THE INVESTIGATION OF SREBP AND C/EBP EXPRESSION DURING GLOBAL ISCHEMIA/REPERFUSION INDUCED OXIDATIVE STRESS IN RAT BRAIN CORTEX AND CEREBELLUM

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THE INVESTIGATION OF SREBP AND C/EBP EXPRESSION DURING GLOBAL ISCHEMIA/REPERFUSION INDUCED OXIDATIVE STRESS IN RAT BRAIN CORTEX AND CEREBELLUM

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ABSTRACT

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Ischemic brain injury causes neurodegeneration. In this study, the mechanism of neurodegeneration was investigated by examining the role of sterol regulatory element binding protein-1 (SREBP-1), CCAAT enhancer binding proteinβ (C/EBPβ), glutathione (GSH), malondialdehyde (MDA), glutathione-S-transferase (GST), and superoxide dismutase (SOD). Carotid artery occlusion (CAO) plus hypotension was produced for 10 minutes. Control groups were sham operated. Animals were sacrificed after 24 hours, 1 week, 2 and 4 weeks of reperfusion periods. The expression of C/EBPβ and SREBP-1 in rat brain cortex and cerebellum were examined by western blotting. C/EBPβ expressions significantly increased in both cytosolic (1.19, 1.58 fold) and nuclear (1.73, 1.81 fold) extracts of brain cortex at 24 hours and 1 week CAO groups, respectively. In cerebellum, C/EBPβ expression significantly increased in 1 week, cytosolic (1.63 fold), and nuclear (1.35 fold)

extracts. SREBP-1 expression increased significantly in both cytosolic (2.07 fold) and nuclear (1.41 fold) extracts of brain cortex in 1 week. SREBP-1 expression significantly increased in cytosolic (2.15 fold) and nuclear (1.79 fold) extracts of cerebellum in 1 week. There were no significant alterations in SREBP-1 C/EBPβ expressions for 2 and 4 weeks in both cytosolic and nuclear extracts of brain cortex and cerebellum. There were insignificant changes in GSH and GST levels in cortex. However, MDA and SOD levels significantly increased by 43.0 % and 47.3 %, respectively, in 24 hours. Our findings indicate that increase in SREBP-1 and C/EBPβ expressions may be related to oxidative stress during ischemic neurodegenerative processes.

Key Words: global brain ischemia, carotid artery occlusion, SREBP-1, C/EBPβ, oxidative stress, GST, GSH, MDA, SOD

ÖZ

SIÇAN BEYİN KORTEKS VE SEREBELLUMUNDA GLOBAL İSKEMİ/REPERFÜZYON TARAFINDAN ARTIRILAN OKSİDATİF STRES SIRASINDA SREBP İLE C/EBP SENTEZİNİN ARAŞTIRILMASI

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İskemik beyin hasarı nörodejenerasyona sebep olur. Bu çalışmada, nörodejenerasyonun mekanizması; sterol düzenleyici elemente bağlanan protein-1 (SREBP-1), CCAAT/tetikleyiciye bağlanan proteinβ'ının (C/EBPβ), glutatyon (GSH), malondialdehit (MDA), glutatyon-S-transferaz (GST) ve süperoksit dismutaz'ın (SOD) rollerinin incelenmesiyle araştırılmıştır. 10 dakikalık karotid arter oklüzyonu (CAO) ve hipotansiyon sıçan modeli uygulanmıştır. Kontrol grupları yalancı operasyona tabi tutulmuştur. Deney hayvanları 24 saat, 1, 2 ve 4 haftalık reperfüzyon süreleri sonucunda öldürülmüştür. SREBP-1 ve C/EBPβ'nın beyin korteks ve serebellumdaki seviyelerindeki değişimler "Western blot" yöntemi ile incelenmiştir. C/EBPβ'nın ekspresyonu beyin kortekste sitozolik (1,19 ve 1,58 kat) ile nüklear (1,73 ve 1,81 kat) ekstraktların ikisinde de, sırasıyla 24 saat ve 1 hafta CAO gruplarında istatistiksel anlamlı artmıştır. C/EBPβ'nin ekspresyonu 1

hafta CAO grubu sitozolik (1,63 kat) ve nüklear (1,35 kat) serebellum ekstrelerinde istatiksel anlamlı artmıştır. 1 haftalık CAO grubunda beyin kortekste hem sitozolik (2,07 kat) hem nüklear (1,41 kat) ekstrelerde SREBP-1 ekspresyonu istatistiksel anlamlı artmıştır. 1 haftalık CAO grubu serebellumunda, hem sitozolik (2,15 kat) hem de nüklear (1,79 kat) ekstraktlarda SREBP-1 ekspresyonu istatiksel anlamlı artmıştır. 2 ve 4 haftalık grupların beyin korteks ile serebellumun hem sitozolik, hem nüklear ekstrelerinde SREBP-1 ve C/EBPβ ekspresyonlarında istatistiksel anlamlı fark yoktur. Global iskemi sonrası sıçan korteks sitozollerinde GSH seviyelerinde ve GST aktivitelerinde anlamlı bir değişiklik bulunamamıştır. Ancak, MDA seviyeleri ve SOD aktiviteleri, sadece 24 saat CAO grubunda kontrole göre, sırasıyla % 43,0 ve % 47,3 anlamlı artış göstermiştir. Sonuçlarımız, SREBP-1 ve C/EBPβ ekspresyonlarındaki artışların, iskemik nörodejeneratif süreçte oksidatif stresten kaynaklanabileceğini düşündürmektedir.

Anahtar kelimeler: global beyin iskemisi, karotid arter oklüzyonu, SREBP-1, C/EBPβ, oksidatif stres, GST, GSH, MDA, SOD

To my Mother and Sister

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

8-OHdG 8-hydroxy-2-deoxyguanosine

ad lib ad libitum

AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA Analysis of Variance

AP Alkaline Phosphatase

AP-1 Activator Protein-1

APAF1 Apoptotic Peptidase Activating Factor 1

APS Ammonium Persulfate

ATP Adenosine Triphosphate

BHT Butylated Hydroxytoluene

bLHL-Zip basic-helix-loop-helix-leucine zipper

BSA Bovine Serum Albumin

bZIP basic leucine Zipper

C Control

ca Circa

CAO Carotid Artery Occlusion

C/EBPα CCAAT Enhancer Binding Protein α

C/EBPβ CCAAT Enhancer Binding Protein β

C/EBPβ-1 CCAAT Enhancer Binding Protein β-1

C/EBPβ-2 CCAAT Enhancer Binding Protein β-2

C/EBPβ-3 CCAAT Enhancer Binding Protein β-3

C/EBPγ CCAAT Enhancer Binding Protein γ

C/EBPδ CCAAT Enhancer Binding Protein δ

C/EBPε CCAAT Enhancer Binding Protein ε

C/EBPζ CCAAT Enhancer Binding Protein ζ

C/EBPs CCAAT Enhancer Binding Proteins

CDNB 1-chloro-2,4-dinitrobenzene

CHOP CCAAT Enhancer Binding Protein Homologous Protein

Cu Copper

CuZnSOD Copper-Zinc Superoxide Dismutase

CYT C Cytochrome c

dATP deoxyadenosine Triphosphate

DHARs Dehydroascorbate Reductase

dH_2O distilled Water

DISC Death-Inducing Signaling Complex

DNA Deoxyribonucleic acid

DTNB 5,5'-dithiobis-(2-nitrobenzoic acid)

DTT Dithiothioreitol

ECL Enhanced Chemiluminescence

ECSOD Extracellular Superoxide Dismutase

EDTA Ethylenediaminetetraaceticacid

EGTA Ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid

ELISA Enzyme Linked Immunosorbent Assay

ER Endoplasmic Reticulum

Fe Iron

FeSOD Iron Superoxide Dismutase

G6PD glucose 6-phosphate dehydrogenase

GAPDH Glyceraldehyde 3-phosphate dehydrogenase

GCS Gamma Glutamyl Cysteine Synthetase

Glu Glutamate

GPx glutathione Peroxidase

GR Glutathione Reductase

GRX Glutaredoxin

GS Glutathione synthetase

GSH L-glutathione reduced

GSNO S-nitrosoglutathione

GSPxs Glutathione Peroxidases

GSSG Oxidized Glutathione

GST Glutathione S-transferase

H_2O Water

H_2O_2 Hydrogen peroxide

HCl Hydrochloric acid

Hg Mercury

HHE 4-hydroxy-2-hexenal

HMG CoA 3-hydroxy-3-methyl-glutaryl-CoA

HPLC High Performance Liquid Chromatography

ht hypotension

IMS Intermembrane Space

IUPAC International Union of Pure and Applied Chemistry

kDa kiloDalton

kg kilogram

K_2HPO_4 di potassium hydrogen phosphate

KH_2PO_4 Potassium dihydrogen phosphate

L• Alkyl Radical

LAP Liver-enriched Activator Protein

LDL Low-Density Lipoprotein

LH Lipids

LIP Liver-enriched Inhibitor Protein

LOO• Fatty Acid Peroxyl Radicals

LOOH Lipid Hydroperoxides

MABP Mean Arterial Blood Pressure

MAPK Mitogen Activated Protein Kinase

MDA Malonedialdehyde

Me Metal

mg milligram

$MgCl_2$ Magnesium Chloride

mm millimeter

MMP Mitochondrial Membrane Permeabilization

Mn Manganese

MnSOD Manganese Superoxide Dismutase

MS Mass Spectrometry

NaCl Sodium Chloride

NADPH Nicotine adenine dinucleotide phosphate

NBT/BCIP Nitro blue tetrazoliumCl/5-Bromo-4-chloro-3-indolyl P

Ni Nickel

NiSOD Nickel Superoxide Dismutase

nm nanometer

NMDA N-methyl-D-aspartic acid

NFκB Nuclear Factor Kappa-B

O_2 Oxygen

OH• Hydroxyl Radical

PDI Protein Disulfide Isomerase

PKC Protein Kinase C

PMSF Phenylmethanesulphonylfluoride

PTP Permeability Transition Pore

PUFAs Polyunsaturated Fatty Acids

PVDF Polyvinylidene Difluoride

RAGE Receptor for Advanced Glycosylation End-products

RNA Ribonucleic acid

ROS Reactive Oxygen Species

S1P Site 1 Protease

S2P Site 2 Protease

S9 Crude Cytosolic Extract

SCAP SREBP Cleavage Activating Protein

SDS sodium dodecyl sulfate

SDS-PAGE Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SEM Standard Error

Ser105 Serine 105 residue

SOD superoxide dismutase

SOD1 Superoxide Dismutase 1

SOD2 Superoxide Dismutase 2

SOD3 Superoxide Dismutase 3

SRE Sterol Regulatory Element

SREBP Sterol Regulatory Element Binding Protein

SREBP-1a Sterol Regulatory Element Binding Protein-1a

SREBP-1c Sterol Regulatory Element Binding Protein-1c

SREBP-2 Sterol Regulatory Element Binding Protein-2

SREBPs Sterol Regulatory Element Binding Proteins

SWI/SNF SWItch/Sucrose NonFermentable

TA Transactivatiton

TBA Thiobarbituric Acid

TBARS Thiobarbituric Acid Reacting Substances

tBID truncated BID

TBST Tris Buffered Saline with Tween-20

TCA Trichloro acetic acid

TEMED Tetramethyl-ethylenediamine

TLR Toll-Like Receptor

tMCAO transient Middle Carotid Artery Occlusion

Tris Tris (hydroxymethyl) aminomethane

TRX Thioredoxin

Tween-20 polyoxyethylene-sorbitan monolaurate

V Volts

v/v volume per volume

w/v weight per volume

CHAPTER I

INTRODUCTION

Mostly ischemia is unpredictable; however ischemia/reperfusion injury is a potential threat for patients who undergo a cardiopulmonary bypass, surgery under tourniquet, organ transplantation, thrombolysis and embolectomy (Sakamoto, Corcoran et al. 2002). The predictable and unpredictable conditions may cause ischemia/reperfusion injury and thus make ischemia complicated and important.

Brain ischemia is the focal reduction of blood flow to the whole brain or solely to a part of brain. Arterial obstruction is the damage of any of the major arteries, such as the carotid artery. One cause of arterial obstruction is the formation of a blood clot in a major artery. Another cause of arterial obstruction can be as a result of a surgical procedure, either accidentally or on purpose (Freeman and Gilfillan, 1950). Cardiac arrest, intraoperative hypoxia/hypotension, obstruction of airway, drug intoxication, or hemorrhagic shock are the other causes of global brain ischemia/reperfusion injury (Li, Zhou et al. 2005).

