primary astrocyte cell culture. Propolis (or bee glue), collected from botanical sources by honey bee, has been used as a popular natural remedies in folk medicine throughout the world. Propolis crude samples were extracted using ethanol. Propolis followed a concentration-dependent increasing and decreasing trend. The results of immunoblot shown an increasing of GFAP expression in primary astrocyte cell culture. The primary cultures of rat astrocytes treated with EEP 25µg/ml induces more intense rising GFAP expression about 89% which was statistically significant (** $p<0.01$) compared to control. Western blot analysis demonstrated significant changes of PARP in primary astrocytes compared to control. In a range of small dose of EEP 10 µg/ml about 150% and EEP 25 µg/ml induced high activation of PARP content 170% compared with control. Otherwise the concentration of EEP 100 µg/ml +LPS 0.01 µg/ml contents about 94% showed total deep decreasing of PARP in primary astrocyte culture. Moreover, the concentration of EEP 100 µg/ml showed high optimum level of Nf-kB activation about 4.88 times compare with control. Furthermore we were observed there was no detectable of p53 expression in normal astrocytes treatment with different concentrations of EEP, thus we used the EEP treatment cognate to astrocytes human glioma cells for verification effect EEP on p53 expression. In this study we determined that EEP treatment can leads to cell reactivity and activate astrocyte through Nf-kB activation. Simultaneous activation GFAP expression and PARP may be just one of plural mechanisms for reactivation astrocytes. In addition, EEP treatment 100 µg/ml is over dose for astrocyte reactivity. All studied parameters are reflect together the one fundamental property of astrocytes, especially reactivation as response for EEP effect. Nf-kB overactivation which leads to depletion of PARP transformation into inactive to active form, because Nf-kB can directly binds PARP and may be main cause for suppression active form PARP.

Key words: Propolis, Astrocyte, Nf-kB, GFAP, PARP, P53.

1. INTRODUCTION

Propolis is the sticky mixture that bees accumulate from various parts of plants such as bark gum and use for sealing the pore and protecting the microbe in the bee hive. Propolis includes several compounds including phenolic, flavonoid, flavones, fatty acid, which have the therapeutic effects such as antimicrobial, antioxidant, immunostimulant and wound healing activities. Propolis gives strong anti-free radical activity, which resulted from the components in propolis including caffeic acid, ferulic acid, and caffeic acid phenyl ester. Propolis can repress the superoxide anion and hydroxyl radical, which are the important and hazardous reactive oxygen species. Free radicals are usually generated from the external source and biological process in the human body, and it can destroy the biomolecule including protein, lipid, and genetic material. Furthermore, free radicals disturb the homeostasis such as DNA repair, inflammation and cell proliferation (Khacha-ananda1 et al., 2013). A natural honeybee product, propolis has been confirmed its antibacterial, antiviral, antiproliferative, hepatoprotective and immunostimulatory activity. The anti-cancer effects of ethanol extract propolis (EEP) have been presented in various cell lines such as mammary carcinoma, epithelial carcinoma, different carcinomas, and human pancreatic cancer. The molecular mechanisms and the effects of honeybee bioactive compounds are actual questions of both cell viability and death regulation (Karpel-Massler et al., 2014) Red Propolis displays more intense cytotoxic action and inhibition of human leukemia cells growth compared with green propolis. The cytotoxic activity of red propolis on six different tumor cell lines was alike to that of anticancer drugs, such as 5-fluorouracil and doxorubicin (de Mendonca et al., 2015).

Astrocytes are specialized glial cells that outnumber neurons by over fivefold. They continuously tile the entire central nervous system (CNS) and exert many fundamental complex functions in the healthy CNS. Astrocytes respond to all forms of CNS insults through a process referred to as reactive astrogliosis, which has become a pathological

hallmark of CNS structural lesions. Substantial progress has been made recently in determining functions and mechanisms of reactive astrogliosis and in identifying roles of astrocytes in CNS disorders and pathologies (Sofroniew & Vinters, 2010).

Inflammation is an essential physiological host response to tissue injury and infection, which is vital for our body when facing invading microbes or the diseases. Several relatively abundant proinflammatory mediators and cytokines are characterized and have been validated their important roles in inflammatory responses, such as nitric oxide (NO), interleukin (IL-), and IL-6. The overproduction of these proinflammatory mediators is typically linked to proinflammatory stimuli which will cause acute or chronic inflammatory responses and be the pathogenesis of many diseases. (K. Wang et al., 2013).The NF-κB serves a family of transcription factors that participate in the regulation of diverse biological processes, including immune, inflammatory and apoptotic responses. Given its ability to regulate expression of inflammatory enzymes, cytokines, chemokines, immunoreceptors, and cell adhesion molecules, NF-κB has often been termed a "central mediator of the immune response". (Palempalli et al., 2009). Cells respond directly to DNA damage with posttranslational modifications of proteins that repair DNA damage, modify gene expression or control passage through the cell cycle. The covalent modification of these proteins induces a dynamic network of protein-protein interactions and regulates enzymatic activities, broadly changing cellular physiology and serving to integrate multiple responses to DNA damage that manage outcomes for DNA repair, cell survival, and responses to chemotherapy. One of the most prodigious posttranslational modifications caused by DNA damage is the poly-(ADP-ribosylation) of proteins, catalyzed by members of the poly-(ADP-ribose) polymerase (PARP) superfamily of NAD+ dependent ADP-ribosyltransferases. Poly-(ADP-ribose) (PAR) is a high, negatively-charged and branched polymer that can exceed the mass of the unmodified protein. PARylation creates binding sites for PAR-specific binding proteins and changes the electrostatic properties of the modified protein, with the notable ability to change DNA binding properties of enzymes, histones, and structural proteins. PARP-1 itself is the target of most of the poly-(ADP-ribosylation) (PARylation) happening in response to DNA damage. Automodification of PARP-1 increases its association with a variety of repair and signaling proteins that are recruited to sites of DNA damage by PARP-1 activity (Pascal & Ellenberger, 2015).

The aim of this study is the elucidation of reactivity cell and inflammatory factors activation that treated with ethanol extract propolis in primary rat astrocyte cell culture.

2. LITERATURE REVIEW

2.1. Propolis

The use of therapeutic plants, phytotherapeutics and organotherapy products in the treatment of diseases is growing universal and thus represents a promising market for the development of new drugs and the treatment of complex diseases, such as cancer (de Mendonca et al., 2015). Propolis is considered an organotherapy product because it carries organic secretions of the bees that produce it. The most popularly known pharmacologically active chemical components in propolis are flavonoids, isoflavonoids, phenolic acid, terpenes, xanthones, propolones and guttiferous, which value for its antimicrobial, anti-inflammatory, antioxidant, antiviral, antifungal and anticancer actions, among other. Climate variations might cause changes in the concentration of bioactive compounds of plants, with consequent alterations in the biological activity of the various types of propolis. Although therapeutic standardization of propolis is challenging, and the relationship between definite types of propolis and specific biological activities is difficult to establish, the presence of a significant amount of one particular compound might lead to the expectancy that the extract has the potential to show bioactivities linked to this possible (de Mendonca et al., 2015).

2.2. Techniques for EEP Preparing

Several approaches have been used for extraction of active component in propolis, for example, maceration, soxhlet extraction, ultrasonic extraction (sonication) and microwave extraction. For maceration technique, the organic solvent is used to dissolve the component in propolis directly without producing heat, so this method is suitable for heat labile and heat stable substance. Soxhlet extraction is made by heating and condensation to evaporate the organic solvent to concentrate the product. Thus, the advantage of this extraction is the use of the low volume of solution, and the extraction is

Suitable for heat stable substance. Sonication technique by an ultrasonic wave can reduce the time and solvent for extraction. This technique is used for dissociation and dissolving propolis. However, during sonication, the heat is produced, so the sonication technique is proper for heat stable substance. Also, the microwave extraction uses the microwave energy to heat solvent for dissociation of propolis. Thus, using microwave extraction also reduces time and solvent (Khacha-ananda, Tragoolpua, Chantawannakul, & Tragoolpua, 2013). Morphological changes of astroglial cell line after treatment with EEP appearing as a changing form of mitochondria with a dense matrix were observed in cells included large amounts of ADP (Dahout-Gonzalez et al., 2006). These morphological changes may indicate overactivity of cells. Morphological and physiological alterations such as cell shrinkage, condensation of chromatin, nuclear fragmentation, cytoskeleton rearrangement, cytoplasmic membrane blisters or apoptotic bodies' formation are the essential component of cell pathology.

2.3. Astrocytes

In the late 1800's, neuroglia was recognized as different cellular elements that included all supporting cells in the central nervous system (CNS). Neuroglial cells are subdivided into categories: astrocytes, oligodendrocytes, and, more newly, NG2 cells (i.e. oligodendrocyte precursor cells). Today, the term glia is commonly used to refer to neuroglia, Schwann cells, and microglia. Occasionally, ependymal cells (also called ependyma glia) are included in the term glia since they are derived from radial glia and share astrocytic properties. Astrocytes have been viewed as a homogeneous cell population that has a star-shaped morphology, extend many processes surrounding neighboring neurons and blood vessels, and include intermediate filaments (glial fibrils). While astrocytes are classically defined by their morphology and expression of glial fibrils, identifying 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 diverse population of cells with numerous functions. Besides, the finding that a subpopulation of GFAP-expressing cells displays neural progenitor or stem cell features and that astrocyte possess neuronal properties (e.g. glutamate vesicular release) further confuses the definition of an astrocyte. Below we summarize the characteristics that collectively would help define a cell as an astrocyte. In

mammals, gliogenesis, which corresponds to the generation of astrocytes and oligodendrocytes, begins late in embryonic development and continues during the neonatal and postnatal period (Figure 2.1.). In the cerebral cortex, one of the best-studied regions for gliogenesis, astrocytes are generated from three different sources: radial glia residing in the embryonic ventricular zone (VZ), progenitors in the postnatal SVZ, and a possible third lineage coming from glial-restricted precursors.(D. D. Wang & Bordey, 2008). Until quite recently, the current view concerning the biology and pathology of astrocytes among those studying clinical disorders and structural lesions of the central nervous system (CNS) was most likely to be that astrocytes are supportive glial cell components in neural tissue and reactive astrogliosis is a reliable and sensitive marker of the diseased tissue. The notion that dysfunctions of astrocytes or reactive astrocytes might offer to the presentation of clinical signs or mechanisms leading to pathological changes in CNS tissue was not considered. However, this viewpoint is gradually changing as a result of steadily increasing interest in and study of the biology and pathology of astrocytes. Over the past 25 years, it has become clear that astrocytes are responsible for a broad kind of complex and essential functions in the normal CNS, including primary roles in synaptic transmission and information processing by neural circuit functions. The functions and mechanisms of reactive astrogliosis and glial scar formation are slowly being elucidated. There is a growing body of proof that points towards the likely for loss of normal astrocyte functions or gain of abnormal effects to contribute to or play primary roles in, disease processes, and there are now numerous examples of astrocyte contributions to clinical and pathological mechanisms. This article summarizes recent advances in astrocyte functions in healthy tissue, the gradations, mechanisms, functions, and effects of reactive astrogliosis, and the appearance and potential roles of reactive astrocytes in a variety of CNS disorders and pathologies (Sofroniew & Vinters, 2010).

2.3.1. Functional Definitions of Astrocytes

An important characteristic of astrocytes that came to light in the last decade is that GFAP expressing cells can contribute to cell genesis both as stem cells and as major cellular elements of the neurogenic microenvironment (also called niche). In the adult SVZ and subgranular zone (SGZ), the multipotent neural stem cells (interchangeably called neural progenitors) express GFAP. These GFAP-expressing cells in the SVZ give

advance to neuroblasts that migrate to the olfactory bulb where they become synaptically integrated olfactory interneurons. Another population of neurogenic GFAP-expressing cells has been found in the SGZ, where GFAP-expressing cells can make newborn granule neurons. While these mature stem cells express GFAP, other cell types also express GFAP, but is not thought astrocytes. It is questionable whether these adult stem cells belong to the astrocyte family. First of all, these GFAP-expressing stem cells express nestin, an intermediate filament marker for embryonic precursor cells that is not present in mature astrocytes. In the adult SVZ, Nestin is also expressed in neuroblasts and intermediate progenitors. Interestingly, nestin is not expressed in the neonatal SVZ but is being in young cortical astrocytes at an early stage of differentiation and in reactive astrocytes, suggesting that nestin expression is not commonly exclusive with an astrocytic identity. GFAP-expressing stem cells do not express S100B, which is expressed in a minority of cells in the SVZ. The following properties of GFAP-expressing stem cells imply that they belong to the astrocyte family. In the SVZ, GFAP-expressing stem cells are derived from radial glia, which is neural progenitors during embryonic growth. GFAP-expressing cells in the SVZ and SGZ have anatomical features in common with astrocytes. For instance, they have long processes that surround and contact blood vessels and neuroblasts. In addition, just like mature astrocytes, they contain glycogen granules and express astrocytic glutamate and GABA transporters (i.e. GLAST and GLT-1, and GAT-3, respectively). Functionally, GFAP-expressing cells of the SVZ studied in acute slices participate properties with radial glia and astrocytes. They have K+ conductance at rest, express connexin 43 gap junctions and hemichannels, have functional glutamate transporters and GABAA receptors, but lack AMPAtype glutamate receptors, which are without in most mature astrocytes (except the specialized Bergmann glia). Nevertheless, GFAP-expressing cells of the SVZ lack barium-sensitive within rectifying K+ currents (KIR), a hallmark of astrocytic differentiation and cell cycle exit. Simultaneously, the recent studies suggest that the GFAP-expressing stem cells have characteristics of embryonic radial glia and mature astrocytes, but exhibit subtle differences and retain properties of neural progenitors. Perhaps these cells are kept in a transitional stage between radial glia and astrocytes, due to the insistence of embryonic extracellular matrix molecules. This permissive environment in the neurogenic niche allows the retention of intrinsic genetic programs to maintain “stemness”. In light data, GFAP-expressing cells of the SVZ have been termed SVZ astrocytes or astrocyte-like

cells. The earlier findings strongly suggest that SVZ astrocytes belong to the large family of astrocytes, but they need a sub-branch to distinguish them from mature astrocytes. Inside the neurogenic niche, it remains unclear whether every GFAP-expressing cell has the potential to behave as a neural stem cell. On the other hand, it is understandable that two types of GFAP-expressing cells, those that are stem cells or those that play instructive roles on neurogenesis, co-exist in addition to the S100B-expressing cells that do not coexpress GFAP. Such a finding would further complicate the nomenclature to distinguish these two types of cells correctly. Considering that astrocytes display an incredible degree of plasticity, it is likely that the same cell can both behave as a stem cell and still direct neurogenesis. One opportunity to address this issue would be to determine the genetic profile of proliferative versus non-proliferative SVZ astrocytes. Such an approach could also help to identify differences between SVZ and mature astrocytes. Future transplant studies into the SVZ of genetically modified mature astrocytes could determine whether they can be reverted to a stem cell phenotype (D. D. Wang & Bordey, 2008).

2.3.2. Synthesis of Extracellular Matrix Proteins, Adhesion Molecules, and Trophic Factors Controlling Neuronal Maturation and Synaptogenesis

Astrocytes are the main source of extracellular matrix (ECM) proteins and adhesion molecules in the CNS. Astrocytes in cultures can either promote or inhibit neurite outgrowth depending on the balance of ECM and adhesion molecules with direction signals navigating neurites during development or in response to injury. Growth-promoting molecules include (but are not limited to) laminin, N-cadherin, neural cell adhesion molecule (NCAM), and fibronectin. More recently, spontaneous calcium oscillations in cultured astrocytes have been shown to regulate neurite growth by controlling the expression of specific growth-enhancing proteins on an astrocytic surface, such as N-cadherin. On the contrary, inhibitory proteoglycans associated with glial boundaries during development provide guidance cues. Astrocytes also synthesize and secrete proteolytic enzymes, especially, the matrix metalloproteinases, which play a crucial role in ECM degradation and remodeling. For example, astrocytes manufacture MMPs of the gelatinase subfamily, MMP-2 and -9, which contribute to extracellular amyloid-β peptide degradation and clearance. Astrocytes are prominent to release growth factors in vitro, including nerve growth factor (NGF), brain-derived neurotrophic factor

(BDNF), neurotrophin-3 (NT-3), and fibroblast growth factor (FGF).These molecules control neuronal maturation and survival. Astrocytes have also been shown to develop neuronal differentiation in vitro via activity-dependent neurotrophic factor release. More specifically, ciliary neurotrophic factor (CNTF) and FGF can be released from cultured astrocytes possibly through a Ca2+-dependent pathway and increase neuronal survival and induce neuronal growth as well as differentiation. Other molecules such as S100B, which stimulates neurite outgrowth as well as astrocytic glutamate uptake, can be released by astrocytes to preserve neurons against glutamate excitotoxicity. These examples offer only a snapshot of the vast repertoire of astrocytic molecules that regulate neuronal maturation and survival under physiological conditions and the following injury. More recently, astrocytes were given to promote synaptogenesis between CNS neurons in vitro and in vivo during development. Interestingly, synaptogenesis coincides with the generation of astrocytes. Retinal ganglion cells cultured in the absence of astrocytes displayed very little spontaneous synaptic activity but showed robust postsynaptic excitatory activity when grown on a layer of feeder astrocytes. In fact, astrocytes were found to induce a 7-fold increase in synapse number between retinal ganglionic cells and a nearly 100-fold increase in synaptic activity. Some of the trophic factors secreted by astrocytes include and thrombospondins. The latter has been shown to be secreted by immature astrocytes in vivo to cause the formation of ultrastructurally normal synapses. (D. D. Wang & Bordey, 2008).

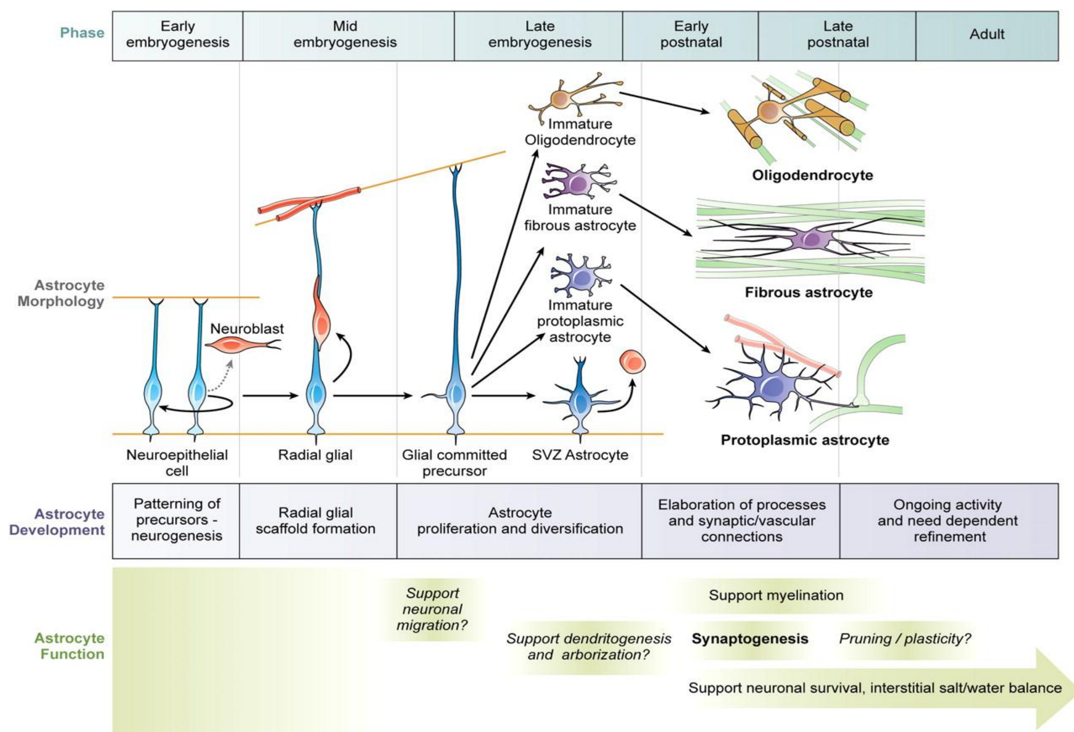

Figure 2.1. Astrocyte morphology and function differences across developmental time. Neuroepithelial cells give increase to radial glia, which generates primary neurons, and then become glial-committed, providing the advance to precursors that proliferate and vary into fibrous and protoplasmic astrocytes, which then go into a protracted stage of postnatal maturation. Astrocyte precursors at these different stages of maturation follow well-established stage-specific roles in assisting myelination and synaptogenesis and may also control other functions, such as neuronal migration, pruning, and so forth. Well-established adult roles for astrocytes, including supporting neuronal survival and homeostasis, likely develop in parallel (Molofsky et al., 2012)

2.3.3. Angiogenesis

Angiogenesis, the production of blood vessels, includes several steps involve basement membrane degradation, endothelial cell proliferation and recruitment, tube formation, and maturation including reconstitution of the basement membrane. When cocultured with astrocytes, endothelial cells form capillary-like structures. Epoxyeicosatrienoic acid (EET), which is the produce of cytochrome P450 epoxygenation of arachidonic acid, is one of the molecules released from astrocytes that can function as a mitogen and morphogen for endothelial cells. Also, astrocytes synthesize laminin to form the astrocyte-endothelial cell interface resembling the basement membrane. An optimal system to study angiogenesis is the retina, which contains normal stellate astrocytes and a specific astrocytic type, called the Muller cell. In the retina, angiogenesis is controlled by an active cooperation between retinal neurons, astrocytes, and endothelial cells. In

particular, retinal neurons release platelet-derived growth factor (PDGF) to stimulate proliferation of astrocytes, which in turn excite blood vessel growth by secreting vascular endothelial cell growth factor (VEGF). In addition, improving vessels provide feedback signals that trigger astrocyte differentiation, including cessation of cell division and upregulation of GFAP. In addition, the final arrangement of retinal blood vessels depends critically on the pre-patterning of astrocytes that direct the width of filopodia budding off the vascular plexus via VEGF receptor activation. Such cooperation may also occur in the CNS, but this remains to be examined. (D. D. Wang & Bordey, 2008).

2.3.4. Astrocyte Biology in Healthy CNS

Since the late nineteenth century, astrocytes have been classified into two main subtypes, protoplasmic or fibrous, by differences in their cellular morphologies and anatomical locations. These two main categories maintain their validity and advantage today. Protoplasmic astrocytes are found during all gray matter and, as first described using classical silver impregnation techniques, exhibit a morphology of several stem branches that give rise to many finely branching processes in a uniform globoid distribution. Fibrous astrocytes are found throughout all white matter and display a morphology of many long fiber-like processes. Classical and modern neuroanatomical studies also indicate that both astrocyte subtypes make extensive contacts with blood vessels (**Figure 2.1.**). Electron microscopic analyses of the mid-twentieth century revealed that the processes of protoplasmic astrocytes surround synapses and that the processes of fibrous astrocytes contact nodes of Ranvier, and that both types of astrocytes form gap junctions between distal processes of neighboring astrocytes (Sofroniew & Vinters, 2010).

2.3.5. Molecular Markers and Proteomic Characterization

Immunohistochemical techniques that allow the detection of specific molecular markers at the single-cell level are essential tools for recognizing and characterizing cells in the healthy and pathological tissue. Expression of glial fibrillary acid protein (GFAP) has become a prototypical marker for immunohistochemical identification of astrocytes. However, it is important to recognize the appropriate uses and limitations of GFAP as an astrocyte marker. GFAP was first separated as a protein highly concentrated in old demyelinated plaques from multiple sclerosis patients and was then found to be

associated immunohistochemically with reactive astrocytes in such plaques and other pathological contexts. In line with this fundamental mode of description, GFAP expression can be marked as a sensitive and reliable marker that labels most, if not all, reactive astrocytes that are responding to CNS injuries. Though, GFAP is not an absolute marker of all non-reactive astrocytes and is usually not immunohistochemically detectable in astrocytes in healthy CNS tissue or isolated from CNS lesions. Although GFAP is immunohistochemically detectable in many astrocytes throughout the healthy CNS, it is evident from double staining with multiple markers (including transgenic reporter proteins) that many developed astrocytes in healthy CNS tissue do not express detectable levels of GFAP and that GFAP expression by astrocytes exhibits both regional and local variability that is dynamically regulated by a large number of inter- and intra-cellular signaling molecules (Figure 2.2.). GFAP has been studied extensively. GFAP is one of a group of intermediate filament proteins, containing vimentin, nestin, and others, that serve largely cytoarchitectural functions. Studies in transgenic mice specify that the expression of GFAP is not essential for the normal appearance and function of most astrocytes in normal CNS of transgenic mice, but is necessary for the process of reactive astrogliosis and glial scar formation. There are different isoforms and join alternatives of GFAP including Gfap-α, Gfap-β, Gfap-γ, Gfap-δ, Gfap-κ, and Gfap-ζ , and these may be expressed in a heterogeneous manner in both healthy CNS and pathological specimens including glioma, but the differential distribution and roles of GFAP isoforms are only beginning to be studied. At the single-cell level, in line with its structural role, GFAP is not present throughout astrocyte cytoplasm, and GFAP immunohistochemistry does not label all portions of the astrocyte but only in the main stem branches. GFAP is absent from the finely branching astrocyte processes and is often not detectably present in the cell body. Consequently, GFAP immunohistochemistry can considerably underestimate the amount of astrocyte branching and territory in comparison with other means of detection such as Golgi staining or expression of reporter proteins such as GFP or b-galactosidase or be filling with fluorescent dyes. Besides, as notes the use of GFAP as an astrocyte marker, it is important to point out that GFAP expression is not exclusive to protoplasmic and fibrous astrocytes. Within the CNS, GFAP is also expressed by some cells that can be considered part of an extended astroglial family that is described in more detail below. Outside of the CNS, GFAP is expressed widely in many tissues by a variety of cell types, also discussed below. Other molecular markers that have been used for

immunohistochemical identification of astrocytes and reactive astrocytes cover glutamine synthetase and S100 b, but these molecules are not entirely restricted to astrocytes. Several recent studies have conducted the large-scale genetic analysis of the astrocyte transcriptome in rodents and humans, and have identified significant numbers of molecules enriched in astrocytes as compared with other neural cells such as neurons and oligodendrocytes. Such studies will provide a rich resource for identifying molecules for mechanistic analyses of functions and roles of astrocytes and reactive astrocytes. Also, such studies are identifying potential candidates for additional, and better, molecular markers with which to identify astrocytes. One such candidate appears to be the protein Aldh1L1, whose promoter reliable targets the expression of reporter molecules such as GFP (green fluorescent protein) to astrocytes and whose identification by immunohistochemistry may give a sensitive chemical marker for most if not all astrocytes in healthy tissue (Sofroniew & Vinters, 2010).