Global ischemia activates cascades of hypoxic events leading to brain edema, cell death, and neurological/neurobehavioral dysfunction (Block, 1999). The major molecular pathogenic mechanisms of neurodegeneration include excitotoxicity, peri-infarct depolarization, inflammation and programmed cell death (Dirnagl, Iadecola et al. 1999).

1.1. Global Brain Ischemia

With an incidence of approximately 250 – 400 in 100 000 and a mortality rate of around 30 %, stroke remains the third leading cause of death in industrialized countries. In the USA alone four-million survivors are coping with its debilitating consequences. Ischemic brain injury results from a complex sequence of pathophysiological events that evolve over time and space (Dirnagl, Iadecola et al. 1999).

Brain tissue has a relatively high consumption of oxygen and glucose, and depends almost exclusively on oxidative phosphorylation for energy production. Focal impairment of cerebral blood flow restricts the delivery of substrates, particularly oxygen and glucose, starting the cascades of events leading to neurodegeneration. The molecular mechanisms involved in neurodegeneration are summarized in Fig. 1.1 (Dirnagl, Iadecola et al. 1999).

Impairment of energy production disturbs the maintenance of ionic gradients (Martin, Lloyd et al. 1994; Dirnagl, Iadecola et al. 1999). Consequently, somatodendritic as well as presynaptic voltage-dependent Ca^{2+} channels become activated and excitatory amino acids are released into the extracellular space. At the same time, the energy-dependent processes, such as presynaptic reuptake of excitatory amino acids, are impeded, which further increases the accumulation of glutamate in the extracellular space resulting in excitotoxicity. Excitotoxicity is the lethal pathology of neurons caused by excessive glutamate receptor stimulation. Activation of NMDA receptors and metabotropic glutamate receptors contribute Ca^{2+} overload (Park, Nehls et al. 1989; Dirnagl, Iadecola et al. 1999), the latter via phospolipase C and inositol-3-phosphate signaling. As a result of glutamate-mediated overactivation, Na^{+} and Cl^{-} enter the neurons via channels for monovalent ions (for example, the AMPA receptor-channel) (Dirnagl, Iadecola et al. 1999).

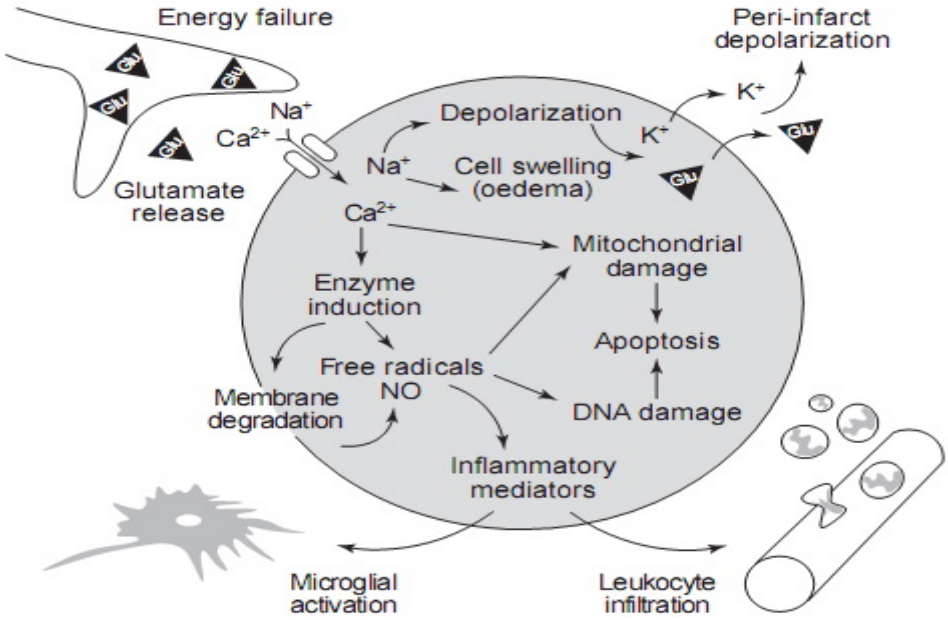

Figure 1.1: Simplified overview of pathophysiological mechanisms in the focally ischemic brain. Energy failure leads to the depolarization of neurones. Activation of specific glutamate receptors dramatically increase intracellular Ca^{2+}, Na^{+}, Cl^{-} levels while K^{+} is released into the extracellular space. Diffusion of glutamate (Glu) and K^{+} in the extracellular space can propagate a series of spreading waves of depolarization (peri-infarct depolarizations). Water shifts to the intracellular space via osmotic gradients and develops edema. The universal intracellular messenger Ca^{2+} overactivates numerous enzyme systems (proteases, lipases, endonucleases, etc.). Free radicals are generated, which damage membranes (lipolysis), mitochondria and DNA, in turn triggering caspase-mediated cell death (apoptosis). Free radicals also induce the formation of inflammatory mediators, which activate microglia and lead to the invasion of blood-borne inflammatory cells (leukocyte infiltration) via upregulation of endothelial adhesion molecules (Dirnagl, Iadecola et al. 1999).

Although calcium is a necessary component of normal intracellular signaling, pathological elevation of intracellular calcium (iCa^{2+}) is the cornerstone of excitotoxicity. Thus, if neurons depolarized by glutamate survive the initial osmotic insult, death by calcium dysregulation will ensue. According to the self perpetuating iCa^{2+} overload is the opening of the mitochondrial permeability transition pore (PTP), which results in Ca^{2+} release from mitochondria and loss of any remaining ATP production. Furthermore, a net increase in ROS occurs with calcium overload, partially due to generation by Ca^{2+}-activated enzymes such as phospholipase A2, xanthine dehydrogenase and nitric oxide synthase. Together, excessive iCa^{2+} and ROS oxidize and/or dysregulate multiple intracellular signaling components such as proteases, kinases, DNA endonucleases, and lipid membranes. Neurons at this stage die by oncosis (necrosis). The loss of cellular membrane activates microglia, which adds to the oxidative stress and widens the area of damage (Smart, et al. 2008).

Apoptosis is a form of programmed cell death that occurs in neurons during development of the nervous system (Mattson, Culmsee et al. 2000). However, apoptotic cell death is also frequently observed in neuronal degeneration after ischemic brain injury in animal models of stroke, although usually this occurs at later time points or at the fringes of a localized area of damage. In this scenario, some cells survive long enough to maintain mitochondrial ATP production and membrane integrity. Then mitochondria orchestrate the programmed cell death (Fig 1.2). The process of apoptosis in neurons is similar to other cells (Smart, et al. 2008).

In neuronal tissue damaged by excitotoxicity, the presence of neuronal death occurring by both apoptosis and oncosis highlights a simple but important message: Under the same toxicological conditions, different neurons can die by different death mechanisms (Smart, et al. 2008).

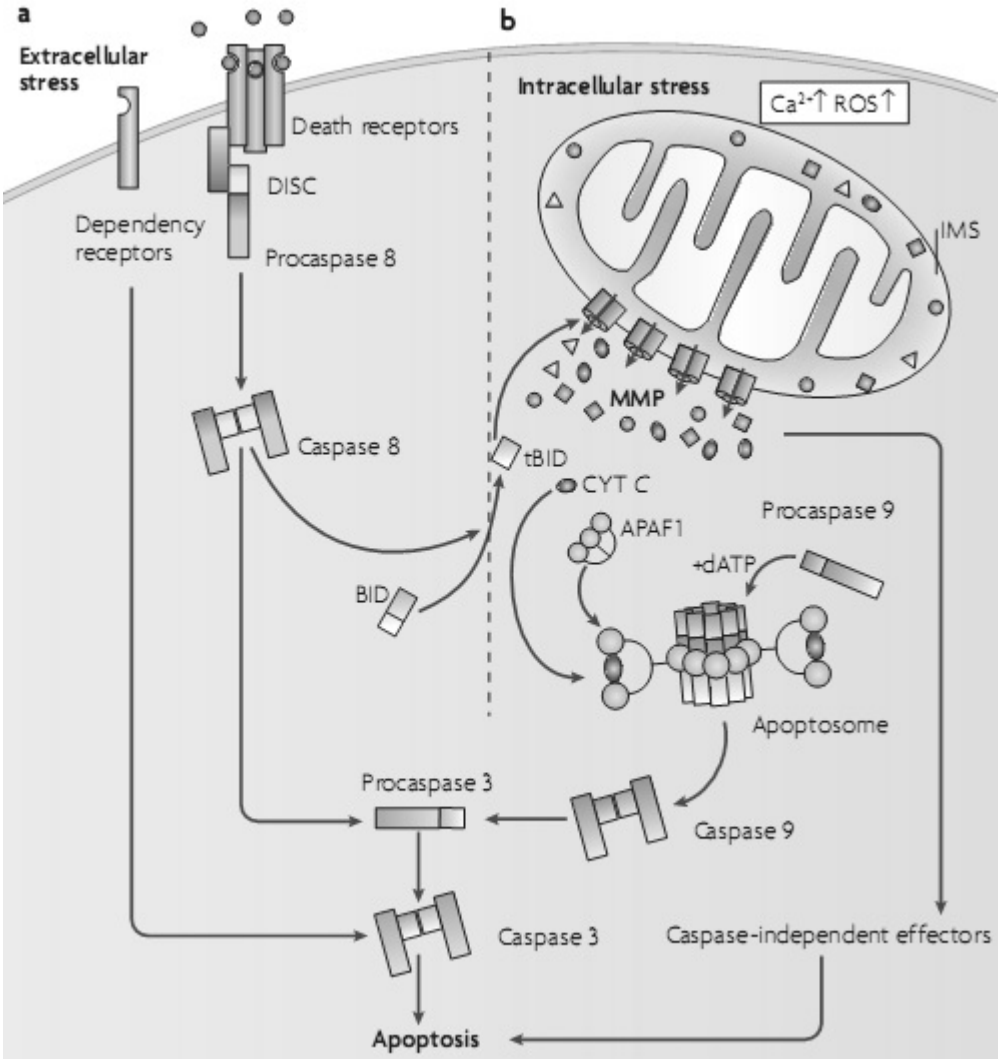

Figure 1.2: Apoptosis can result from the activation of two biochemical cascades, which are known as the extrinsinc (part a) and the intrinsic (or mitochondrial, part b) pathways. The extracellular apoptotic pathway is initiated at the plasma membrane by specific transmembrane receptors, whereas mitochondrial apoptosis is triggered by intracellular stimuli such as Ca^{2+} overload and overgeneration of reactive oxygen species (ROS). MMP: mitochondrial membrane permeabilization, IMS: intermembrane space, CYT C: cytochrome c, APAF1: apoptotic peptidase activating factor 1, tBID: truncated BID, DISC: death-inducing signaling complex, dATP: deoxyadenosine triphosphate (Galluzzi, Blomgren et al. 2009).

Inflammatory mechanisms play an important role in the risk of stroke and during the acute phase of brain ischemia, which contributes to functional outcome of patients. Systemic inflammatory mechanisms are associated with higher risk of stroke and vascular recurrences after a first ischemic stroke (Yi, Park et al. 2007). Several inflammatory molecules are implicated during the acute phase of ischemic stroke, such as cytokines, adhesion cell molecules and metalloproteinases, which are related to the presence of early neurological deterioration and infarct volume (Fig 1.3). Molecular markers of inflammation can be useful tools for the management of patients with ischemic stroke, both during the acute phase and to predict prognosis and prevent the risk of a new vascular event (Rodriguez-Yanez and Castillo, 2008). Moreover, as a part of the cellular response to traumatic injury, gene expression has prompted a considerable amount of research interest. Gene family members that cause cellular response encode several types of proteins including growth factors, growth factor receptors, cytoskeletal proteins and transcription factors. The ability to regulate gene expression may enable cells to proliferate, change phenotypically, or in the case of neurotrauma, to be protected and repaired (Kapadia, Tureyen et al. 2006).