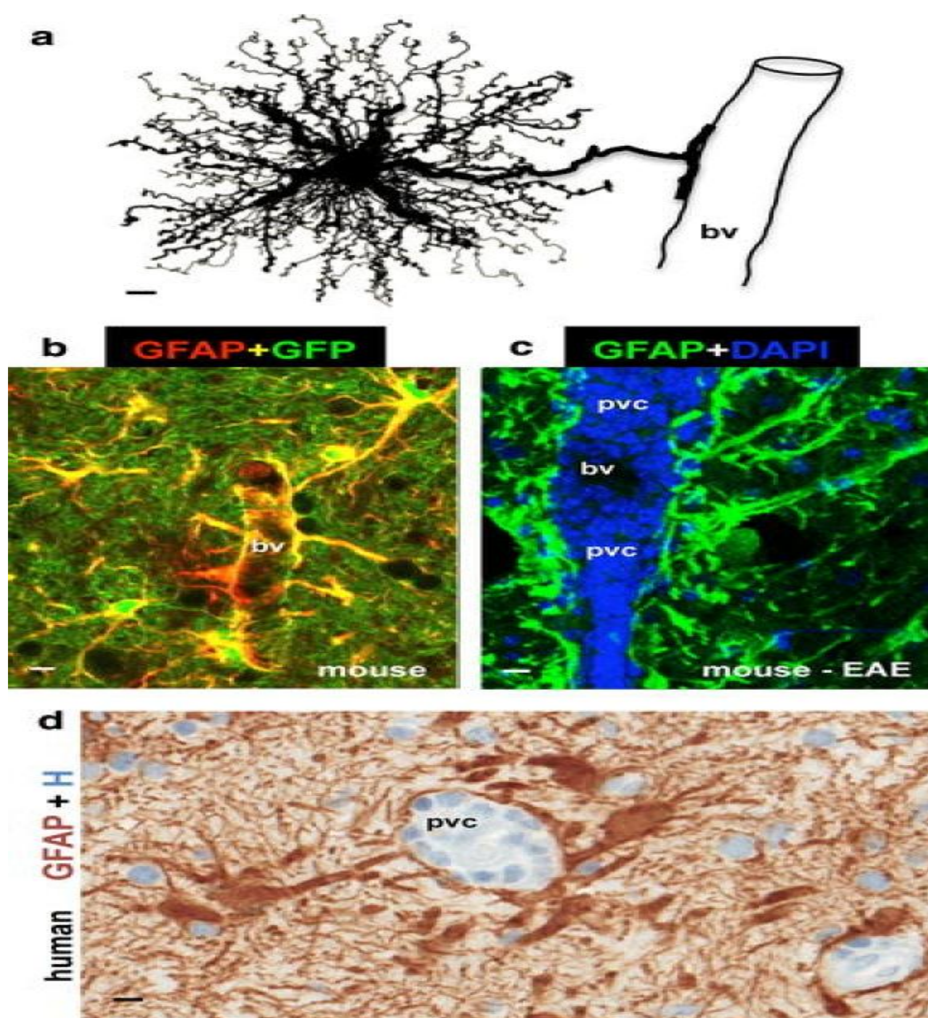

Figure 2.2. Astrocyte morphology and interactions with blood vessels in healthy and diseased tissue.

A The protoplasmic astrocyte is giving rise to a dense network of finely branching processes during 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 normal mouse gray matter stained immunohistochemically for GFAP (red) as well as the transgene-derived reporter molecule GFP (green). See that in these transgenic mice, GFP reporter exists in all of the fine processes of the protoplasmic astrocytes throughout the neuropil, whereas the GFAP is present only in the high stem astrocyte processes and end feet (which appear yellow where green and red staining overlap). Note that end feet from many astrocytes contact and envelop bv. C Two-color fluorescence is showing dense collections of GFAP-positive (green) endfeet and processes of reactive astrocytes lining up along perivascular cuffs or clusters (PVC) of inflammatory cells stained with DAPI (blue) in a mouse with test autoimmune encephalomyelitis (EAE). Transgenic disruption of this reactive astrocyte barrier leads to great invasion of inflammatory cells away from perivascular clusters into CNS parenchyma during EAE. D Two color brightfield staining is showing human necropsy specimen with reactive astrocytes lining their processes along perivascular cuffs of inflammatory cells as if forming perivascular scar-like barriers similar to those observed in experimental animal models. Scale barsa 3 µm, b 7.5 µm, c 15 µm, d 5 µm (Sofroniew & Vinters, 2010)

2.3.6. GFAP-expressing Stellate Cells within Liver, Pancreas, and Kidney

There is now growing evidence that stellate-shaped, GFAP-expressing cells in various tissues may have functions that are similar to those of astrocytes. For example, there is rising evidence that GFAP- expressing pancreatic stellate cells play important roles in tissue repair, fibrosis, and scar formation. Hepatic stellate cells not only express GFAP and have the morphological appearance related to astrocytes but also contact hepatic sinusoids and blood vessels and may take part in hepatic immune processes. In this opinion, it is particularly interesting that the liver, like the CNS, has a certain level of immune privilege, and that both CNS and liver have resident populations of macrophage-related cells (Kupfer cells in the liver and microglia in the CNS) as well as of stellate cells that take part in immune and inflammatory processes. Also, there is the indication that GFAP-expressing mesangial cells in the kidney function as local modulators of innate and adaptive immune responses. The developing recognition that tissue-specific cells like astrocytes and stellate cells play essential roles in regulating local immune and inflammatory processes is likely to impact considerably on concepts about organ-specific vulnerability to autoimmunity and other inflammatory conditions (Sofroniew & Vinters, 2010).

2.3.7. Reactive Gliosis

Reactive gliosis is a pathological process that astrocytes take on in diseased conditions such as ischemia, traumatic injury, infection, and systemic inflammation. Reactive astrocytes divide and convert hypertrophic with long and thick processes. The expression of the glial fibrillary acidic protein (GFAP) in reactive astrocytes increases in response to a kind of insults, such as lipopolysaccharide (LPS), and causes glial scar tissues to form around the injury site. Consequently, the injury site is sealed, and the spread of inflammation or damage to the adjacent intact tissues is minimized. However, reactive astrocytes are detrimental because they physically block neuronal regeneration and hence inhibit functional recovery. The mechanisms underlying the morphological changes in astrocytes under injury conditions, and their biological implications in relation to neuronal regeneration, remain unclear (Kang, Lee, Han, Choi, & Song, 2014).

2.3.8. Reactive Astrocytosis

The CNS responds to neural injuries with a rise in the number and size of cells expressing the glial fibrillary acidic protein (GFAP), a phenomenon referred to as reactive astrocytosis. GFAP is an intermediate filament cytoskeletal protein expressed primarily by astroglia and represents the prototypic marker of astroglial activation. Nevertheless, despite its prominent up modulation in response to various injuries, the proper function of the GFAP molecule remains uncIear. Suppression of GFAP expression in glial cell lines with antisense mRNAs suggests that GFAP may be necessary for the formation of stable glial processes in response to neuronal signals (Figure 2.3.) (Eddleston & Mucke, 1993).

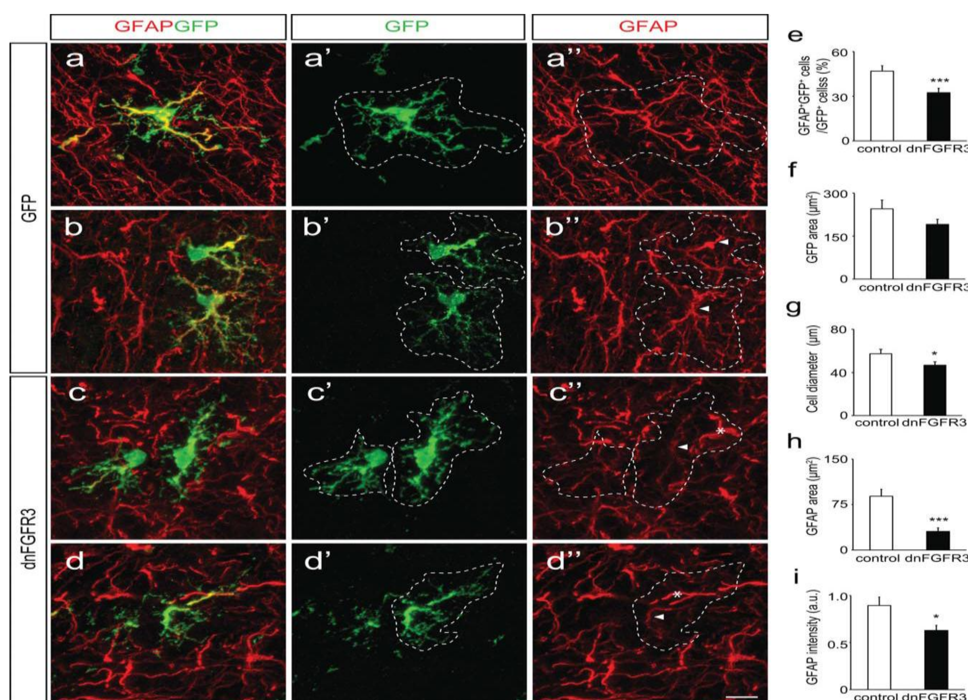

Figure 2.3. Altered morphology and GFAP expression in reactive astrocytes by blocking FGFR3 activity. (a–d00) Two different examples of astrocytes infected with the GFP (a–b00) or dnFGFR3 virus (c–d00), labeled with GFP (green) and GFAP (red). Dotted lines mark the boundaries of astrocytes. Note that GFAP expression of GFP1 processes (arrowheads, b00–d00) was weaker than the GFAP1GFP- processes of adjacent astrocytes that invade the zone of a GFP-labelled astrocyte (asterisks, c00, d00). (e–i) Quantification of the percentage of astrocytes, GFP area, diameter, GFAP area and GFAP intensity in GFP (n511) or dnFGFR3 (n517)-infected astrocytes (Kang, Lee, Han, Choi, & Song, 2014)

2.4. NF-κB (Nuclear Factor Kappa-Light-Chain-enhancer of Activated B Cells)

Nuclear factor kappa-light-chain-enhancer of activated B cells (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 induce the expression of IL 1β, which enhances the expression of proinflammatory molecules (Caccamo et al., 2005). NF-κB was found by Sen and Baltimore in 1986 as a nuclear factor that ties to the enhancer region of the κB chain of immunoglobulin in B cells. It has been shown since to be a ubiquitous transcription factor present in all cell types. It consists of homo- or heterodimers of the Rel family proteins, p50/NF-κB1, p52/ NF-κB2, p65/RelA, and c-Rel. In most cell types studied to date, in resting stage NF-κB dimers are retained in the cytoplasm through a physical association with inhibitor proteins, termed IκBα (Yadav, Prasad, Sung, & Aggarwal, 2011). In normal conditions, the inhibitor of B binding protein (IB), such as IB, interacts with p50/p65 heterodimer in the cytoplasm and masks nuclear localization sequence, so the NF-kB dimers failed to bind to B sites. In inflammatory situations, IB will be phosphorylated then degraded by 26 s proteosome. This leads to the free NF-kB to translocate to the nucleus, binding specifically to the B sites in the genome. AP-1 is a family of DNA-binding transcription factors composed of dimers with two proteins. The best-characterized AP-1 consists of two proteins namely Jun and Fos (Chang et al., 2010). Various genes encoding for proinflammatory mediators, cytokines, chemokines, and some inducible enzymes contain B sites and AP-1 binding sites. The transcription of these genes is organized, in a sense, by the NF-kB and AP-1 activity (Vallabhapurapu & Karin, 2009). The NF-κB signal transduction pathway is dysregulated in a kind of human cancers. In most cancer cells, NF-κB is constitutively active and remains in the nucleus. NF-κB activity not only protects cancer cells from apoptotic cell death but may even improve their growth activity. Inhibition of NF-κB activation produces a corresponding increase in apoptosis, indicating that the balance of cell viability versus cell death is protected by the degree of NF-κB activation. Agents that can down-modulate the activation of NF-κB have possible for therapeutic intervention (Onori et al., 2009).

2.5. Overview to the Inflammatory Response

Inflammation is an immunological response to pathogens and damage that is initiated to preserve the body and provides to physiological and pathological processes, such as wound healing and infection at the compromised site. The process is characterized by adhesion, migration, and chemotaxis of leukocytes to the inflammatory environment. The transcription factor NF-κB plays a central role in regulating inflammatory, immune and anti-apoptotic responses. It is constituted of homodimers and heterodimers of the Rel family of proteins, including p65/RelA, RelB, c Rel, p50/p105 and p52/p100. 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. Therefore, agents that can downregulate the activation of NF-κB have a potential for therapeutic interventions, whereas the activation of NF-κB advance 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 by increasing the expression of several inflammatory cytokines that participate in 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 associated with inflammation.

The transcription factor NF-κB has an essential role in a variety 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. Several in vitro and in vivo studies have reported various 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 recorded that CAPE acts to suppress the NF-κB activation induced by ROS-generating agents in human histiocytic and coronary artery endothelial cells. It is considered that, rather than preventing the degradation of κB inhibitor α (IκB α), CAPE suppresses NF-κB activation by inhibiting the interaction between NF κB proteins and

DNA (Figure 2.4.). The anti-inflammatory effect of CAPE is most expected due to the inhibition of ROS production at the transcriptional level, through the suppression of NF-κB activation, and the direct inhibition of the catalytic activity of iNOS. CAPE displayed an anti-inflammatory action on rat burn healing by reducing MPO activity, NO levels and the number of CD68 positive cells. CAPE decreased 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.