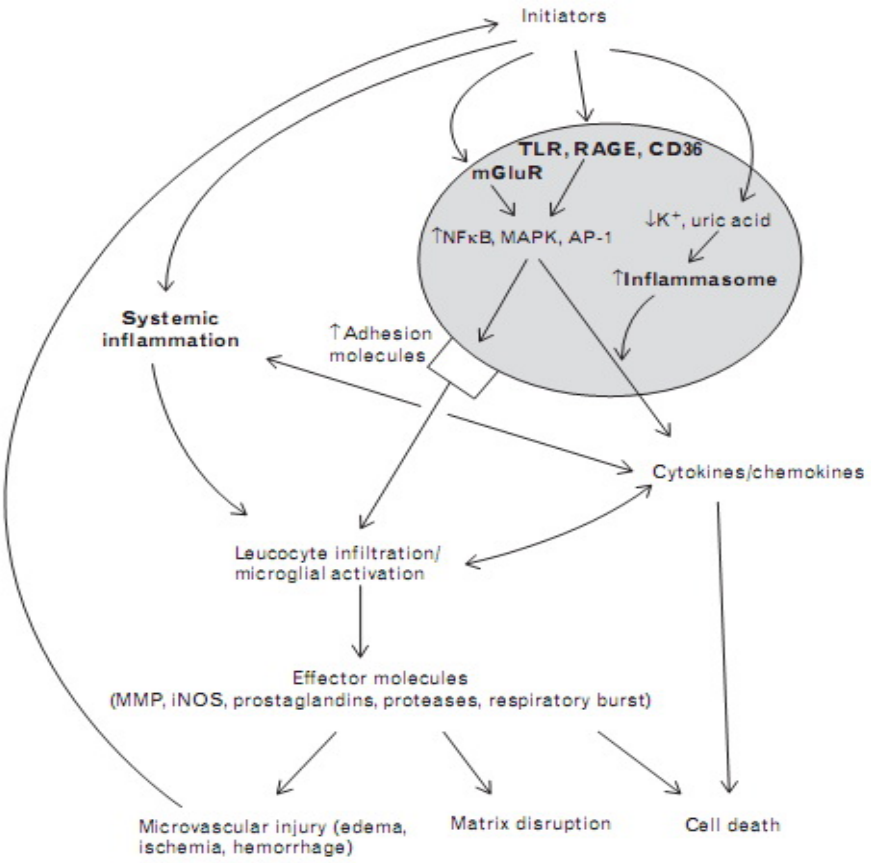

Figure 1.3: Initiation of the inflammatory cascade by a variety of factors leads to systemic inflammation or activation of cellular (shaded) proinflammatory pathways in a typical cell (neuron, astrocyte, endothelial cell, etc.). AP-1: activator protein-1, MAPK: mitogen activated protein kinase, MMP: matrix metalloproteinase, NFκB: nuclear factor kappa-B, RAGE: receptor for advanced glycosylation end-products, TLR: toll-like receptor (Kleinig and Vink, 2009).

C/EBPs are well known mediators of injury and inflammation responses (Lekstrom-Himes and Xanthopoulos, 1998). SREBP levels are altered under stress conditions, and are mediator of a neuroprotective enzyme aromatase (Kelicen and Nordberg, 2006). Aromatase neuroprotection are most likely mediated by local estrogen synthesis (Saldanha, Duncan et al. 2009). These transcription factors become a target for studies which investigate the mechanisms of neurodegeneration.

1.2. Role of Transcription Factors in Neurodegeneration During Global Ischemia

Transcription factors are proteins; which bind to DNA and regulate the gene expression (Herdegen and Leah, 1998). It is possible to say that they are the switches of genes.

Some transcription factors forms dimers with other transcription factors to bind to DNA, by their leucine zipper (Landschulz, Johnson et al. 1988). Also some transcription factors need cleavage or other arrangements before binding to DNA (Cahill, Ernst et al. 1994; Boulikas, 1995). When transcription factors bound to DNA, they activate or suppress the expression. The activation or suppression depends on the properties of the gene promoter (Herdegen and Leah, 1998).

There are lots of other proteins and enzymes necessary for transcription of a gene, especially chromatin structure arrangement is needed (Lee and Young, 2000). However the key role of transcription factors in gene expression is essential.

1.2.1. Sterol Regulatory Element Binding Protein (SREBP)

Sterol regulatory element binding proteins (SREBPs) are members of a basic-helix-loop-helix-leucine zipper (bLHL-Zip) transcription factor family. SREBPs are specialized on the regulation of lipid homeostasis by playing a key role for the transcriptional regulation of cholesterol and fatty acid mechanism involved enzymes' genes. This regulation keeps cholesterol, fatty acid, triacylglycerol and phospholipid levels under control (Fig 1.4) (Brown and Goldstein, 1997; Horton, Goldstein et al. 2002; Eberle, Hegarty et al. 2004).

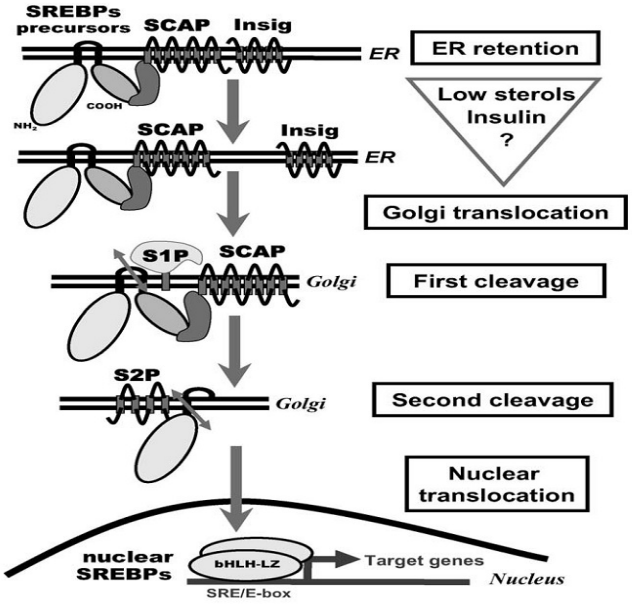

Figure 1.4: SREBPs activation by proteolytic cleavage. SREBPs are tightly associated with the SCAP/SREBP complex in the ER compartment. Upon appropriate conditions (low sterol concentrations or possibly insulin), the interaction between Insig and SCAP decreases and allows the escorting by SCAP of SREBPs to the Golgi apparatus where the S1P cleaves SREBP at a lumenal site. The S2P then cleaves releasing the NH2-terminal SREBPs domain (nSREBPs). This domain containing the bHLH-LZ region is then translocated to the nucleus where it will bind its target genes on SRE or E-box sequence as a homodimer (Eberle, Hegarty et al. 2004).

SREBPs are composed of three main domains; first N-terminal domain contains the transactivation domain and bHLH-Zip region for DNA binding and dimerization. In the middle hydrophobic transmembrane domain which has a role in binding to endoplasmic reticulum and golgi membrane (Fig 1.5). Third domain; C-terminal one was including the regulatory domain. SREBPs constitutes of three members; which are SREBP-1a, SREBP-1c, and SREBP-2. SREBP-1c is functionary for fatty acid synthesis and insulin induced glucose mechanism. SREBP-2 is functionary for cholesterol synthesis. Whereas SREBP-1a can potentially bind all SREBP related genes (Eberle, Hegarty et al. 2004).

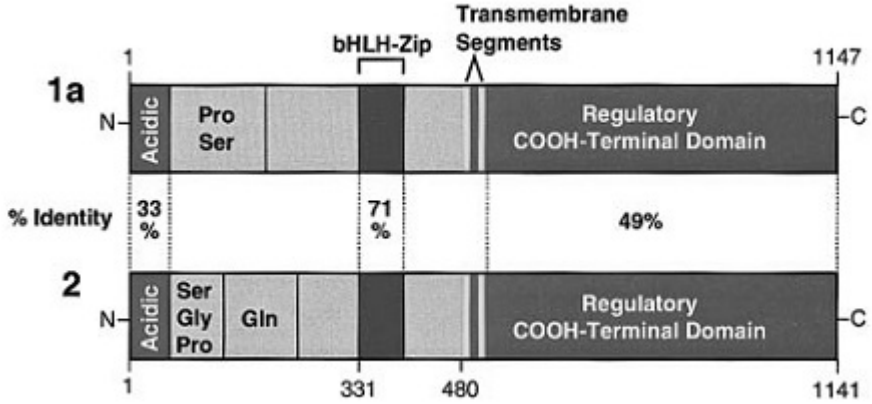

Figure 1.5: Domain structures of human SREBP-1a and SREBP-2. The sequence of SREBP-1c (not shown) is identical to that of SREBP-1a except for a shortened NH2-terminal acidic domain (Brown and Goldstein, 1997).

As mentioned above SREBPs are bound to ER membrane and needed cleavage for activation. SREBPs have a pivotal role in cholesterol feedback mechanism. SREBPs are synthesized as a precursor protein and located in ER and also nuclear envelope (Fig 1.6). This precursor SREBP has a 120 – 130 kDa molecular weight. SREBPs make a complex with SREBP cleavage activating protein (SCAP). In sterol withdrawal this SREBP precursor transported to the golgi via a vesicle carrier. In golgi two site-specific cleavage occur by site-1 protease (S1P) and site-2 protease (S2P) then N-terminal transactivation domain were disengaged (Nohturfft, Brown et al. 1998; Brown and Goldstein, 1999). The nuclear active form is derived from this cleaved N-terminal, then transcription factor binds to genes with sterol regulatory element (SRE) sequence (Edwards, Tabor et al. 2000; Shimano, 2001). LDL receptor gene, HMG CoA synthase gene, fatty acid synthase gene and some other genes are targets for SREBPs (Shimano, 2001).

Mostly it is thought that nuclear active form is about 60 – 70 kDa, notwithstanding it was shown that a 42 – 47 kDa nuclear active form is available (Kaul and Kaur, 2001; Chang, Wang et al. 2005; Chang, Edeen et al. 2006).

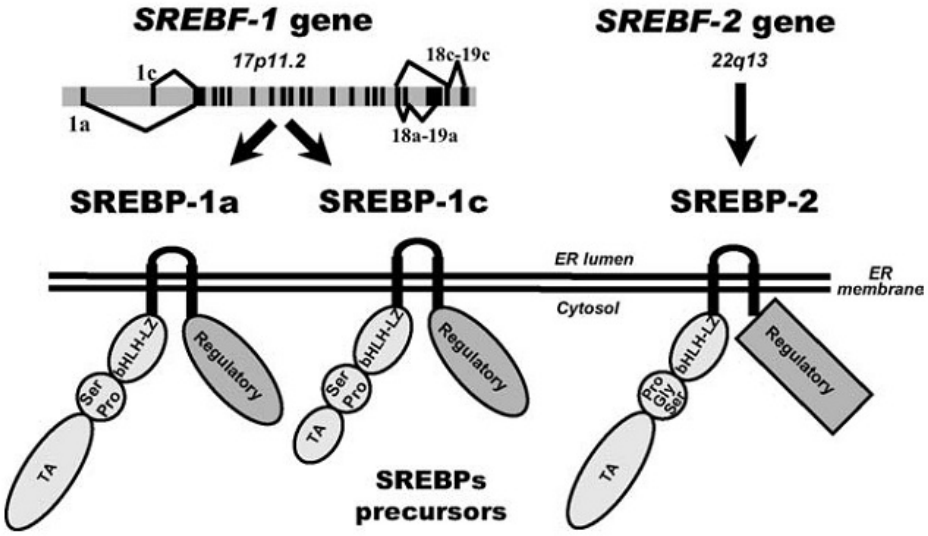

Figure 1.6: SREBPs genes and structure. SREBPs family is composed of three members. Each SREBP precursor is organized into three domains: *i)* an NH_2-terminal domain that contains the transactivation (TA) domain, a region rich in serine and proline and the bHLH-LZ region for DNA binding and dimerization; *ii)* two hydrophobic transmembrane spanning segments interrupted by a short loop that projects into the lumen of the ER; and *iii)* COOH-terminal segment regulatory domain (Eberle, Hegarty et al. 2004).

Because of the importance of cholesterol and fatty acid levels in neuronal homeostasis; SREBPs have become a potential target for neurobiological studies (Mielke and Lyketsos, 2006; Korade, Kenworthy et al. 2009). However it was not well established the interaction between SREBPs and neurodegeneration. In some studies the distribution of SREBP-2, age relation of SREBP-1 and expression of SREBP-1 in brain during neonatal term were reported (Okamoto, Kakuma et al. 2006; Kim and Ong, 2009; Saito, Chakraborty et al. 2009). However not much studies valid for the interaction between neurodegeneration and SREBPs. Under these conditions it becomes important to detect the interrelations between SREBPs and neurodegeneration.

1.2.2. CCAAT Enhancer Binding Protein (C/EBP)

CCAAT-enhancer binding proteins (C/EBPs) are a family of transcription factors which exist in all mammals. C/EBPs are characterized by specificity in binding to CCAAT motifs in DNA sequences. This family is a subfamily of basic region of leucine zipper (bZIP) transcription factors. This C/EBP family consists of six members; C/EBPα, C/EBPβ (also known as NF-IL6, IL-6-DBP, LAP, AGP/EBP, CRP2), C/EBPδ (also known as CRP3, CELF, NF-IL6β), C/EBPγ (also known as Ig/EBP), C/EBPε (also known as CRP1), and C/EBPζ (also known as CHOP, Gadd153). All C/EBPs, except C/EBPζ, have three domains; first N-terminal region which has regulatory function, in the middle there is a canonical basic domain which has DNA binding function, and third C-terminal leucine zipper domain has heterodimer or homodimer formation function (Maytin and Habener, 1998; Cardinaux, Allaman et al. 2000; Wang, Lee et al. 2008).

C/EBPβ is transcribed from an intronless gene, however after some modifications isoforms occur (Eaton and Sealy, 2003). For human there are three isoforms of C/EBPβ, although very similar of these three isoforms are valid for mice and rats. The large size of human C/EBPβ protein has 346 amino acids while this number is 297 for rat. Three isoforms of C/EBPβ are result from these -346 aa and 297 aa long- pioneer proteins. C/EBPβ-1, C/EBPβ-2, C/EBPβ-3 are human isoforms while LAP*, LAP and LIP represent the rat equivalents (Fig 1.7). The longest isoform C/EBPβ-1 is 51-59 kDa for human (LAP*45-55 kDa), a shorter form C/EBPβ-2 is 45-50 kDa for human and LAP is 35-38 kDa for rats, finally shortest one C/EBPβ-3 and LIP are 20-21 kDa for both organisms. An important point occur for C/EBPβ-1 and LAP*; a possible longer form. This form is thought to be a more highly modified form of C/EBPβ-1 and LAP*. This form can be detected at 65 kDa band.

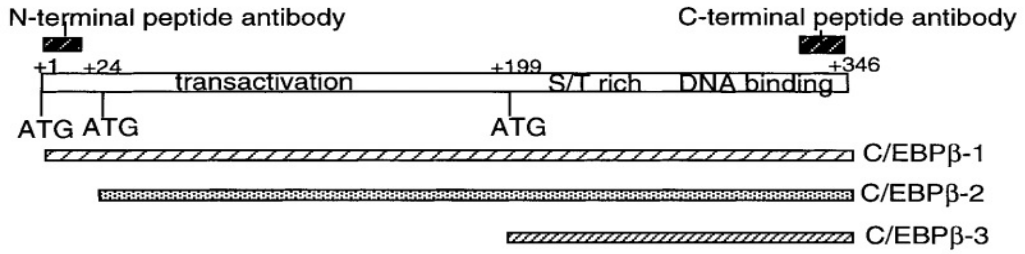

Figure 1.7: Schematic representation of the C/EBPβ protein isoforms, showing ATG start sites of the three protein isoforms (Eaton, Hanlon et al. 2001).

Nevertheless these isoforms has different characteristics. C/EBPβ-1 (LAP*) and C/EBPβ-2 (LAP) are known as activator forms while C/EBPβ-3 (LIP) is oppositely has a repressor role.

Recently in human it was demonstrated that C/EBPβ-1 and C/EBPβ-2 works at different phases of cell cycle or different type of tissues. For instance in non-proliferated cells both C/EBPβ-1 and C/EBPβ-2 exist, however in neoplastic cells C/EBPβ-1 is absent. The kDa of these isoforms vary in a range and can be different by the type of the tissue (Eaton, Hanlon et al. 2001).

C/EBPβ is highly involved in inflammation. C/EBPβ and some other C/EBPs are activated by the inflammatory events in the brain. Expression of many pro-inflammatory genes including the cytokine genes (such as interleukins) is known to be controlled by C/EBPs (Cardinaux, Allaman et al. 2000; Yi, Park et al. 2007). In some studies it was shown that there is an important interaction between inflammation and C/EBPβ. In this interaction the phosphorylation of C/EBPβ is significant. Also to have special sites for phosphorylation makes C/EBPβ a target for inflammation related proteins. These inflammation related proteins are; stress activated protein kinases, such as p38 or protein kinase C (PKC) cause C/EBPβ phosphorylation at Ser105 residue (Fig 1.8). This process increases the transactivation potential of C/EBPβ and then C/EBPβ bind to target genes. There is a domino effect in this cascade which begins with inflammation or stress (Trautwein, Caelles et al. 1993; Engelman, Lisanti et al. 1998; Cassel and Nord, 2003).

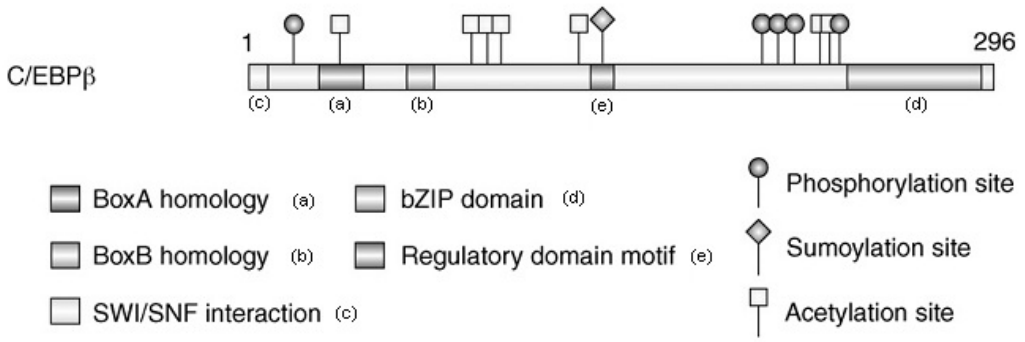

Figure 1.8: Post-translational modification sites in C/EBPβ. The position of functionally defined domain involved in DNA binding and dimerization (bZIP domain), TBP/TFIIB binding (BoxA and BoxB), SWI/SNF interaction, and intramolecular inhibition (RDM) is indicated (Nerlov, 2008).

1.3. Role of Oxidative Stress in Global Brain Ischemia/Reperfusion Injury

In rodent cerebral ischemia models, reoxygenation during reperfusion after carotid artery occlusion provides oxygen as a substrate for numerous enzymatic oxidation reactions and for mitochondrial oxidative phosphorylation to produce adenosine triphosphate. Reperfusion after ischemia causes overproduction of reactive oxygen species (ROS) in mitochondria, and consumption of endogenous antioxidants by these radicals may lead to a dramatic rise in intracellular ROS. Oxygen radicals, the products of biochemical and physiological reactions (excitotoxicity, apoptosis, inflammation), are known to damage cellular lipids, proteins, and nucleic acids (Sugawara, Fujimura et al. 2004; Saito, Maier et al. 2005).

Although superoxide anion radicals and hydrogen peroxide (H_2O_2) are produced by mitochondria constantly, reperfusion enhances their production. These constantly produced (ROS) are scavenged by superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase. Other small molecular

antioxidants, including glutathione (GSH), Vitamin C, and Vitamin E, are also involved in the detoxification of free radicals (Sugawara, Fujimura et al. 2004).

1.3.1. Biomarkers of Oxidative Stress

When reactive oxygen species (ROS) including superoxide anion and nitric oxide; overbalance the equilibrium between oxidation and reduction of intracellular substances, oxidative stress eventuates. ROS fasten on membrane lipids, nucleic acids, proteins and carbohydrates (Tsukahara, 2007). It is important to demonstrate the relation between oxidative stress and alterations in cells and tissues. Oxidation products of DNA, lipids and proteins are essential biomarkers of oxidative stress (Blumberg, 2004).

Free radicals affect DNA by oxidizing bases, creating abasic sites, breaking strands and forming cross-links between DNA and proteins. Hydroxyl radical (OH•) oxidize guanine to 8-hydroxy-2-deoxyguanosine (8-OHdG); an important biomarker for DNA oxidation. This base modification situation increase mutations in DNA (Shigenaga and Ames, 1991; Gate, Paul et al. 1999). DNA glycosylase has an important role in repair unless the higher degree of oxidation is irreversible (Gate, Paul et al. 1999).

When a protein's side chains of lysine, proline, arginine and threonine are oxidized, protein carbonyl groups emerge. Protein carbonyl groups are biomarkers of protein oxidation. Nevertheless 3-nitrotyrosine, 3-chlorotyrosine and 2-oxohistidine are remarkable products of protein oxidation and used to detect oxidative stress effects on proteins (Gate, Paul et al. 1999; Beal, 2002).

Membrane lipids were a general target for ROS because of unsaturated fatty acid content (Behn, Araneda et al. 2007). The peroxidation of polyunsaturated fatty acids (PUFAs) initiated by hydroxyl radical ($OH^{\bullet}$). PUFAs undergoes a molecular rearrangement after initiation with $OH^{\bullet}$; as a result fatty acid peroxyl radicals (LOO•) were formed. These peroxyl radicals reacts with other membrane lipids (LH) to form lipid hydroperoxides (LOOH)

and alkyl radical (L•) (Fig 1.10). Also alkyl radicals react with O_2 again and continue this course as a vicious circle. The increase in the lipid hydroperoxide level, alters the membrane proteins (Gate, Paul et al. 1999). As a result of oxidation of membrane lipids; malonedialdehyde (MDA) was produced (Behn, Araneda et al. 2007). It is important to determine the concentration of MDA, because it supplies us a considerable data about lipid peroxidation and oxidative stress.

Most of the MDA in the cells were produced by peroxidation of polyunsaturated fatty acids with two or more methylene-interrupted double bonds (Del Rio, Stewart et al. 2005). Withal peroxidation of polyunsaturated fatty acids there are other mechanisms possibly producing MDA. For instance one of the theories suggests the bicyclic endoperoxide as precursor of MDA. In this pathway methyl linoleate turn into prostaglandin-like endoperoxides, under stress conditions MDA was formed (Pryor and Stanley, 1975; Del Rio, Stewart et al. 2005). Other two theories were both based on possible hydroperoxide formation and β-cleavage of the fatty acid chain which produces a hydroxyperoxyaldehyde (Fig 1.9). To form an MDA; β-scission of hydroxyperoxyaldehyde or hydroxyl radical reaction with acrolein radical is needed (Esterbauer, Schaur et al. 1991; Del Rio, Stewart et al. 2005).

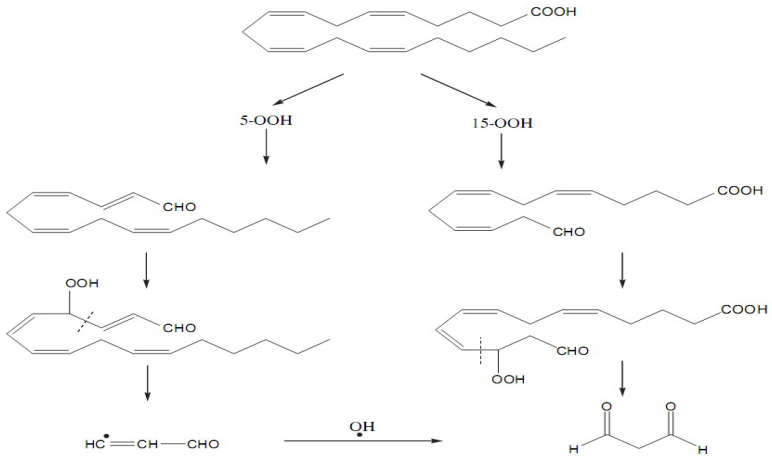

Figure 1.9: The two mechanisms based on the successive hydroperoxide formation and β-cleavage of the fatty acid chain to give a hydroperoxyaldehyde;

MDA is then generated by β-scission or by reaction of the final acrolein radical with a hydroxyl radical (Del Rio, Stewart et al. 2005).