NF-κB signaling also has central roles in chronic precancerous inflammation and cancer-induced inflammation. CAPE acts to down regulate inflammation by blocking NF-κB and affects a kind of mediators, including adhesion molecules, cytokines, and iNOS. CAPE is a well-documented inhibitor of NF-κB, which may be an action mechanism for the CAPE mediated anti-inflammatory and anticancer effects. Although CAPE has been described to behavior its anti-inflammatory activities by modulating various inflammatory pathways, containing inhibition of the transcription factors NF-κB and signal transducer and activator of transcription 3 (acute-phase response factor), the compound has already been evaluated for antitumor efficacy in numerous in vitro and in vivo studies (Armutcu, Akyol, Ustunsoy, & Turan, 2015). Certainly, it has been reveal that oxidative stress leads to activation of the transcription nuclear factor NF-kB, which, in turn, induces the expression of several genes (growth factors, cell adhesion molecules, and cytokines) regularly regulating biochemical pathways involved in astroglial cell response to various stimuli (Caccamo et al., 2005). The brain is highly susceptible to being damaged by hypoxia because of its high order for oxygen supply. Microglia is resident innate immune cells in the brain, establishing the first line of defense against brain insults. It is commonly accepted that hypoxia is one of the neuroinflammatogens in the brain because hypoxia activates microglia to produce the excessive secretion of proinflammatory cytokines, including interleukin-1 (IL-1) and tumor necrosis factor-(TNF-α). It is also known that proinflammatory cytokines secreted by microglia improve cognitive deficits in aged people and Alzheimer’s disease (AD) patients. In previous studies, were observed that enhanced production of reactive oxygen species (ROS) due to the increased mitochondrial DNA damage in microglia is responsible for exaggerated inflammatory responses in aged animals after treatment with lipopolysaccharide, because the increased intracellular ROS level activates nuclear factor (NF-kB) which regulates the

expression of several proinflammatory cytokines. Hypoxia can stimulate microglia to generate ROS. Therefore, it is reasonable to suppose that hypoxia activates NF-kB to induce the overstated inflammatory response by microglia for enhanced production of ROS due to the mitochondrial DNA damage. Propolis is a resinous substance produced by honeybees as a defense against intruders. It has important therapeutic properties that have been used since ancient times. The chemical composition of propolis depends on the local floral at the site of collection. Regarding the fact that propolis has hepatoprotective, antitumor, antioxidative, and anti-inflammatory effects, propolis may have protective effects against the hypoxia-induced neuroinflammatory responses. In the recent study, was supply the first evidence that propolis can significantly inhibit the secretion of IL-1, TNF-, and interleukin-6 (IL-6) by microglia through inhibition of the NF-kB activation in microglia. Furthermore, propolis significantly inhibits the increased generation of ROS from the mitochondria that are responsible for the NF-kB activation. These observations recommend that propolis may be useful to prevent hypoxia-induced neuroinflammation (Wu et al., 2013).

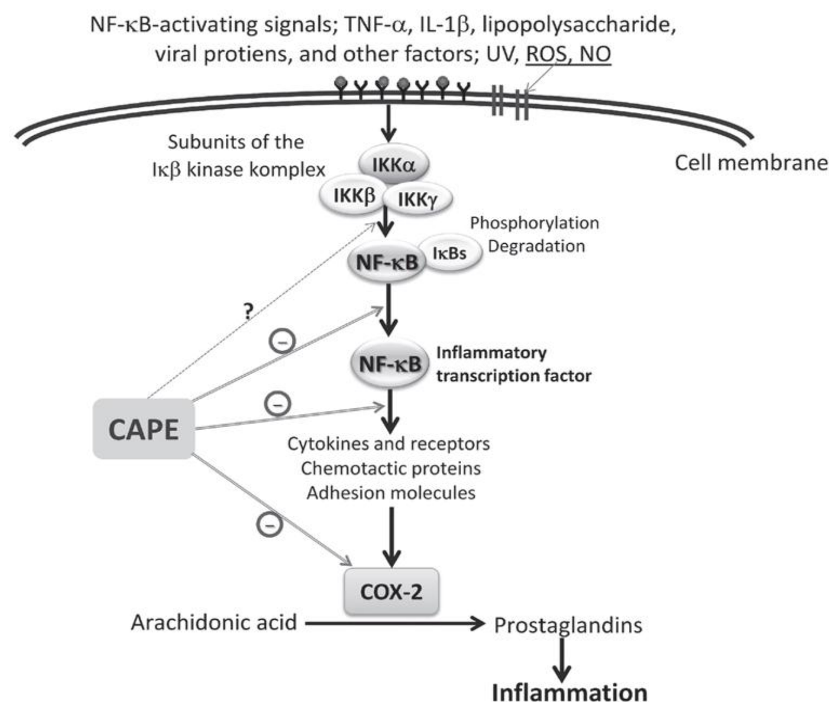

Figure 2.4. Schematic presentation of the anti-inflammatory effects of CAPE in the inhibition of inflammation. COX 2, cyclooxygenase 2; IκB α, κB inhibitor α; IKK, IκB kinase; IL 1β, interleukin 1β; NF κB, nuclear factor κB; NO, nitric oxide; ROS, reactive oxygen species; TNF α, tumor necrosis factor α; UV, ultraviolet (Armutcu, Akyol, Ustunsoy, & Turan, 2015)

2.6. Poly (ADP-ribose) Polymerase

The development of poly (ADP-ribose) polymerase (PARP), or as it was called then ADP ribosyl transferase (ADPRT), goes hand-in-hand with anticancer therapy. The first declaration, before the enzyme was determined, was that the initial chemotherapy agents, the DNA alkylating agents, caused a profound decrease in glycolysis due to depletion of cellular NAD+. ADP-ribose polymers were recognized shortly afterward and finally, the enzyme responsible, PARP was discovered. The PARP reaction catalyzes the cleavage of NAD+ into nicotinamide and ADP-ribose leading to the rapid expending of NAD+ when DNA is damaged by alkylating agents. The second product of the reaction, nicotinamide, causes a modest product inhibition of the response. Based on this information the first PARP inhibitors were the nicotinamide analogs where the heterocyclic nitrogen at the three position was replaced with carbon to generate a benzamide analog. Replacements at this three positions were ameliorated solubility and the 3-substituted benzamides, e.g. 3-aminobenzamide (3-AB) helped elucidate the function of PARP. A pivotal study exhibited that 3-AB inhibited the repair of DNA breaks induced by the DNA alkylating agent, dimethyl sulfate (DMS), and enhanced DMS cytotoxicity. This study suggests a probable advantage of PARP inhibitors in combination with DNA alkylating agents to treat cancer. Of course we now know that there is a family of PARP enzymes but, in terms of DNA repair and its exploitation in cancer therapy, PARP1 and PARP2 are the targets, as these enzymes have imbrication function in the repair of DNA breaks by the base excision repair/single strand break repair (BER/SSBR) pathway. More lately, PARP3 has been shown to co-operate with PARP1 in response to DNA double-strand breaks (Curtin & Szabo, 2013). The PARP enzyme family consists of PARP-1 and many of recently identified poly (ADP-ribosyl) using enzymes. PARP-1 or PARP also mentioned to as poly (ADP-ribose) synthetase or poly (ADP-ribose) transferase is activated by DNA single-strand breakage caused by free radicals and the strong oxidant peroxynitrite, the product of superoxide reaction with nitric oxide. PARP is a rich (approximately one enzyme molecule per 50 nucleosomes) enzyme constitutively expressed in most cell types. This energy-consuming enzyme, which has a high binding affinity for right ends of DNA and 3’ single-base overhangs, is thought to be implicated in DNA repair. Its catalytic activity is strongly excited after binding to broken DNA ends – only about 1% of the total PARP molecules is active under physiological conditions, in

the absence of massive DNA strand breaks (Obrosova & Julius, 2005). DNA repair enzyme poly (ADPribose) polymerase (PARP) is expressed at higher levels in tumor cells when compared to benign tissues and cells, PARP may, therefore, represent a tumor-specific treatment target. Moreover, while assisting rapid dividing cancer cells with DNA-repair, PARP prevents apoptotic cell death (Karpel-Massler et al., 2014). Poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme that is involved in the cellular response to DNA injury. DNA breaks are believed to be compulsory triggers for the activation of PARP. These DNA breaks are provoked by a variety of environmental stimuli, such as oxidative and nitrosative stress. Upon confronting DNA strand breaks, PARP catalyzes the cleavage of NAD into nicotinamide and ADP-ribose and then uses the latter to synthesize polymers of ADPribose, covalently connected to nuclear proteins, including PARP itself. When DNA damage is mild, poly (ADP-ribosyl) action promotes cell survival. When DNA damage is severe, PARP activation can induce cellular energetic disturbances, leading to cell dysfunction or death. A genetic disorder of PARP or pharmacologic inhibition of this enzyme has beneficial effects on inflammation, shock, stroke, myocardial ischemia/reperfusion, and prevents the onset of autoimmune diabetes. PARP is now identified to play a role also in the regulation of gene transcription. Several transcription factors, including nuclear factor-kB (NF-kB), p53, and AP-1, interact with PARP and are regulated by it. By using PARP inhibitors or knocking out PARP gene in cells or mice, both NF-kB activation and transcription of NF-kB–dependent genes, such as inducible nitric oxide synthase or intracellular adhesion molecule (ICAM)-1, can be reduced, propose that inhibition of poly(ADP-ribosyl)action might prevent the consequences of inflammation or stress by modification of NF-kB–dependent pathways. Evidence suggests that oxidative and nitrosative stress are higher than normal in retinas from diabetic animals, hence potentially activating PARP and contributing to the pathogenesis of diabetic retinopathy. In the recent study, describe that PARP activity in the retina is increased in diabetes and that inhibition of PARP inhibits the growth of early lesions of diabetic retinopathy. These beneficial effects of PARP inhibition are mediated at least in part via its regulation of NF-_kB (Zheng, Szabo, & Kern, 2004).

2.6.1. Structure and Mechanism of DNA Damage-Dependent PARPs

Three members of the PARP superfamily are catalytically activated through interaction with DNA damage: PARP-1, PARP-2, and PARP-3. PARP involvement in the cellular response to DNA damage has long been appreciated and continues to improve actively. A general model that has collectively appear indicates that the DNA-damage-dependent PARPs act early in the process of harm detection, which promptly results in PARP catalytic activation and an explosion of PAR generation. PARP presence and activity at the damage site then can offer to the efficiency of the repair process and the repair pathway choice. A fundamental role of the DNA-damage-dependent PARPs and the PAR modification they produce is to recruit repair factors to the site of damage. Several motifs and domains have been recognized in repair proteins that mediate the interaction with PAR and the recruitment to sites of PAR synthesis. In addition to PAR serving as a conscript platform, PAR modification of repair and chromatin-associated factors in the nearness of a damage site is anticipated to change the catalytic properties of targeted proteins, and the local structure of chromatin. However, detailed insights into the PAR-mediated regulation of protein function lack in general. And although a general model for PARP participation to the DNA damage response has formed, the molecular details of PARP involvement are not clearly established, which has limited our understanding of PARP's participation to specific steps of repair, and the contribution of several PARPs to repair pathway choice. Over recent years, structural and biochemical studies have provided fundamental insights into the early stages of PARP-1 involvement in DNA repair: the discovery of DNA damage, and the allosteric coupling of injury detection to acute levels of PAR production. The DNA-damage-dependent PARPs have similar catalytic domain structures, but they vary from somewhat in the domains that contact DNA damage. In the catalytic domain, they share a protected structural feature known as the helical domain (HD) (also referred to as the PARP regulatory domain – PRD). The HD is only found in the DNA-damage-dependent PARPs, and it plays an important role in regulating PARP catalytic activity, as described later. The HD is proximate to the ADP-ribosyl transferase (ART) fold, which is common to all PARP family members. The ART contains the binding site for NAD+, which provides ADP-ribose, and a second binding site for an ADP-ribose unit, which accepts the next ADP-ribose during the PAR extension reaction that can result in both linear and branched polymers (Figure 2.5.).

Detailed structural views of PAR biosynthesis (NAD+ binding, beginning on the target protein, polymer extension) have not been obtained. Thus our complete understanding of PAR synthesis is limited. The NAD+ binding sites for the DNA-damage-dependent PARPs are similar and have the saved His-Tyr-Glu (HYE) amino acids that define catalytically active PARP members able of forming PAR (as opposed to mono-ADP-ribose). The acceptor binding sites differ between PARP-1, PARP-2, and PARP-3 and this is expected to influence the type of polymer formed (e.g. polymer length, the number of branch points). For example, PARP-3 has an Arg residue in the acceptor site where PARP-1 and PARP-2 have a Met residue, which is supposed to contribute to the binding pocket for the adenosine base of an acceptor ADP-ribose modification. Probably, this change in sequence perturbs the binding site and contributes to the smaller size of polymer produced by PARP-3 (Vyas et al., 2014).

2.6.2. Mechanism of PARP-1 Activation

Outside of the catalytic domain, the DNA-damage-dependent PARPs also share a Trp-Gly-Arg (WGR) domain that is essential for damage-dependent activation and is the most defining feature of the DNA-damage-dependent PARPs. A crystal structure that contained the basic domains of PARP-1 in complex with DNA damage rendered the first views of the WGR domain contacts with DNA (Figure 2.5.). The structure showed that conserved regions of the WGR make sequence-independent contacts with the DNA backbone near the 5′ terminus. The importance of these contact residues to catalytic activation was established through mutagenesis. Although there are no structures for PARP-2 and PARP-3 in complex with DNA damage, it is worth noting that their activation levels are sensitive to modifications to the 5′ terminus of the DNA, such as phosphorylation, suggesting that their WGR domains have specialized interactions with the 5′ terminus. PARP-1, in contrast, is comparatively insensitive to the detailed composition of the break site, compatible with the PARP-1 complex structure in which the 5′ terminus is not immediately contacted. The biochemical results for PARP-2 and PARP-3 suggest that they are most powerful activated at confirmed stages of the repair process, for example, when a DNA break has been processed to the point of containing a 5′ phosphorylated Nick that is responsible for DNA ligation. Indeed, the efficiency of recovery from a DNA double-strand break depends on PARP-3, which is suggested to aid

recruitment of the DNA ligation complex that completes the NHEJ repair pathway. In the mechanism of activation for each of the DNA-damage-dependent PARPs that offer to their specialization toward distinct repair pathways, and the stage at which they act within a given repair pathway. Further structural and biochemical studies will help to more define the specifics of the particular PARP involvement in the DNA repair response (Rulten et al., 2011). Maybe most notably, the structural basis for PARP-1 engaging a single-strand break is a definite gap in understanding of PARP-1 function as a "nick" sensor. PARP-2 and PARP-3 lack the extensive regulatory domains seen in PARP-1, but yet have extensions N-terminal to the WGR that at limited play a role in DNA binding and activation, though, there are inadequate structural and mechanistic insights into their N-terminal regions and how they might specialize the function of PARP-2 and PARP-3.positioning of the Zn1 and Zn2 domains requires that they originate from separate polypeptides, which is in contention with a number of recent biophysical studies indicating that PARP-1 interacts as a monomer with DNA. Thus, the relevance of the reversed binding mode of Zn1 needs further investigation. Additional structural studies are needed to help clarify this discrepancy and to understand how PARP-1 engages different types of DNA damage fully. Furthermore, it will be important to explain how PARP interaction with DNA damage is distinct from its interaction with undamaged DNA, and the functional consequences of these differences. There has been serious recent progress in the identification of PARP auto modification sites using mass spectrometry. However, there is still much to learn regarding the functional consequences of change at a given site, and either modification at different residues could lead to different outcomes (e.g. PARP-1 mediated recruitment versus PARP-1 release). Understanding the mechanism of PARP-1 release from DNA damage has significance to the effects of certain clinical PARP inhibitors that prevent the release mechanism to differing degrees and "trap" PARP molecules on DNA damage (Pascal & Ellenberger, 2015).

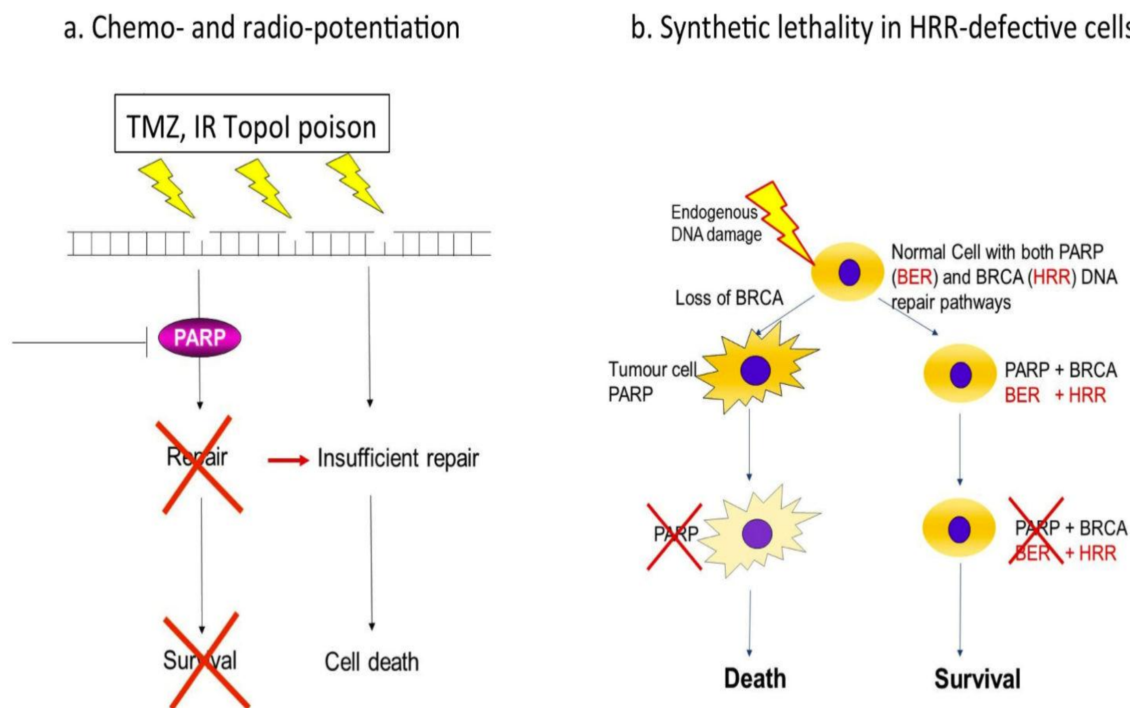

Figure 2.5. Mechanism of action of PARP inhibitors in cancer therapy.
(a) PARP promotes the repair of DNA damage induced by IR, TMZ and topo I poison allowing the cell to survive. If PARP is inhibited then repair is insufficient, and DNA damage persists leading to cell death. (b) BER and HRR complement each other in the repair of endogenous DNA damage. Loss of BRCA1 or BRCA2 (or any other component of HRR) can lead to genomic instability and tumor development. Such tumor cells become more reliant on PARP for repair of endogenous DNA damage such that when PARP is inhibited the cell cannot repair its DNA sufficiently and dies. Healthy cells that still retain functional HRR will survive even though PARP is inhibited (Curtin, 2013)

2.6.3. Turnover of Poly-(ADP-ribose) is Required for Normal Responses to DNA Damage

The enzymatic synthesis of poly-(ADP-ribose) and its degradation are commensurately necessary for normal responses to DNA damage. In mammals, the enzyme poly-(ADP-ribose) glycohydrolase (PARG) is the main activity that removes poly-(ADP-ribose) from proteins by separating ribose-ribose bonds. PARG is a sufficient enzyme that degrades PAR by a combination of endo- and exo- glycohydrolase activity, removing most of the PAR polymer but leaving a single ADP-ribose attached to the protein. The remaining ADP-ribosyl modification can be eliminated by one of several recently identified mono-(ADP-ribose) glycohydrolases. The genetic disorder of the PARG gene caused embryonic lethality and decreased PARG activity sensitizes cells to a spectrum of DNA damaging agents resembling that caused by genetic knockdown of PARP-1

expression or pharmacologic inhibition of PARP activity. For example, BRCA2-deficient cells that are particularly sensitive to PARP inhibitors are also hypersensitive to PARG inhibition by the nonselective inhibitor, gallotannic (Pascal & Ellenberger, 2015).

2.7. The p53 Tumor Suppressor Gene

The ubiquitously expressed tumor suppressor p53 is a multifunctional protein that regulates cellular stress replies such as cell cycle arrest, apoptosis, and senescence. Owing to the final nature of the two following responses, the activity of p53 is tightly regulated. In normal cells, p53 is present at the low level that is preserved by its constant proteasomal degradation. Upon cellular stresses, such as oncogene overexpression or DNA damage, the expression level of p53 is enhanced. The rise in p53 protein level results from reduced degradation rather than increased transcription or translation and let for the rapid activation of p53-mediated stress responses. The tumour-suppressive function of p53 predominantly depends on its function as a transcription factor, while several controversial Transcription-independent pro-apoptotic activities of p53 have been described. As a transcription factor, p53 can positively or negatively regulate the expression of many target genes, encoding, for instance, pro-apoptotic Bcl-2 proteins (e.g. Bax, Bak, Puma, Noxa), caspases, death receptors (e.g. Fas), DNA repair proteins or the cell cycle inhibitor p21. The net result of p53 activation seems to be dictated by the balance of p53 target gene expression and visible as transient cell cycle arrest, cell death by apoptosis or induction of senescence. Each of these stress responses tends to inhibit cellular transformation and tumor genesis by eliminating damaged cells from the proliferative cycle. It is thus obvious that inactivation of p53 by either mutation or dysregulation is commonly noted in human cancer. Mutations in p53 that disturb its proper function are found in about 50% of human cancers, and the surviving half of tumors seem to have malfunctions of the p53 pathway (Essmann & Schulze-Osthoff, 2012).

2.7.1. The Bright Side of P53 in Cancer Therapeutics

Cells are continuously submitted to conditions and stimuli that can result in genotoxic stress, containing oxidative metabolism and irradiation. Thus, the ability to detect and repair consequent DNA damage is a critical function of healthy cells. Failure to

efficiently repair DNA damage can eventually result in malignant transformation. Induction of a cell cycle arrest (a transient or permanent block of cell proliferation) or the activation of cell death pathways in response to genotoxic stress involves the major arms of the survival-death axis governed by p53. Because of these biological features, inactivation of wild-type p53 is a crucial step in tumor evolution and progression, reflected by the high incidence of TP53 mutations in a variety of human cancer types. p53 is generally expressed at low levels so that it does not disrupt the cell cycle or cause the cell to undergo unfortunate death. Such low concentrations are obtained through the operation of a negative feedback loop consisting of wild-type p53 and the MDM2 gene and its product. MDM2 is a p53 transcriptional target whose product ubiquitinates p53, thus marking it for proteosome-mediated degradation. Nevertheless, p53 is stabilized and accumulates upon stresses such as DNA damage or oncogene activation, producing in cell cycle arrest, senescence, and/or cell death through transactivation of its target genes, comprising those encoding p21 (which promotes cell cycle arrest) and the pro-apoptotic proteins Bax (Bcl-2-associated protein), PUMA (p53 up-regulated modulator of apoptosis), and Noxa (phorbol-12-myristate-13-acetate-induced protein 1) (Figure 6). Because the downstream targets that have been recognized play a critical role in the p53 tumor suppression response, these objectives should be a major avenue for therapeutic intervention in p53 activation in cancer cells. It has also been demonstrated that in a strong rate of human tumors, p53 is inactivated by MDM2 overexpression and amplification. Besides, it is well established that MDMX/MDM4—a nonredundant homolog of MDM2—also regulates p53 and is overexpressed in many cancers. However, unlike MDM2, MDMX expression is not regulated by p53 and its product lacks intrinsic ubiquitin ligase activity; thus, it is not a fundamental part of the negative feedback loop described above. However, MDMX forms heterodimers with MDM2, which improves the ability of MDM2 to induce p53 degradation. Interrupting the interactions between p53 and its negative regulators such as MDM2 to activate or maintain p53 is a promising therapeutic strategy for the treatment of cancers retaining wild-type p53. Tumors that provide mutations in TP53 often overexpress mutant p53, resulting in increased resistance to traditional chemotherapy and radiotherapy as compared with cells that do not overexpress mutant p53. This finding indicates that such mutant p53 provides a particular selective advantage for tumor development—an oncogenic gain-of-function phenotype. Tumor cells containing mutant p53 should become sensitive to chemotherapy

upon restoration of the wild-type p53 pathway (Figure 2.6.). This creates mutant p53 an attractive target for selective cancer therapy that would not affect healthy cells because normal cells do not contain mutant p53 (Mandinova & Lee, 2011).