Generally there are two approaches in MDA determination methods; one is about precipitating the proteins by acidic or basic pre-treatment, other is about organic solvent pre-treatment. Initial one detects total MDA, latter detects the free MDA (Del Rio, Stewart et al. 2005). Some techniques are spectrophotometric (Jain and Levine, 1995; Madhava Rao and Sresty 2000), some of them use High Performance Liquid Chromatography (HPLC) combined Ultraviolet-Visible detection (Agarwal and Chase, 2002; Karatas, Karatepe et al. 2002), and some other use Mass Spectrometry (MS) (Cighetti, Debiasi et al. 1999). In addition to these TBARS or MDA detecting techniques; some other lipid peroxidation products are used to determine oxidative stress. For instance isoprostanes (Sakamoto, Corcoran et al. 2002) and 4-hydroxy-2-hexenal (HHE) (Shibata, Yamada et al. 2004). These are more sensitive methods but very expensive when compared to TBARS or MDA assays.

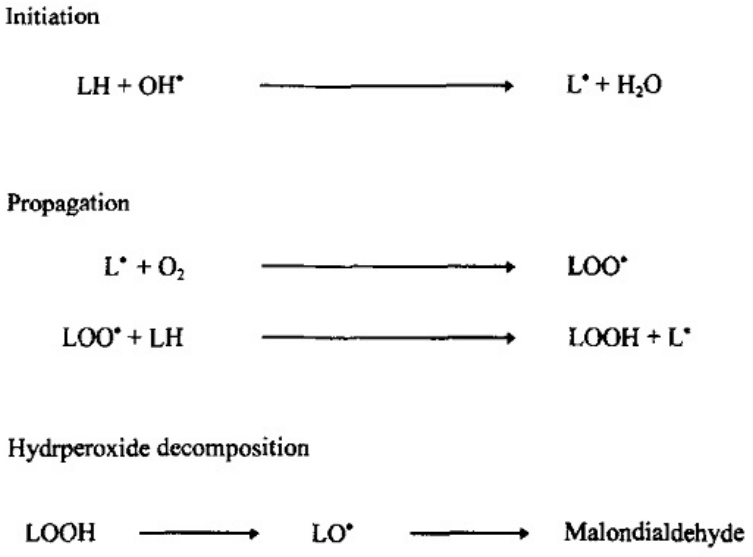

Figure 1.10: Mechanism of lipid peroxidadation. (LH: polyunsaturated fatty acid, L•: alkyl radical, LOOH: lipid hydroperoxide, LO•: alkoxyl radical) (Gate, Paul et al. 1999).

There are numerous techniques existing to determine the lipid peroxidation from cells, tissues or liquid samples. Most widely used and cheapest method is thiobarbituric acid reacting substances (TBARS) method. This assay gives us data about lipid peroxidation by all thiobarbituric acid reacting substances not only MDA. Finally it was shown that determining the MDA level is an important biomarker for oxidative stress (Yagi, 1976).

1.3.2. Non-Enzymatic Antioxidants

Antioxidants are molecules which have the ability to neutralize the oxidized molecules. Non-enzymatic antioxidants are divided into two categories as water soluble or lipid soluble. Ascorbic acid (vitamin C), glutathione (GSH), lipoic acid and uric acid are water soluble ones, while carotenes, α-tocopherol (vitamin E) and ubiquinol (coenzyme q) are lipid soluble (Seifried, Anderson et al. 2007).

One of the most important non-enzymatic antioxidants vitamin E is needed to be supplied in diets for human since it can't be synthesized. Vitamin E generally protects membrane lipids from oxidation. Also it has a role on protein and DNA prevention (Ikeda, Negishi et al. 2003).

Vitamin C donates an electron since it is a reducing agent. Vitamin C is very effective in reducing the oxygen, sulfur or nitrogen-oxygen radicals. Also it has a role on reducing the tocopheroxly radical to vitamin E (Padayatty, Katz et al. 2003).

Glutathione is the most abundant and important non-enzymatic antioxidant in the cell (Evelson, Travacio et al. 2001). Glutathione (GSH) is a tripeptide. γ-glutamylcysteinylglycine is the IUPAC name of the molecule. It is the most abundant thiol and non-enzymatic antioxidant in the cell, with around a 0.5 – 10 mM concentration. Glutathione synthesized in two steps, both depending on adenosine triphosphate. First glutamic acid and cysteine bind, the glycine bound this dipeptide to construct a tripeptide (Chu, 1994).

As an antioxidant GSH was present in reduced form in normal conditions. When GSH is oxidized, GSSG form ensues. GSSG consists of two reactive GSH, since GSH donated an electron to a free radical in the cell. The GSSG level in the cell would rise. At this point glutathione reductase (GR) enzymatically converts GSSG to GSH, to maintain the balance between oxidized and reduced forms. In this GR reaction NADPH is used as a cofactor (Skaper, Floreani et al. 1999).

Glutathione redox cycle is an important mechanism for antioxidant defense system of the cell. The ratio between GSH and GSSG is around 10:1 – 100:1 inside a normal cell. If this ratio decreases, it is an evidence of oxidative stress. GSH level is always higher within the cell because of *de novo* synthesis of GSH, GR activity and GSSG efflux. Most of the GSSG was resulted by the activity of glutathione peroxidase (GPx). This enzyme converts hydrogen peroxide to H_2O via oxidizing GSH. As mentioned above the balance of GSH/GSSG ratio kept by GR. Additionally GSH is involved in dehydroascorbate reductase system, also this makes GSH more important as an antioxidant. By this enzyme and GSH, ascorbic acid levels should be kept stable in the cells. One of the most important enzymatic antioxidant systems; is glutathione-S-transferase (GST) activity (Fig 1.11). This enzyme has an essential role for detoxification of drugs and xenobiotics. GSTs are also GSH dependent (Pompella, Visvikis et al. 2003; Muller, 2004).

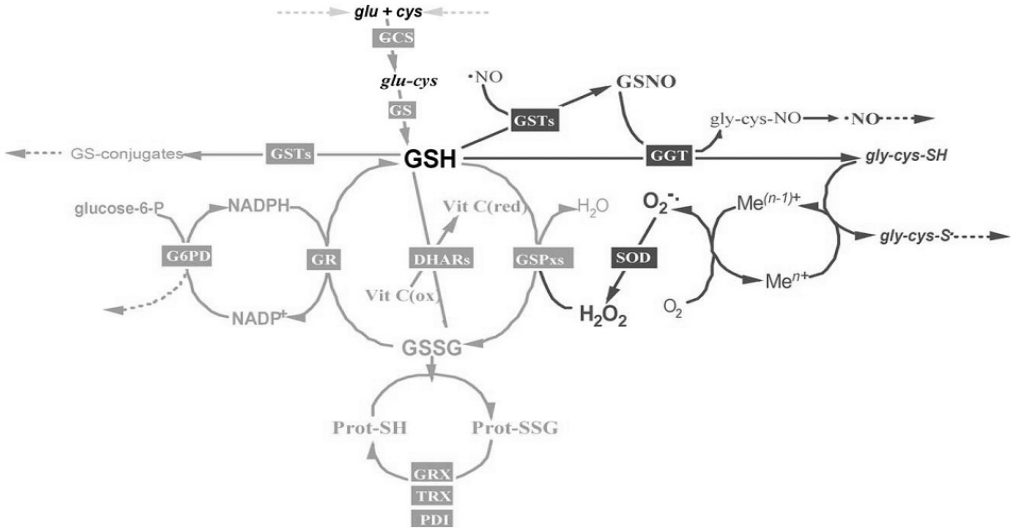

Figure 1.11: Outline of the interrelations connecting the various roles played by GSH in cellular homeostasis. (GCS: gamma glutamyl cysteine synthetase, DHARs: dehydroascorbate reductase, G6PD: glucose 6-phosphate dehydrogenase, GSPxs: Glutathione peroxidases, GR: glutathione reductase, GRX: glutaredoxin, GS: glutathione synthetase, GSNO: S-nitrosoglutathione, GSTs: glutathione S-transferases, Me: metal, PDI: protein disulfide isomerase, SOD: superoxide dismutase, TRX: thioredoxin) (Pompella, Visvikis et al. 2003).

GSH has a special position in cellular antioxidant systems. In ischemic brain GSH levels were used to detect the oxidative stress. More than that, glutathione involved molecules proposed as protective agents (Khan, Jatana et al. 2006).

1.3.3. Antioxidant Enzymes

Non-enzymatic antioxidants are very important in defense system of cell; they are protective against reactive oxygen species. However, superoxide anion or hydroperoxides can not be discarded by non-enzymatic defense system. Enzymatic antioxidants should take place against highly stable ROS. Antioxidant enzymes have remarkable role in protecting cells from oxidative

stress. Especially aerobic cells produce reactive oxygen species continuously during energy metabolism. To keep the homeostasis of an aerobic cell, enzymatic antioxidant system must be very active.

The most important antioxidant enzymes are; SOD, catalase, and glutathione system enzymes like; GR, GPx and GST. SOD degrade superoxide radical, catalase decompose hydrogen peroxide to water and oxygen. Glutathione reductase; reduces GSSG to GSH. Glutathione peroxide catalyses GSH and hyrogen peroxide reaction and form GSSG. GST conjugates GSH to toxins or xenobiotics and transfer out of the cell (Przybylska, Albera et al. 2007; Zafir and Banu, 2009).

1.3.3.1. Glutathione S-transferase (GST)

The GSTs are found in all eukaryotes and prokaryotic systems, in the cytoplasm, in the microsomes, and in the mitochondria. Soluble forms of GSTs are homo or heterodimers of different subunits with distinct substrate specificities and monomers are having molecular weight from 20 kDa to 25 kDa (Mannervik and Jensson, 1982). Sequences and the known three-dimensional structures suggest that these proteins share a common ancestry. GSTs are expressed at high levels in mammalian tissues, for instance 4 % of the total soluble proteins of human liver is GSTs (Eaton and Bammler, 1999). Seven different classes of soluble GSTs have been identified as; alpha (α), mu (μ), pi (π), sigma (σ), theta (θ), kappa (κ) and zeta (δ). They differ from each other by their substrate specificity, chemical affinity, structure, amino acid sequence and kinetic behavior of the enzyme (Landi, 2000). But later studies have shown that there also beta (β), delta (δ), tau (τ), phi (φ), lambda (λ) and omega (ω) forms exist (Dixon, Lapthorn et al. 2002). Alpha, mu and pi forms are active in drug metabolism and sigma form is functioning in the prostoglandin synthesis (Jowsey, Thomson et al. 2001). Zeta and theta are found in both animals and

plants but thau and phi are plant specific (Dixon, Lapthorn et al. 2002) (Fig 1.12).

They are essential enzymes for detoxification system; this family of enzymes conjugates reactive molecules to scavenging molecule GSH. These reactive molecules are especially toxins and xenobiotics. After GSH – reactive molecule conjugation, this conjugate sent to extracellular area. GSTs have a major role in transporting harmful molecules out of cell (Jemth and Mannervik, 1999). GSTs are predominantly expressed in the cytosol, where their GSH dependent catalytic functions include the conjugation and resulting detoxification of herbicides, the reduction of organic hydroperoxides formed during oxidative stress and the isomerization of maleylacetoacetate to fumarylacetoacetate, a key step in the catabolism of tyrosine (Marrs 1996; Edwards, Dixon et al. 2000).