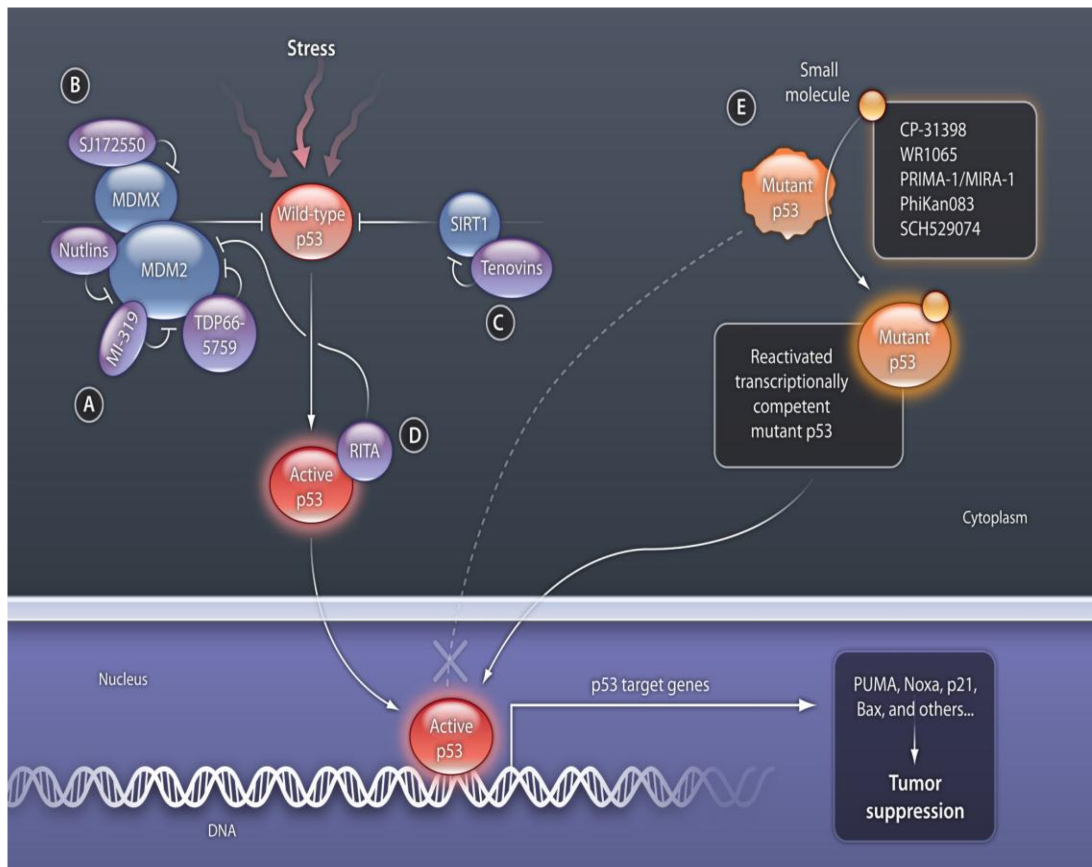

Figure 2.6. Modulating the p53 pathway with small molecules.
Wild-type p53 is activated by a variety of stressors, including DNA damage, oncogene expression, nutrient starvation, oxidative stress, and depletion of ribonucleotide triphosphates (used in RNA synthesis). (A) Small molecules that target MDM2 and block p53 binding stabilize p53. (B) SJ172550 targets the p53 binding pocket of MDMX, also promoting p53 stabilization. (C) Tenovin-6 inhibits the protein deacetylase activity of SIRT. Acetylation results in the stabilization of p53 and interferes with MDM2-mediated degradation. (D) RITA binds to p53 and interferes with the interaction of MDM2 and p53, activating p53 function. (E) Small molecules designed to bind transcriptionally inert mutant p53 proteins stabilize the core domain, restore the native state, and eventually enable binding to DNA

2.7.2. P53 in Normal Tissue

DNA damage-induced cell death within the activity of chemotherapeutic drugs is the most widely used strategy for cancer therapy. Though, selectivity remains a great interest because most such drugs kill both cancer cells and the normal enclosing cells, which is an important cause of the side effects of cancer chemotherapy that severely limit modern

treatment regimes. While the key to successful anti-cancer therapies is to target critical nodes that are ordered for the survival of cancer cells, such therapies should not be dangerous to healthy cells. The idea of returning wild-type p53 pathways (apoptosis and cell cycle arrest) by inhibiting proteasomal degeneration of p53 (for example, via MDM2 inhibition) is a promising therapeutic strategy. So far, the identification of small molecules that either (i) inhibit the E3 ligase action of MDM2 or (ii) can occupy the hydrophobic p53-binding pocket/cleft in MDM2 is entirely feasible. These MDM2 inhibitors all cause tumor regression through cell death in xenograft models, although it is unclear how such molecules would affect healthy cells and tissues in humans (Mandinova & Lee, 2011).

2.7.3. P53 as a Guardian of Cancer Cells

Wild-type p53 can perform cancer cells to a survival path in response to stress such as DNA damage. Survival-promoting activities of wild-type p53 include the induction of a wide domain of cellular responses, including cell cycle regulation, facilitation of DNA repair pathways, the preservation of the cellular redox state, and activation of genes with anti-apoptotic activities. The major role of p53 is to guard healthy cells against malignant transformation and to protect the genome under stress situations. For example, p53 can directly share in repair processes by binding to DNA and resolving abnormal DNA structures or by activating p53 target genes that are implicated in the regulation of DNA repair. Because of its functions in DNA repair and cell cycle regulation, wild-type p53 might also operate as a “guardian” in cancer cells to promote cancer cell survival in the presence of stress, thereby elevating the view that wild-type p53 activity may not always be helpful in preventing tumorigenesis. Indeed, some cases, the maintenance of wt-p53 has been shown to protect cancers from some forms of cytotoxic chemotherapy and so can be correlated with a poor response to treatment. Furthermore, the use of p53 inhibitors has been suggested for chemoprotection of normal cells since much of the toxicity seen in response to traditional genotoxic chemotherapies is due to the activation of p53. Therefore inhibition of p53 in normal cells may preserve them from cell death. More recently, accumulating evidence indicated that p53 engages strong pro-survival pathways which are important for maintenance of a normal, healthy cell/tissue but may offer to tumor cell survival against cytotoxic damage. A growing number of p53-induced

survival genes have been recognized, which function through several various mechanisms. Many p53 downstream or interacting target proteins function to inhibit apoptosis, including genes implicated in DNA repair, cell cycle control, metabolism, oxidative stress response, transcription, growth factors and receptors, etc. It is clear that wild-type p53 is not only a powerful proapoptotic induced in response to cytotoxic stress but also a robust (Mandinova & Lee, 2011).

3. MATERIALS AND METHODS

3.1. Reagents

The following chemicals were used in this study: medium for cell cultures Dulbecco's modified Eagle's medium (DMEM) (Biowest), Fetal bovine serum (FBS), trypsin-EDTA (GibCo), Puck's-D1 solution, antibiotics (penicillin, streptomycin) and calcium-free phosphate buffered saline (PBS) (SIGMA) were purchased. Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich). Acrylamide/ bis-acrylamide solution, 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 p53 (SantaCruz bio, Anti-p53 antibody (ab31333), Antibodies against Nf-kB (ABCAM, Anti-NF-kB p105/p50 antibody [E381] (ab32360), Antibodies against GFAP (ABCAM, Anti-GFAP antibody [EP672Y] (ab33922).

3.2. Description of Raw Propolis

Bee Propolis is a sticky material that bees produce from plant resins and their body secretions. The chemical composition of propolis shows the botanical sources from which the bees forage. Several bioactivities have been ascribed to some of the 300+ compounds present in honeybee propolis, including antioxidant, antimicrobial, anti-inflammatory and antitumor effects. Over the past five years, much interest has focussed on the possible use of propolis for the therapy of injury and disease, and this has been studied mainly using animal models or cell lines. For example, propolis and its components have been reported to produce benefits in models of hypertension, wounds, burns, cancer and septic arthritis (Massaro et al., 2013).

3.3. The Preparation of Extract Propolis for Treatment Cell lines

Propolis extraction can be performed by several techniques. Different components in propolis were shown from each extraction method. The organic solvents were used to extract the chemical composition from propolis such as water, ethanol, methanol, hexane and acetone (Khacha-ananda, Tragoolpua, Chantawannakul, & Tragoolpua, 2013). In our investigation we were used ethanol extracted propolis method, in this study work we obtained collected green propolis of bee hive from Kurdistan Region of Iraq during winter 2016. Furthermore, propolis composition is entirely changeable make a problem for the medical use and standardization. The propolis have been cleaned and saved with protection from the light. Ethanolic extraction was prepared by extraction of 30g of bee glue with 450ml of ethanol 98% for 12h of a darkened cabinet in the shaker machine, then it was filtered by qualitative filter paper (125mm and 110mm), vacuum dried, and evaporated in rotary evaporation the output of prepared extracted (% w/w) as that original product. The EEP was put in to a glass container and left for approximately three days for the remaining solvent to evaporate; as a result, a solid mass (162 g) with viscous manifestation was obtained and saved. Although, the extracts were filtered twice, dried and stored in sealed bottles. The ethanol extracts were kept at 4 °C until use.

3.4. Surgically

Normal brain rat pups tissue samples were obtained from the animal house of Biology Department in Bingol University –Turkey, about 24h aged (newborn). The tissue specimens were collected promptly after a surgical resection. The tissues were separated, homogenized, filtered with 150 μm sieve and cultivate into tissue culture plates with a medium containing Dulbecco's modified Eagle's medium (DMEM) complete 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 per a week. Under these situations, 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.

3.5. Cell Cultures and Treatments

Primary cultures of astrocytes were prepared from newborn rat brains (24h) and checked for purity (Caccamo et al., 2005). All efforts were made to minimize both suffering and number of animal used. So as to reach proper astrocyte density it is necessary to use 4 rat pup cortices per T75 tissue culture flask. Astrocytes were seed in 75-cm^2 flasks at a beginning density $6 \cdot 10^5$ cells/ml (20 ml/flask) in Dulbecco's modified Eagle's medium (DMEM) completed 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 were washed 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) was added to stop the action of trypsin. After the cells were centrifuged at 500g about 5 minutes, the cells were plated for the experiments in DMEM with 10% (vol/vol) (FBS). For experimentation cells were used between day 11 and 13 in culture. Cell cultures were distinguished to contain more than 90% astrocytes. After dispose of the supernatant, the pellet of cells was again suspended in a complete DMEM medium and then the inoculum of cells corresponding to the final concentration of 1×10^5 cells/ml was calculated. After distributing the culture medium in plaques of 24 wells, the compounds were added to two concentrations of 50 μg/ml and 100 μg/ml, and the DMSO was applied as a diluting control and statistic parameter (Machado et al., 2016). Astrocytes at 13 days in vitro (DIV) were treated with ethanol extracted propolis for 24 hr as previously reported (Caccamo et al., 2005). All experimental procedures adhere to the "Guidelines for Proper Control of Animal Experiments" approved via the local ethics regulations.

Human glioma U373 was used as referent-object for determination EEP effect on P53 expression in glial family cells. Doxorubicin treatment in human glioma U373 used for creation the model of cytotoxic effect of anticancer therapy. Accordingly treatment EEP combined with Dox was performed for study anti-cytotoxic effect of propolis.

3.6. Preparation of Protein Samples

Tissue preparation can be performed at cold temperatures to prevent denaturation and degradation of protein. Cells should be quickly frozen to avoid protease degradation of proteins or collected and lysed as quickly as possible. Solid tissue is mechanically broken down, usually using a homogenizer or via sonication in a lysis buffer. Lysis buffer should be used to make able lysis of cells and to solubilize proteins. Lysis buffer include 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). Importantly, quantification and comparison with other samples in western blot analysis is dependent on the protein lysates prepared for polyacrylamide gel electrophoresis. 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 proceedings can be slowed down enormously if samples are kept on ice or at 4°C at all times and appropriate inhibitors are added fresh to the lysis buffer.

3.7. Immunoblot Method

Polyacrylamide gel electrophoresis (PAGE) gives a versatile, gentle, high-resolution method for fractionation and physical-chemical characterization of molecules by size, conformation, and net charge (Chrambach & Rodbard, 1971). For optimal separation, it is important to determine 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 applied 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-methylene bisacrylamide (Bis, for short). Bis is a cross-linking agent for the gels. The polymerization is initiated via the addition of ammonium persulfate along with either DMAP or TEMED. The gels are neutral, hydrophilic, three-dimensional networks of long hydrocarbons cross linked via methylene groups. The separation of molecules within a gel is determined via the relative size of the pores

formed within the gel. The pore size of a gel is determined via 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 requisite 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. Transfer 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 (PVDF) (Kurien & Scofield, 2006). Transferring proteins from a gel to a membrane should realize 90 – 120 min with constant current 150 – 200 mA. Its major advantages are speed and completeness of transfer. This process uses 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 sorption onto membranes: To check for success of transfer, wash the membrane in TBST. 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 distained completely via repeated washing in TBST or water. When using a PolyVinylidene Difluoride (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 applies for western analyses, with advantages and disadvantages in using either type (MacPhee, 2010). Detection, the probes that are labeled and bound to the protein of interest need to be detected on the western blot. Chemiluminescent detection is applied most often and therefore, will be briefly described. Increased chemiluminescence (ECL) is a sensitive method and can be applied for relative quantitation of the protein of interest (Kurien & Scofield, 2006). The primary antibody binds to the protein of interest and the secondary antibody linked to horseradish peroxidase, is applied to cleave a chemiluminescent agent. The reaction product produces luminescence, which is related to the amount of protein (MacPhee, 2010). 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 several more times. Moreover, subsequent reprobing can interfere with protein antigens, resulting in a decreased signal (Alegria-Schaffer, Lodge, & Vattem, 2009).

Relative quantification and analysis. The blot results can be analyzed via densitometry to measure the relative amount of a specific protein on the blot via 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-phosphate dehydrogenase [GAPDH]) or a band of known protein concentration. Relative optical density units can be plotted in a graph, and the appropriate statistical analysis can be performed on the samples that have been converted to optical density units (Jensen, Wood, & Keller-Wood, 2007).

3.8. Determination of Protein Concentration

The protein concentrations of the samples to be loaded on a gel need to be determined. Quantification for total protein can be achieved via 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 used where a standard curve is created to determine unknown sample concentrations.

3.9. Statistical Analysis

The statistical analysis was performed with the Statistic software (GraphPad Prism, v.6.01). The acquired results were shown in the form of the mean and standard variations. A $P < 0.05$ was considered statistically significant, data groups that are significant at different levels: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4. RESULTS

4.1. GFAP

Primary astrocyte cell cultures were treated with EEP and with LPS as stimulation factor. The treatment primary astrocyte with EEP 10 µg/ml induces small increasing GFAP expression. The rising of GFAP expression was 22% compared with control. Nevertheless, the treatment with EEP 25µg/ml concentration induces more intense rising GFAP expression about 89%. In contrast, treatment primary astrocyte with EEP 100 µg/ml leads to increasing GFAP expression content on 67%. It is known that LPS activates astrocyte for inducing proinflammatory factors and reactivation. Treatment primary astrocytes with EEP 10 µg/ml +LPS 0.01 µg/ml induced rising GFAP expression about 81% compared with control. The treatment with concentration of EEP 25 µg/ml + LPS 0.01 µg/ml induced more high increasing GFAP expression about 143% and the concentration of EEP 100 µg/ml + LPS 0.01 µg/ml about 110% compared with control. The maximum effect for GFAP expression treated with EEP has in concentration 25µg/ml with and without LPS (Figure 4.1.).