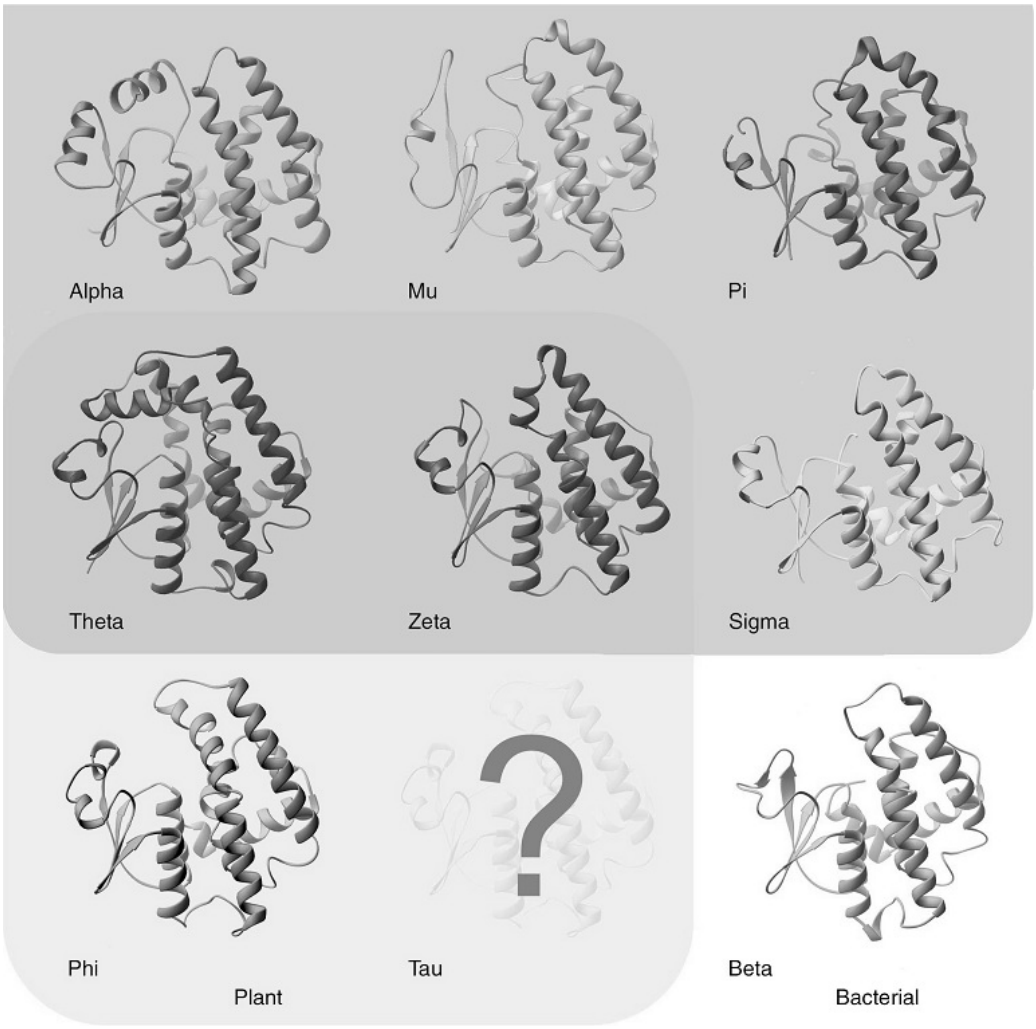

Figure 1.12: The structure of GST subunits alpha, mu, pi, theta, zeta and sigma are found in animals; phi and thau are plant; beta is bacteria specific (Dixon, Lapthorn et al. 2002).

GSTs also have non-catalytic roles, binding flavonoid natural products in the cytosol prior to their deposition in the vacuole (Edwards, Dixon et al. 2000). In a study, it was demonstrated that GSTs have other potential roles; for instance GSTs act as a component of ultraviolet-inducible cell signaling pathways and as potential regulators of apoptosis (Loyall, Uchida et al. 2000).

1.3.3.2. Superoxide Dismutase (SOD)

Superoxide dismutases are extenvise enzymes catalyzes the dismutation of superoxide anion (O_2•) to H_2O_2 and O_2. Superoxide dismutases exists nearly all aerobic cells, since the antioxidant capacity of this enzyme is very crucial. SOD catalyzes the reaction 104 times faster than spontaneous dismutation of substrate at neutral pH (Gate, Paul et al. 1999). SODs are metalloenzymes, by this property a two step reaction was catalyzed by SOD. Cu, Mn, Fe or Ni in the enzyme take an electron from superoxide and forms O_2, in the next step a superoxide anion and 2H are catalyzed to H_2O_2, and then SOD returns back to the initial form (Goldstein, Michel et al. 1988).

Four different kinds of SODs have been characterized in several organisms; in cytosol a copper- and zinc- containing form (CuZnSOD) is localized, in mitochondria a manganese-containing form (MnSOD) exists, in some prokaryotes and plants' outer mitochondrial membrane iron containing form (FeSOD) exist, and extracellular SOD (ECSOD) a copper- and zinc-containing form exist in the extracellular matrix (Marklund, 1982). Also a possible another from exist for prokaryotes a nickel containing form (NiSOD) (Barondeau, Kassmann et al. 2004). In human and in all other mammals there are three genes encoding SOD, these are SOD1, SOD2, and SOD3. CuZnSOD is encoding by SOD1, MnSOD is encoding by SOD2, and ECSOD is by SOD3. Not only the gene names these SOD1, SOD2 and SOD3 are also used to describe the enzymes (Niizuma, Endo et al. 2009).

CuZnSOD is a homodimer with a molecular weight of 32 kDa, it contains both Cu (II) and Zn (II) at its active sites. MnSOD is a homotetramer with a molecular weight of 88 kDa. MnSOD, having manganese (III) at its active site, is localized in the matrix of the mitochondria, this location is important for removal of O_2• produced by the respiratory chain. ECSOD is a secretory tetrameric Cu/Zn containing glycoprotein, with a molecular weight of

around 135 kDa (Marklund, 1982; Kurobe, Inagaki et al. 1990; Kurobe, Suzuki et al. 1990).

The activity of SOD is very important because of the spontaneous dismutation of superoxide radical is very slow in low concentrations of this anion. It is needed to be catalyzed this reaction by an enzyme, so SOD takes place (Goldstein, Michel et al. 1988). Also SOD is vital for development; SOD1 and SOD2 knock out mice studies indicated that the lack of this enzyme cause death or wide range of pathologies. But SOD3 knock out mice lived without any obvious defects except sensitivity to hyperoxic injury (Li, Huang et al. 1995; Elchuri, Oberley et al. 2005; Sentman, Granstrom et al. 2006) .

SOD is a very important antioxidant enzyme and has a role in oxidative stress defense system in ischemic brain (Sugawara, Fujimura et al. 2004).

1.4. Experimental Models of Brain Ischemia

Although cats, rabbits, dogs and non-human primates have been used for animal experiments on cerebral ischemia most of these experiments were performed with rodents. The fact that the anatomy of the cerebral vasculature doesn't differ grossly between rodents and higher species including humans supports this choice.

In the beginning of the 1980s three models of global ischemia were described which are mainly in use today:

1. The four vessel occlusion model in the rat where the vertebral arteries are permanently occluded and the carotid arteries are clamped transiently for 10 – 30 minutes.

2. The two-vessel occlusion plus hypotension model in the rat which is produced by transient occlusion of both common carotid arteries during 10 – 30 minutes and simultaneous arterial hypotension (ca 50 mm Hg) due to the withdrawal of blood.

3. In gerbils a transient occlusion of both common carotid arteries during 3 minutes is sufficient to induce a global cerebral ischemia as these animals display an incomplete circle of Willis.

There are two other rarely used models of global ischemia in rats. One of these models is interruption of the cardiac output by compression of the major cardiac vessels. The other is done by the elevation of the intracranial pressure close to the mean arterial pressure. Both result global cerebral ischemia (Saito, Maier et al. 2005).

1.5. Scope of the Study

Since global ischemia is unpredictable, and tissue damage following cerebral ischemia results from very complex pathophysiological processes such as excitotoxicity, peri-infarct depolarization, inflammation and apoptosis, all four are potential targets for therapy. Interventions may combine strategies that enhance early reperfusion and neuroprotection. There has been a recent explosion of interest in this field with over 1000 papers and over 400 clinical articles appearing in last 6 years (Ginsberg, 2008). Global brain ischemia has been studied for three decades in defining mechanisms; however there are still many unclarified aspects in this issue. The interaction of oxidative stress parameters and transcription factor expressions are not studied clearly. Ischemia is an unpredicted condition except some cases, so it is very hard to propose a preventive procedure. Nevertheless, most of the studies are concentrated on the underlying mechanisms or therapies and chemicals to recover the situation. In this study our aim is to shed a light on the mechanism of neurodegeneration by investigating the role of transcription factors such as C/EBP and SREBP, and oxidative stress in carotid artery occluded rat brain cortex and cerebellum. To reach our goal the changes in MDA and GSH levels, GST and SOD activities, SREBP and C/EBP expressions in global ischemic rat brain cortex and cerebellum were investigated. We envisaged detecting the interaction between

two different mechanisms during a time course. The time course designed as including 24 hours, 1 week, 2 weeks and 4 weeks of recovery periods. Animals were planned to sacrifice after these time intervals, brains removed and dissected. Cytosolic (S9) and nuclear fractions of each tissue were prepared. Nuclear fractions are important because transcription factor expressions needed to be detected also in the nuclear fractions. Oxidative stress will be emerged by ischemia/reperfusion injury. To create ischemia/reperfusion injury, both bilateral carotid artery occlusion and hypotension applied. Control groups are sham operated.

CHAPTER II

MATERIALS AND METHODS

2.1. Chemicals

Enhanced chemiluminescence kit (ECL-plus) and films were obtained from Amersham International (Buckinghamshire, UK). Butylated hydroxytoluene (BHT), Trizma base, bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB), L-Glutathione reduced (GSH), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), polyoxyethylene-sorbitan monolaurate (Tween-20), sodium dodecyl sulfate (SDS), ammonium persulfate (APS), N,N,N',N'-Tetramethyl-ethylenediamine (TEMED), magnesium chloride ($MgCl_2$), Phenylmethanesulphonylfluoride (PMSF), glycerol, thiobarbituric acid (TBA), 1,2,3-Trihydroxybenzene (Pyrogallol) were purchased from Sigma (St. Louis, USA). Antibody to C/EBPβ and SREBP-1, polyvinylidene difluoride (PVDF) membrane, enhanced chemiluminescence lighting system and protein molecular marker were products of Santa Cruz Biotechnology Inc. (Santa Cruz, USA). DC Protein assay reagents A and B for Lowry protein assay, 30 % acrylamide/bisacrylamide solution 29:1 (3.3 % C) were obtained from Bio-Rad Laboraties (Richmond, USA). Coomassie brilliant blue G-250, dithiothioreitol (DTT), Nonidet P-40 was purchased from AppliChem (Darmstadt, Germany). Ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA) was supplied from Bioworld (Dublin, Ireland). Protease inhibitor cocktail tablets

were obtained from Roche Diagnostics (USA). Potassium dihydrogen phosphate (KH_2PO_4), di potassium hydrogen phosphate (K_2HPO_4), ethylenediaminetetraaceticacid (EDTA), hydrochloric acid (HCl), sodium chloride (NaCl), glycine, 2-mercaptoethanol were supplied from Merck (Darmstadt, Germany). Ethanol and methanol were purchased from Riedel-de Haën (Germany). Trichloro acetic acid (TCA) was obtained from Carlo Erba (Milan, Italy). Ketamine (Alfamine), xylasine (Alfazyne) were supplied from Egevet (Izmir, Turkey). Heparine (Nevparin) was obtained from Mustafa Nevzat (Istanbul, Turkey).

2.2. Experimental Animals

Male Wistar rats (4 - 4.5 months old, 345 ± 45 g weight) were used in the experiments, rat chow and water supplied *ad lib*. Animals were kept under standard colonial conditions (4/5 animal per cage, 20-22 °C, 12 hours day/night cycle). 48 animals were grouped into two, first group being sham operated control (C) group, and the second group was exposed to carotid artery occlusion (CAO). Animals in control and CAO groups were killed by decapitation at different reperfusion/recovery periods: 24 hours (24h C, 24h CAO), 1 week (1w C, 1w CAO), 2 weeks (2w C, 2w CAO), and 4 weeks (4w C, 4w CAO). Each reperfusion/recovery period group contained 6 rats.

Animals were treated according to European Communities Council Directive's (86/609/EEC) preventing animals and experimental usage instructions and all the procedures were approved by Hacettepe University Committee for Ethics in Animal Research (2002/63-5).

2.3. Methods

2.3.1. Carotid Artery Occlusion

Bilateral carotid artery occlusion + hypotension (CAO + ht) was produced by using the method originally described by Smith et al. (1984) (Smith, Auer et al. 1984) with some modifications as previously described by Zhou et al. (2003) (Zhou, Li et al. 2003). Animals were anesthetized with ketamin-xylasine (90/10 mg/kg). A feedback-controlled warming blanket, heating pad and lamps were used above the body to maintain the body temperature throughout the experiment, during surgery and recovery period within the normal range (37.0 – 37.5 °C). The femoral artery was exposed and catheterized with a polyethylene catheter to allow continuous recording of arterial blood pressure. Simultaneously jugular vein catheterized for withdrawal of blood samples in order to create hypotension (ht). Bilateral common carotid arteries were temporarily occluded by arterial clips, after exposure in the neck, and blood was gradually withdrawn from the jugular vein into a heparinized syringe to reduce the mean arterial blood pressure (MABP) to 30 – 35 (from 45 – 50) mm Hg for 10 min. After 10 minutes of ischemia, the arterial clips were removed; the withdrawn warmed shed blood was reinfused (Fig 2.1) into the jugular vein to restore normotension. The wounds were sutured, and the animals placed in the cage until they woke up. Full physiological monitoring was continued for 3 hours into the postischemic period. Sham-operated rats received similar operative preparation but were not subjected to carotid artery occlusions or blood withdrawal.