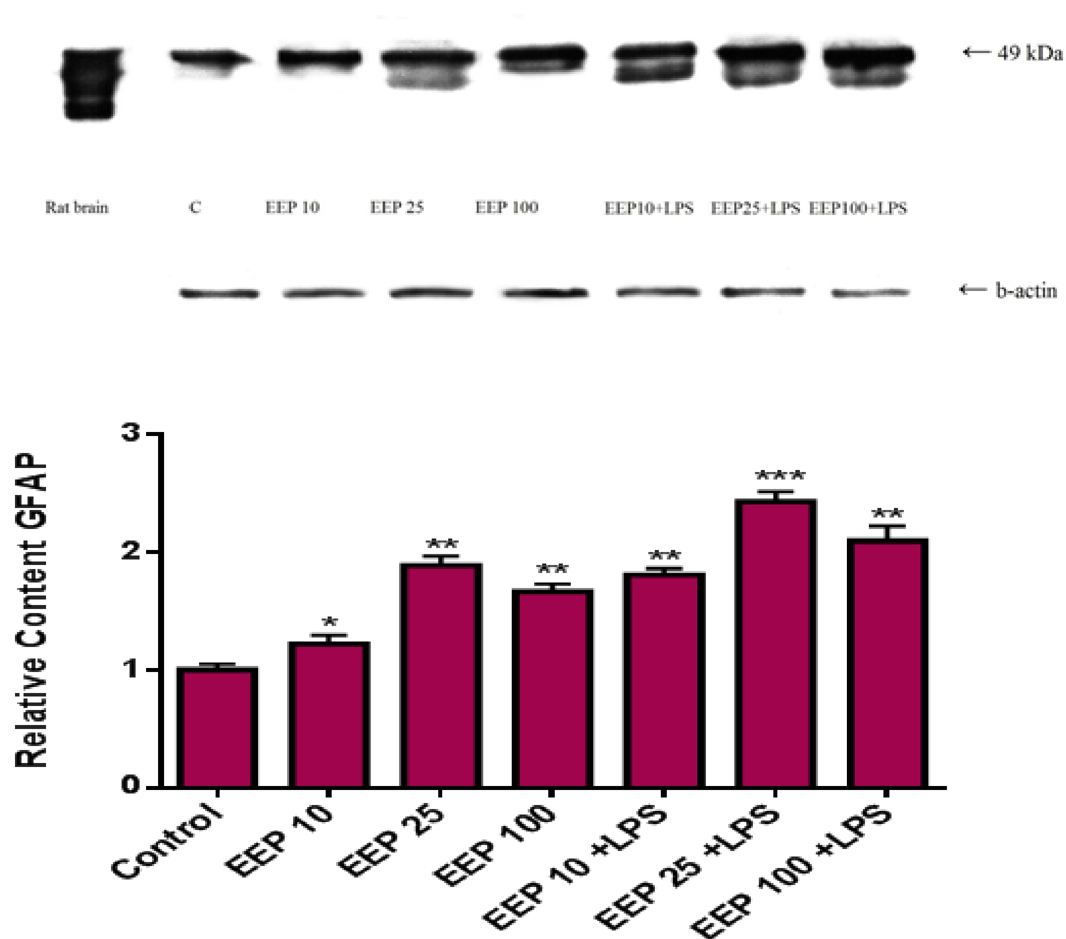

Figure 4.1. Apportion content of GFAP in control and primary astrocyte cell culture treated with EEP and EEP+LPS Significant of differences: * $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.2. PARP

Western blot analysis demonstrated significant changes of PARP in primary astrocytes compared to control. The groups of astrocytes were treated with different concentrations EEP for 24 hours. In a range of small dose of EEP 10 µg/ml the rising of PARP expression was about 150% compared with control. Moreover, the treatment astrocytes culture with EEP 25 µg/ml induced more high activation of PARP contents about 170% compared with control. On the contrary, the concentration of EEP 100 µg/ml contents about 92% and concentration EEP 100 µg/ml +LPS 0.01 µg/ml contents about 94% showed total deep decreasing of PARP in primary astrocyte culture. The treatment with EEP 10 µg/ml + LPS 0.01 µg/ml contents about 87% and EEP 25 µg/ml + LPS 0.01 µg/ml contents about 64% induced the rising of PARP compared with control. The

combined treatment with EEP and LPS induced very similar change in two studied groups. Accordingly observed results may be related with indirect effects of bioactive EEP compounds on multifactorial pathways that modulate of activation of PARP (Figure 4.2.).

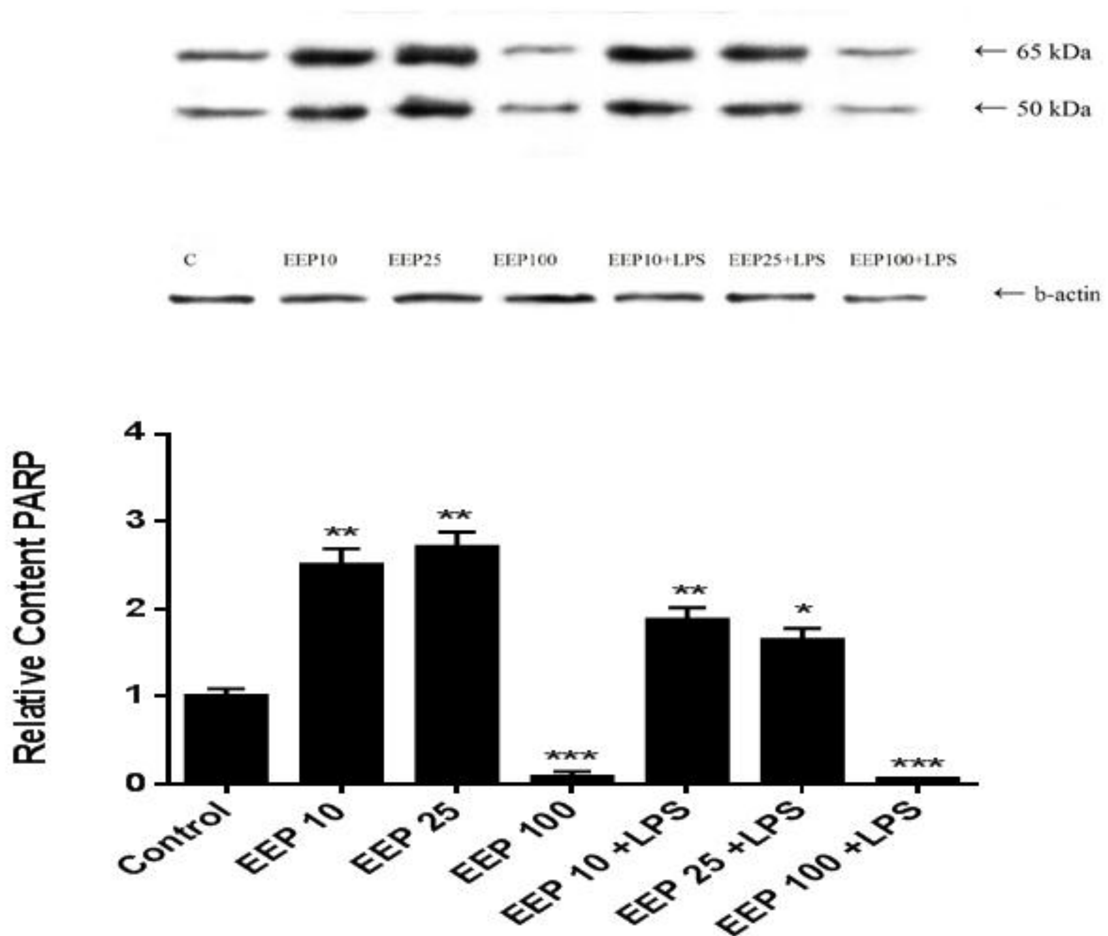

Figure 4.2. Relate content of PARP in control and primary astrocyte cell culture treated with EEP and EEP+LPS Significant of differences * $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.3. Nf-kB

Our experimental model employed primary cultures of rat astrocytes. At this time, cell cultures were enriched in differentiated astrocytes, consistently with Nf-kB. In a range of small dose of EEP 10 μg/ml about 78% and EEP 25 μg/ml contents 118% induced elevating activation of Nf-kB to compare with control. Moreover, the concentration of EEP 100 μg/ml showed high optimum level of Nf-kB activation about 3.88 times

compare with control. The treatment with EEP 10 µg/ml +LPS 0.01 µg/ml contents about 119% and EEP 100 µg/ml +LPS 0.01 µg/ml induced the rising of Nf-kB contents about 87% and treatment with EEP 25 µg/ml +LPS 0.01 µg/ml be visible more increase content about 2.75 times. The combined treatment with EEP and LPS induced very similar change in two studied groups. Thus the maximum effect for Nf-kB activation treated with EEP has in concentration 100µg/ml (Figure 4.3.).

In our present study extends recent observations on the effects of EEP in primary cultures of astrocyte cells for 24 hours, showing that EEP treatment can activate astrocytes through Nf-kB activation, GFAP expression and PARP can be just one blocked mechanism and enhance activation.

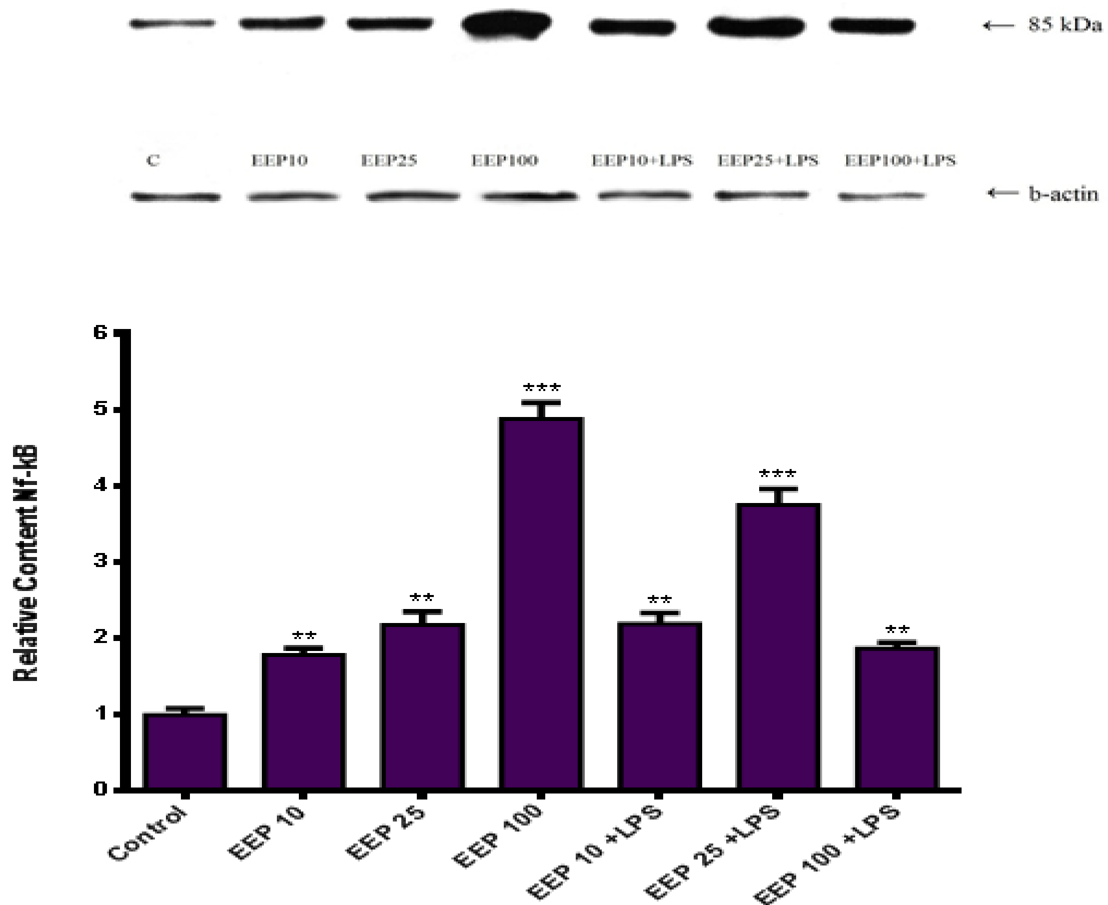

Figure 4.3. Showing the competition of NF-kB in control and primary astrocyte cell culture treated with EEP and EEP+LPS Significant of differences ** $p<0.01$, *** $p<0.001$

4.4. P53

In our experimental it has been reported that primary astrocyte cultures treatment with EEP as well control group which had no detectable p53 expression, due to EEP does not induce of p53 expression. Thus we used the EEP treatment cognate to astrocytes human glioma cells for verification a possible effect of EEP on p53 expression. Human glioma U373 is known as a referent-object for determination the effect different kind anticancer treatment on both the state and the response of glial family cells. Doxorubicin treatment has a cytotoxic effect for a cancer and normal cells. This chemical is wide used for anticancer therapy. Accordingly treatment EEP combined with Dox was performed for study anti-cytotoxic effect of propolis. Besides we used human gliomas cell lines (U373) which treated with different concentration of EEP have been response on increase expression of p53 protein. For the treatment of human gliomas cell lines with concentration of EEP 10 µg/ml induced strong expression of p53 protein about 92% and concentration of EEP 25 µg/ml revealed moderate expression about 78%, but concentration of EEP 100 µg/ml induces few raises expression about 15% according to control. All concentration of EEP demonstrated detectable p53 protein (Figure 4.4.).