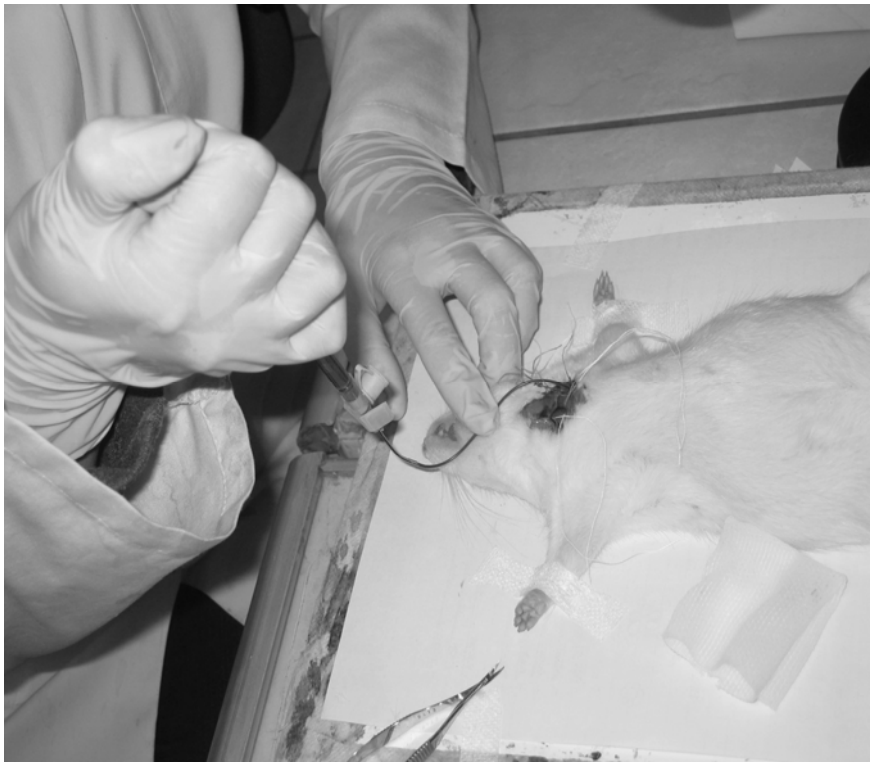

Figure 2.1: A photograph during two vessel carotid artery occlusion operation at the blood reinfusion stage.

2.3.2. Dissection of Brain and Homogenization of Tissues

Animals were sacrificed by decapitation at designated time intervals (24 hours, 1, 2 and 4 weeks). To open the skull, a cut was made starting from foramen magnum ends at the frontal bone with a sterile surgical scissors. The bones of the skull were grabbed up by surgical forceps. Then brain removed and washed out in physiological saline to get rid of the blood. And whole brain was placed on ice for dissection. Cerebral cortices and cerebella were separated with a surgical spatula, then tissues placed in liquid nitrogen and stored at - 80 $^{\circ}$C.

Tissues were homogenized by using Potter-Elvehjem teflon-glass tissue homogenizer connected to a drill at speed of 2500 rpm with 2 – 3 passes in 3 volumes of ice-cold homogenization buffer per 1 gram of tissue. The

homogenization buffer is 50 mM phosphate buffer pH 7.4, containing 1 mM EDTA, 5 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF, and 1:25 (v/v) protease inhibitor cocktail. All the procedures were done in cold room (+ 4 °C).

Homogenates were centrifuged at 1000 × *g* for 10 minutes at + 4 °C, then 100 µl supernatant were removed and 1.4 µl of 40 mM BHT added to this aliquot for TBARS assay. Then the rest of the supernatant were transferred to another 1.5 ml micro-centrifuge tube. The supernatant from 1000 × *g* was centrifuged at 14000 × *g* for 20 minutes at + 4 °C. The supernatant collected from 14000 × *g* is crude cytosolic extract (S9). S9 was removed and stored at – 80 °C in aliquots for GST activity, total SOD activity, total thiol group determinations, protein concentration determinations by Lowry assay and Western blots.

Pellet from the 1000 × *g* were used to prepare the nuclear extracts. For cortex 310 µl or for cerebellum 250 µl buffer (50 mM Tris-HCl buffer pH 7.5, containing 10 % (v/v) glycerol, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 % (w/v) Nonidet P-40, 1 mM DTT, and 1:25 (v/v) protease inhibitor cocktail) added to each pellet. Then the mixture was incubated and vortexed at every 10 minute for an hour in cold room (+ 4 °C). After incubation; mixture was centrifuged at 14000 × *g* for 20 minutes at + 4 °C. Supernatant (nuclear extract) was removed and stored in aliquots at – 80 °C for protein concentration determinations by Bradford assay and Western blotting experiments.

2.3.3. Protein Concentration Determination

Determination of protein concentration in crude cytosolic extracts, was performed Lowry (Lowry, Rosebrough et al. 1951) method. BioRad DC protein assay kit was used for protein determination and measurements were at ELISA plate reader (Bio-Tek ELx800). Lowry method depends on the reactions between proteins and copper in an alkaline medium which reduces the Folin reagent and forms a detectable color.

Standards prepared by dissolving of crystalline bovine serum albumin (BSA) in dH_2O. Standard concentrations were 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 1.0, 1.2, and 1.4 mg/ml protein.

5 µl of standards, dH_2O as blank, or sample was added to each micro plate well as triplicates. Then 25 µl of reagent A and 200 µl of reagent B were added. Mixture incubated 30 minutes at room temperature and plate was placed in reader, gently mixed for 5 seconds by reader. Absorbance of standards and samples were measured against blank at 630 nm. Protein concentrations in S9 fractions were calculated using the standard curve obtained from BSA (Appendix A).

Determination of protein concentration in nuclear extracts were performed by Bradford method (Bradford 1976). 5 × Stock Bradford reagent was prepared by dissolving 0.1 g Coomassie brilliant blue G-250 in 50 ml of 99 % ethanol, and then completing the volume to 200 ml with phosphoric acid.

The same standard set of Lowry assay (0.1 – 1.4 mg/ml BSA) were used for Bradford assay. 5 µl of standard, sample or dH_2O for blank and 250 µl of Bradford reagent were added per well. All the standards and samples were studied as triplicates. Measurements were done against blank at 630 nm with ELISA micro plate reader (Bio-Tek ELx800). Protein concentrations in nuclear extracts were calculated using BSA standard curve (Appendix B).

2.3.4. Determination of Total GST Activity

GST enzyme activity was determined spectrophotometrically by monitoring the thio-ether formation at 340 nm using CDNB as substrate basically according to the method of Habig (Habig, Pabst et al. 1974) as modified previously (Boyoglu, 2004; Oztetik, 2005). The procedure was adapted previously for ELISA plate reader by Yilmaz (Yilmaz, 2006).

Each reaction mixture contained 100 mM potassium phosphate buffer, pH, 7.4 containing 1 mM GSH, 1 mM CDNB and crude cytosolic extract in

final volume of 250 µl in 96 well plate. The reaction was started by the addition of sample into each well. The plate was placed into ELISA plate reader (Bio-Tek ELx808) at room temperature. After machine mixing, measurement was started automatically and measurement done at every 20 seconds for 10 minutes. The blank wells contained all the constituents except S9 enzyme source. Instead S9, phosphate buffer was added to complete the volume to 250 µl.

Slopes of the best lines drown for each well separately by the software of the instrument was used as the rate of reaction (dA/dt) and the further calculations were completed. GST specific activity results were given as the amount of thioether (nmol) formed by 1 mg total protein of sample in one minute of reaction. Extinction coefficient (ε) was 7.20 mM^{-1} with a corrected path length of 0.70 cm according to the volume of reaction mixture in plate well (Perera, Hemingway et al. 2008).

$$SA = \frac{dA/dt}{\varepsilon\,(mM^{-1})} \times \frac{0.250\ ml}{1000\ ml} \times \frac{1000}{12.5} \times DF \times \frac{1}{mg\ prot\ /\ ml} = (nmoles \times min^{-1} \times mg\ prot^{-1})$$

2.3.5. Determination of Total SOD Activity

Determination of total SOD activity depends on the inhibition of autoxidation of pyrogallol. Atmospheric oxygen provides the autoxidation of pyrogallol in alkaline solution which produces superoxide radical. As superoxide builds in the solution, the formation of yellow chromophore of oxidized pyrogallol accelerates because superoxide also reacts with pyrogallol. SOD neutralizes this radical and therefore slows down the pyrogallol oxidation and inhibit color formation (Marklund and Marklund, 1974).

As reagents; Tris-EDTA buffer pH, 8.2 containing 50 mM Tris, 10 mM EDTA, for an alkaline medium, and 15 mM pyrogallol dissolved in dH_2O (light sensitive, freshly prepared) were used.

Total SOD enzyme activity was modified for ELISA plate reader. Sample wells were added, in the order, 228 µl Tris-EDTA buffer, 8 µl of pyrogallol and 4 µl of sample (same protein concentration for each well). For each blank well, 232 µl Tris-EDTA buffer were added to replace sample. Then kinetic determinations were recorded for 3 minutes at 415 nm in ELISA plate reader (Bio-Tek ELx808). Slopes (ΔOD/min) of kinetic readings were determined, and slopes of samples were subtracted from the slopes of blanks (ΔΔOD/min) to express the degree of inhibition of pyrogallol autoxidation. Then the group averages were calculated and the results were expressed in percentages.

2.3.6. Total Thiol Group Determination

Cytosolic total thiol amount of each sample was determined by the method defined by Sedlak and Lindsay (Sedlak and Lindsay, 1968) which adapted to ELISA plate reader. This method is based on the reduction of DTNB by sulfhydryl groups, to produce a characteristic yellow color which gives its maximum absorbance at 412 nm. Each free –SH group reduces the DTNB into 1 molecule of 2-nitro-5-mercaptobenzoic acid that creates the detectable yellow color.

As reagents: 200 mM Tris buffer pH, 8.2 containing, 20 mM EDTA; 10 mM freshly prepared DTNB; 0.1, 0.2, 0.5, 0.75 and 1 mM GSH standards and methanol were used for this assay.

20 µl diluted rat brain cortical cytosolic crude extracts or GSH standards were added to each 1.5 ml micro-centrifuge tube, for blank tubes Tris-EDTA buffer added instead of sample or standard. Then 60 µl of Tris-EDTA buffer, 40 µl of DTNB, and 280 µl of methanol were added to tubes. Reaction mixtures were incubated at room temperature in dark for 30 minutes, and then centrifuged at 3000 × g for 15 minutes. 200 µl of supernatant were taken and

read at 415 nm with ELISA plate reader (Bio-Tek ELx808). All the samples, standards or blanks were run as triplicates.

Total thiol concentrations of samples were calculated using the calibration curve obtained from GSH standards. Results were expressed as μmole GSH per mg protein.

2.3.7. Determination of TBARS Concentration

TBARS assay (Madhava Rao and Sresty, 2000) was modified for ELISA plate reader. Basically, the method depends on the colored complex formed from the reaction of TBA with MDA. This colored complex gives an absorbance at 532 nm.

20 % (w/v) TCA and 0.5 % (w/v) TBA were the reaction mixture, both dissolved in dH_2O. 40 mM BHT was added previously to 1000 × g supernatants of samples to prevent artificial increase of MDA by suppressing autoxidation of lipids until experiments were done.

100 μl sample and 400 μl TBA + TCA mixture were added to 1.5 ml micro-centrifuge tubes. Then tubes were left incubation in boiling water for 30 minutes. Then samples were centrifuged at 10000 × *g* during 15 minutes at + 4 °C, and then supernatants were removed to 96 micro plate wells as duplicates. 150 μl of supernatant was removed by avoiding unprecipitated particles. TBA + TCA mixture were used as blanks. All the readings were done at 532 nm and 600 nm. All the samples and blanks were run as duplicates

TBARS concentrations were calculated by subtracting absorbencies of the samples at 600 nm from 532 nm. And the difference obtained from subtraction divided to 155 mM^{-1} was the extinction coefficient (ε) of colored complex. The results were expressed as mM/ml.