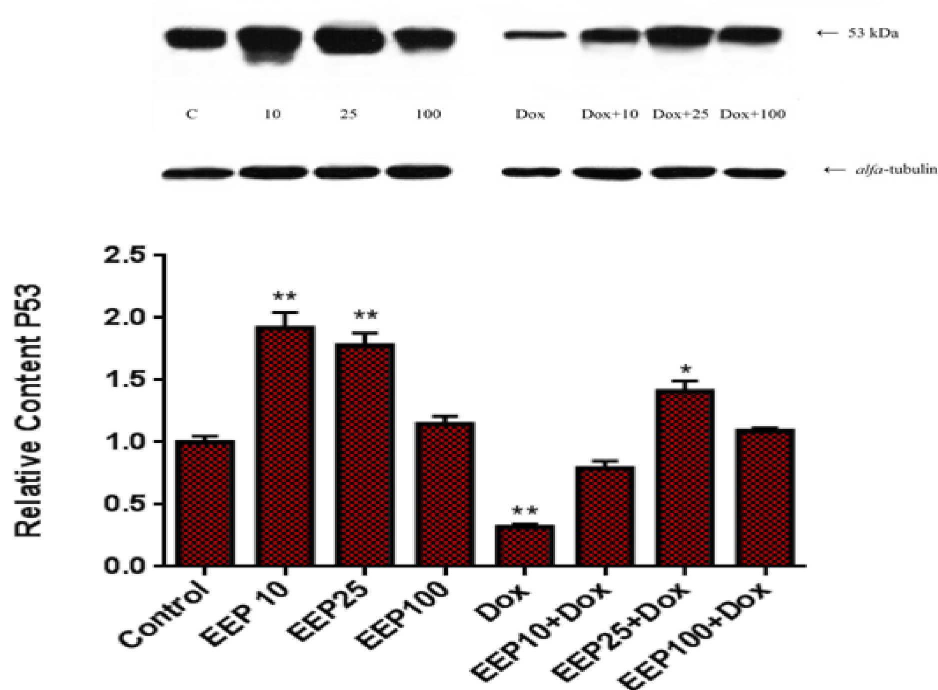

Figure 4.4. Relate content of p53 protein in control and glioma cell culture treated with EEP and EEP+ Dox Significant of differences * $p<0.05$, ** $p<0.01$

Furthermore, the treatment of human glioma cells with concentration of EEP 10 µg/ml+Dox have been suppress of p53 protein about 21% compared with control, and concentration of EEP 25 µg/ml+Dox moderate of p53 protein expression about 41%, also concentration of EEP 100 µg/ml+Dox less expression of p53 protein about 9% compared with control. But the treatments of malignant glioma with Dox have been deep suppression of p53 protein about 68% according to control.

5. DISCUSSION

Taking together observed results show the significant effects EEP on activation of primary astrocytes for cell response. Therefore, the reactivation of primary rat astrocyte was associated with cytoskeleton changes, especially, GFAP overexpression and hypertrophy cell body.

GFAP is an extremely regulated protein, whose expression is influence by multiple factors such as brain injury and disease. All type III intermediate filament (IF) proteins are implicated in the structure and function of the cell's cytoskeleton. Possibly the most well-known function of IFs is to supply 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 notion to help maintain mechanical power, as well as the shape of cells. The exact function of GFAP remains a mystery, despite the huge number of studies using it as a marker for astrocytes.

Astrocytes are implicated in a wide range of CNS pathologies, including trauma, ischemia, and neurodegeneration. In response to essentially any CNS pathology, astrocytes go through a characteristic change in appearance, i.e. reactive gliosis. A well-known advantage of reactive astrocytes is increased production of IFs, a result of the increased expression of GFAP. GFAP expression is highly regulated e.g. due to an injury or in the trajectory of a disease. It is important to perceive that the increase in GFAP protein and the expression of novel GFAP isoforms, which would not be expressed under normal circumstances, can change the function of the astrocyte. Abundant in vitro studies have shown that a change in GFAP expression alters the capability of astrocyte proliferation or other features of astrocyte transformation. Both the GFAP transgenic and GFAP mutant astrocytes reveal measurable default of growth in culture, which manifest

to reflect a combination of increased cell death and decreased proliferation. Studies in glioma cell lines have shown an converse relation between levels of GFAP expression and proliferation (Middeldorp & Hol, 2011). Here in our experiment reported that primary astrocytes treated with EEP and LPS via different concentrations showed dramatically increase expression of GFAP throughout 24 hours compared with control. As well the elevating of GFAP expression prompt to enhance cell reactivity and there is not linear depend effect/dose for 24 hours later treatment with EEP. In addition GFAP over expression into activated astrocytes at same time proinflamator factors always produced activated astrocytes more intensive than normal cell astrocytes (Figure 4.1).

Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme (Zheng, Szabo, & Kern, 2004). PARP is regulated numerous cellular functions comprising DNA repair, cellular division and differentiation, DNA replication, transformation, gene expression and amplification, mitochondrial function, and cell death. Modified activity of PARP is reported under several pathological conditions, including diabetes. Extensive experimental data generated in both tissue culture and animal models imply that diabetes-induced PARP activation or its overexpression in the retina by DNA damage causes cell death; an appearance that precedes the development of histopathologic change. Newly, it was recorded that PARP activation contributes to superoxide anion radical and peroxynitrite formation in peripheral nerve, vasa nervorum, and aorta of STZ-induced diabetic rats and high-glucose-exposed human Schwann cells. Furthermore, PARP inhibition counteracted diabetes-induced systemic oxidative stress and 4-hydroxynonenal adducts accumulation in peripheral nerves and improved nerve fiber function (Mohammad, Siddiquei, & Abu El-Asrar, 2013). Several transcription factors, including nuclear factor-kB (NF-kB), p53 and AP-1, interact with PARP and are regulated by it. By using PARP inhibitors or knocking out PARP gene in cells or mice, both NF-kB activation and transcription of NF-kB–dependent genes, such as inducible nitric oxide synthase or intracellular adhesion molecule (ICAM)-1, can be reduced, suggesting that inhibition of poly-(ADP-ribosyl)-ation might prevent the consequences of inflammation or stress by modification of NF-kB–dependent pathways (Zheng et al., 2004). PARP inhibition or PARP insufficiency is known to downregulate several mechanisms of cell death, including mitochondrial permeability transition, mitochondrial oxidant generation, and the release of the cell death mediator apoptosis-inducing factor. Recently, it was

reported that PARP regulated several genes of apoptotic regulators, including caspase-1 and caspase-3 (Zheng et al., 2004).

In this experiment, we investigated the treatment of primary cultures rat astrocytes with EEP, the concentration of EEP 100 µg/ml and concentration EEP 100 µg/ml+LPS 0.01 µg/ml shown extensive suppression of PARP activation that may reflects the rising of intensive cell death. However the concentration of EEP 10 µg/ml and concentration EEP 25 µg/ml with LPS 0.01µg/ml appear more activation compared with control. Thus there is contrary activation between EEP 100 µg/ml with EEP 100 µg/ml + LPS 0.01 µg/ml and EEP 25 µg/ml has over activation cell reactivity. In comparison with NF-kB related of PARP that has contrast significant activation, in the primary astrocytes treatment with EEP 100 µg/ml leads to intensive suppression PARP and over activation of NF-kB. Also that has divergence activation between EEP 100 µg/ml PARP and EEP 100 µg/ml on GFAP, high expression of GFAP and deep suppression of PARP (Figure 4.2). Recently were shown the positive effects an inhibitor of PARP and fullerene C60 for the prevention over activity PARP and GFAP overexpression (Guzik et al., 2016, Nedzvetsky et al., 2016). Thus, the effect of EEP may be associated with regulation of cell reactivation and its metabolic activity.

Obtained results are the evidence the effect of EEP on activation NF-kB dependent pathways for cell reactivation. The significant rising of NF-kB content may activate transcription some antioxidative enzimes and proinflammation factors. Thus, EEP effect as antioxidant may be combined with direct scavenging activity and indirect way that can be realized with transcriptional regulation of antioxidative enzyme system.

The transcription factor NF-kB has long been known to be involved in oxidative stress in many cell types. Additionally, NF-kB nuclear translocation has been hypothesized as a key factor in oxidative stress triggered under different conditions in astroglial cells. NF-kB, a dimer complex composed fundamentally of p50 and p65 subunits, was first reported as a central regulator of gene expression in immune cells; however, eventually a role for NF-kB in both neuronal and glial cells has been reported (Caccamo et al., 2005). The activation of NF-kB is multifaceted. In an inactive state, this molecule composed of either homo-or hetero dimers of various sub unit compositions (i.e., p50, p52, p65, which is also called RelA,RelB,andc-Rel) in the cytoplasm, with dimers bound by the inhibitory

protein IkB. In neurons, the most common composition consists of the p65, p50, and IkBa subunits. In the canonical activation pathway, NF-kB turns into activated by a series of neurochemical events that are begin upon activation of IkB kinase (IKK). IKK activation outcome in degradation of IkB via phosphorylation and upon release of IkB, the resulting dimer becomes activated, translocates to the nucleus, and binds to the DNA consensus sequence of its gene targets, where it can either induce or suppresses gene expression. One such target of NF-kB transcription is IkB , thereby supplying a negative feedback mechanism to tightly regulate NF- kB-dependent gene transcription. Assemble evidence suggests that NF-kB activation in astroglial cells may occur through different pathways, including ROS production and activation of muscarinic receptors (Caccamo et al., 2005).

In light of these observations, it is possible to hypothesize that in our experimental model EEP induced NF-kB activation. High levels of NF-kB activity could in turn, sustain a central pathway in cell defense against disease associated mechanisms in the inflammation process. Treatment of primary astrocytes, with concentration of EEP 100 μg/ml it has over activation of NF-kB so may lead to activation other bioactive mechanisms. Furthermore we report that NF-kB was more increased with EEP treatment than treatment with EEP + LPS compared with controls. A value of ** $p<0.01$, *** $p<0.001$ were considered to indicate statistical significance (Figure 4.3).

The ubiquitously expressed tumor suppressor p53 is a multifunctional protein that organizes cellular stress responses such as cell cycle arrest, apoptosis and cell senescence. Consequently, the aim to restore p53 activity in human cancer has helped in developing a number of antitumor therapies in preclinical and clinical trials (Geng et al., 2013). Inactivation of cell-death pathways is a middle component of cancer advancement. Different mechanisms exist in normal human cells to invoke cell death and eliminate damaged cells that may otherwise expand and form a tumor. Thus a number of known death regulators are mutated or lost in cancer. In particular, the p53 tumor suppressor, a vigorous inducer of apoptotic cell death, is mutated in approximately 50% of all tumors. The induction of cell death by p53 occurs via both target gene activation and transactivation-independent mechanisms at mitochondria. In response to various forms of cellular stress, the levels of p53 elevate and, after rapid localization of a proportion of

p53 to mitochondria, p53 collect in the nucleus where it transactivates a number of proapoptotic target genes (Crighton et al., 2006).

In the current study, we determined the treatment of human gliomas cell lines U373 with ethanol extract propolis by activation and stabilization of p53 protein *in vitro*. Our data demonstrated that EEP has effect on regulation p53 protein expression. Moreover, the treatment of glioma cell lines with EEP shown over expression of p53 protein and treat-ment of glioma cell lines with Dox leads to prevention cytotoxic effect of Dox and to restore p53 expression. Furthermore the treatment of human gliomas cell lines with EEP+Dox more effectively expression of p53 protein than the Dox without EEP that suppressed of p53 protein according to the control group (Figure 4.4).

CONCLUSION

1. The results presented study shown that EEP treatment induce astrocyte cell reactivity and activate astrocyte through Nf-kB activation, activation of GFAP expression and PARP expression. These cytoskeleton and transcriptional activation may be one of important mechanism for reactivation astrocytes.

2. Obtained results evident that EEP treatment with 100 µg/ml is over dose for astrocyte reactivity. 100 µg/ml EEP may lead to over regulation between Nf-kB-PARP, Nf-kB-GFAP and PARP-GFAP.

3. Nf-kB over activation which was determinate in treated with EEP astrocytes can lead to depletion of PARP transformation into inactive to active form. It supported with ability of Nf-kB directly binds PARP and may be main cause for suppression active form PARP.

4. The absence of p53 expression in normal rat astrocytes and in astrocytes treated with EEP are the evidence that EEP in range concentration 10 – 100 µg/ml didn't induce changes which may provoke switching for p53 expression. This findings show non toxicity EEP in used range concentration 10 – 100 µg/ml.

5. We determined the changes p53 expression in human gliomas cell lines U373 treated with EEP. EEP induce in glioma cells an activation and normalization of p53 protein *in vitro*. The treatment with Dox induce dramaticaly suppress p53. Furthermore combining treatment of human gliomas cell lines with EEP + Dox effectively restore the expression p53 protein. More effective dose was observed 25 µg/ml compared to 10 and 100 µg/ml EEP.

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