2.3.8. Western Blotting

SDS-PAGE: Polyacrylamide slab gel electrophoresis, in the presence of the anionic detergent sodium dodecyl sulfate (SDS), was performed on 4 % stacking gel and 10 % separating gel in a discontinuous buffer system (Laemmli, 1970). Fermentas prestained protein ladder or Cruz marker was used as molecular weight standards. Stacking buffer is 500 mM Tris-HCl, pH, 6.8, separating buffer is 1500 mM Tris-HCl, pH, 8.8, and 30 % Acrylamide/Bisarylamide solution 29:1 (3.3 % C) were obtained from Bio-Rad, 20 % SDS solution, 10 % APS were prepared, and TEMED used for SDS-PAGE. Vertical slab gel electrophoresis was carried out using the EC120 Mini Vertical Gel System (E-C Apparatus Corp. NY, USA) that can be used to run two gels simultaneously. The dimensions of the glass are 8.3 × 7.4 cm. Aliquots from the protein samples to be analyzed were diluted 5:1 with 6 × sample buffer (5 parts sample, 1 part sample buffer). Sample buffer is commercially available. Then the samples were placed in boiling water bath for 5 minutes. Afterwards equal amounts of protein per lane (100 μg for C/EBPβ, 200 μg for SREBP-1) were loaded onto a 10% polyacrylamide gel and separated by electrophoresis at 50 V for 45 minutes until passing the stacking gel, then 100 V for 2 hours. 5 × stock running buffer is; 25 mM Tris buffer pH, 8.3 contain 192 mM Glycine.

Transfer of proteins: Transfer was carried out using EC140 Mini Blot Module of the EC120 Mini Vertical Gel System (E-C Apparatus Corp., NY, USA), and Polyvinylidene difluoride (PVDF) was used as a blotting membrane. PVDF membranes were incubated with methanol for 2 minutes to overcome the hydrophobicity of the membrane. Proteins on gels obtained from the SDS-PAGE were directly transferred to PVDF membrane (Santa Cruz, Biorad) in the presence of transfer buffer in transfer apparatus at 60 V for 60 minutes. Transfer buffer contains 25 mM Tris, 192 mM glycine and 20 % methanol.

Immunostaining: 10 × stock Tris-buffered saline contains 100 mM Tris-HCl, pH 7.6 – 8.0, 1500 mM NaCl. Then 0.1 % Tween-20 added 1 × TBS to

obtain TBST. Membranes were blocked with 5 % nonfat dry milk dissolved in TBST. The PVDF membranes were then incubated with target antibodies at room temperature: polyclonal C/EBPβ antibody diluted 1:5000 in TBST containing 5 % nonfat dry milk and 1 % BSA, SREBP-1 antibody diluted 1:2000 in TBST containing 5 % nonfat dry milk. Bound antibodies were detected using a goat-anti rabbit Ig (Santa Cruz). *a. enhanced chemiluminescence method*: The reaction has been developed with an enhanced chemiluminescence lighting system (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Immunoblots were exposed to film (Kodak, Amersham) 30 seconds for C/EBP and 45 minutes for SREBP at room temperature. *b. NBT/BCIP method*: In NBT/BCIP method also same incubations were done for antibodies. During the secondary antibody incubation period, the streptavidin - biotinylated AP complex was prepared by the addition of streptavidin to biotinylated AP (both 1/7,500 diluted in TBST) and allowed to stand at least 1 hour and not more than 3 hours at room temperature. After the incubation with secondary antibody, the membrane was washed again with TBST (five times, each 5 min) and then incubated for 1-2 hours in the previously prepared streptavidin - biotinylated AP complex. Afterwards, the membrane was washed three times, 5 min each, again with TBST and the AP color developing solution (BCIP/NBT) was added. The specific protein bands started to appear after 10 - 30 min. Finally, the membranes were carefully dried.

For both techniques the images were obtained using a scanner connected to the computer. And the bands were analyzed by "Scion image" image analyzer.

2.3.9. Statistical Analysis

Values were expressed as mean ± SEM and were obtained from six independent animals. Statistical analyses were carried out by one way ANOVA

and statistically significant at $p < 0.05$. Also data acquired from control and operated subjects were assessed by Student t-test with Prism program.

CHAPTER III

RESULTS

3.1. Effects of CAO on SREBP Expression in Cortex and Cerebellum

The effects of carotid artery occlusion (CAO) on SREBP-1 active form (42 – 47 kDa) expression in the rat brain cortex and cerebellum parts were examined by western blotting in two subcellular fractions, namely cytosolic (S9) and nuclear fractions. The fractions were prepared as described in "Materials and Methods". Protein determinations were done and same amount of protein were applied to each well. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard in cytosolic samples. SREBP-1 and GAPDH antibody stained bands were observed (as shown in Appendices) and quantified with Scion image program. The results were expressed as fold differences in comparison to their control groups after normalizing with respect to internal standard of each group.

As shown in Fig. 3.1 and Table 3.1, there was a statistically ($p < 0.05$) significant increase (2.07 fold) in the expression of SREBP-1 active (42 – 47 kDa) form for 1 week CAO group compared to 1 week control group in cortex S9 fractions. There was also an increase in SREBP-1 expressions for 24 hours group (1.30 fold), however it wasn't statistically significant. After 1 week SREBP-1 expressions tend to decrease back to control levels, although remains

higher than controls (Fig 3.1 and Appendix C). Similar results were obtained from rat brain cortex nuclear extracts, except an insignificant (0.81 fold) decrease in SREBP-1 levels was observed at 24 hours (Fig 3.2 and Table 3.2) (Appendix D). Nevertheless, in the nuclear fractions of cortex, the statistically significant ($p < 0.05$) increase (1.41 fold) was observed in the expression of SREBP-1 in 1 week CAO group when compared to its control. Similar to S9 fractions of cortex, after 1 week, SREBP-1 levels decreased back to control levels.

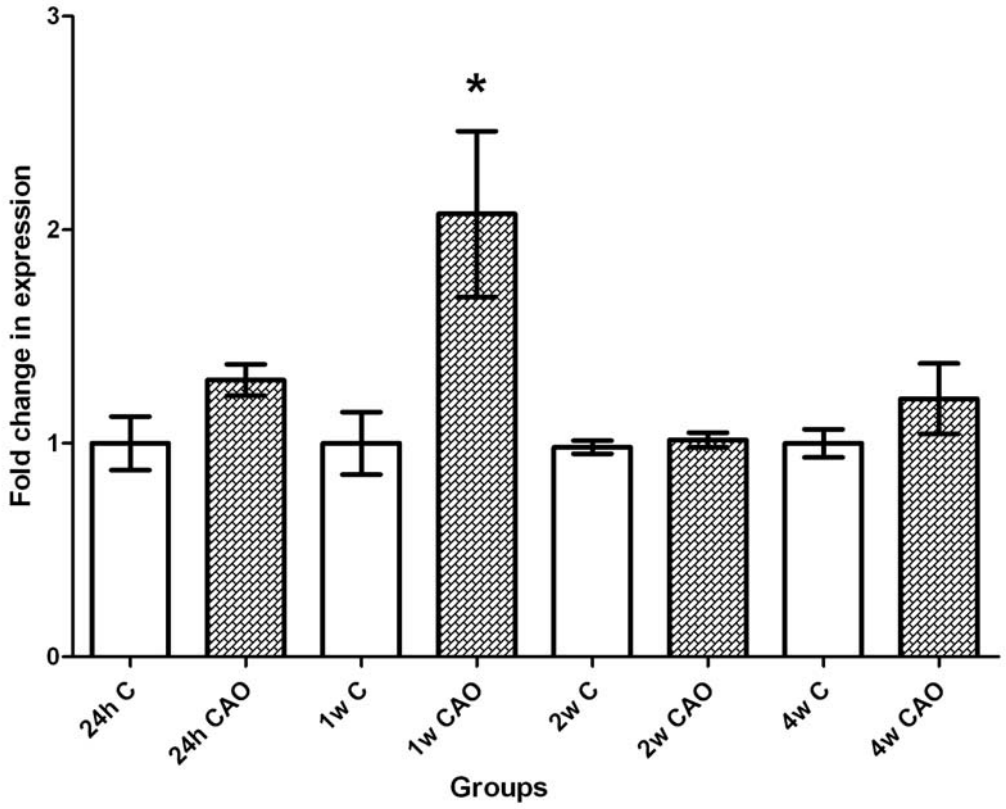

Figure 3.1: Variations in expression of SREBP-1 of rat brain cortex as determined by western blotting in S9 fractions prepared separately from rat brains at different recovery periods. Each bar represents average of 6 rat brains.
* Significantly different ($p < 0.05$) than 1w C group (Student t-test)

Table 3.1: Fold change in SREBP-1 expressions in rat brain cortex S9 fraction

	24h C	24h CAO	1w C	1w CAO	2w C	2w CAO	4w C	4w CAO
Mean	1.00	1.30	1.00	2.07	1.00	1.02	1.00	1.21
± SD	0.31	0.18	0.36	0.95	0.07	0.08	0.16	0.40

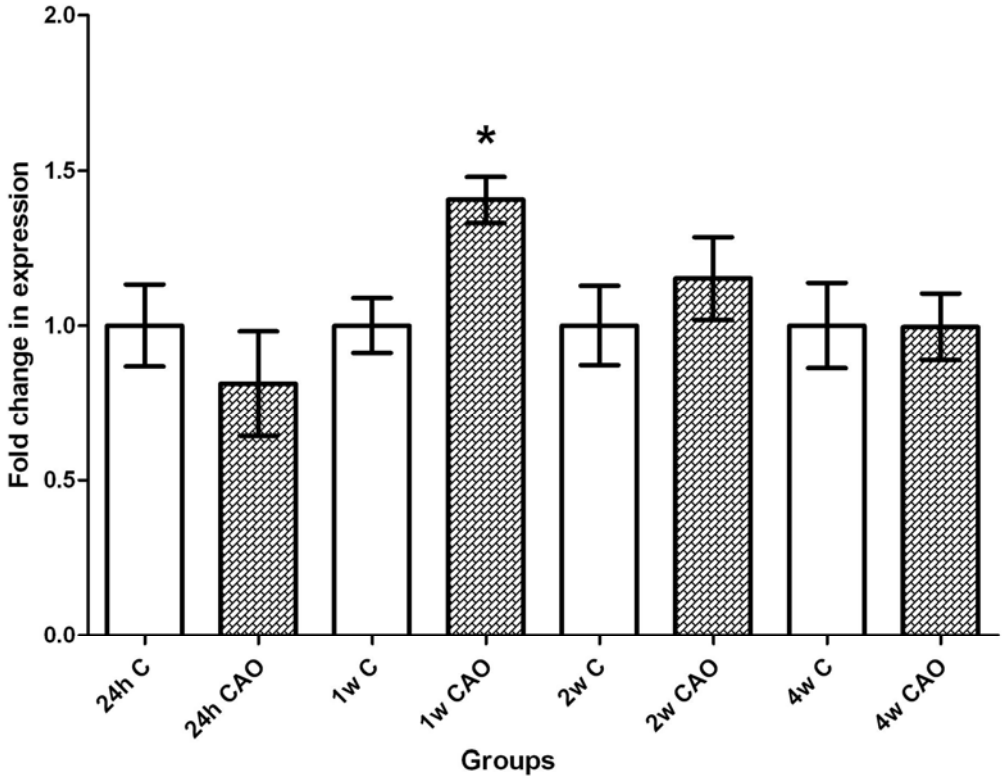

Figure 3.2: Variations in expression of SREBP-1 of rat brain cortex as determined by western blotting in nuclear fractions prepared separately from rat brains at different recovery periods. Each bar represents average of 6 rat brains.
* Significantly different ($p < 0.05$) than 1w C group (Student t-test)

Table 3.2: Fold change in SREBP-1 expression in rat brain cortex nuclear fraction

	24h C	24h CAO	1w C	1w CAO	2w C	2w CAO	4w C	4w CAO
Mean	1.00	0.81	1.00	1.41	1.00	1.15	1.00	1.00
± SD	0.32	0.41	0.21	0.18	0.31	0.32	0.33	0.26

In cerebellum S9 fractions, SREBP-1 active (42 – 47 kDa) form expressions was significantly ($p < 0.005$) increased 2.15 fold only in 1 week CAO group when compared to its control. Nearly there were not any alterations at other time intervals CAO and control groups (Fig 3.3 and Table 3.3) (Appendix E).

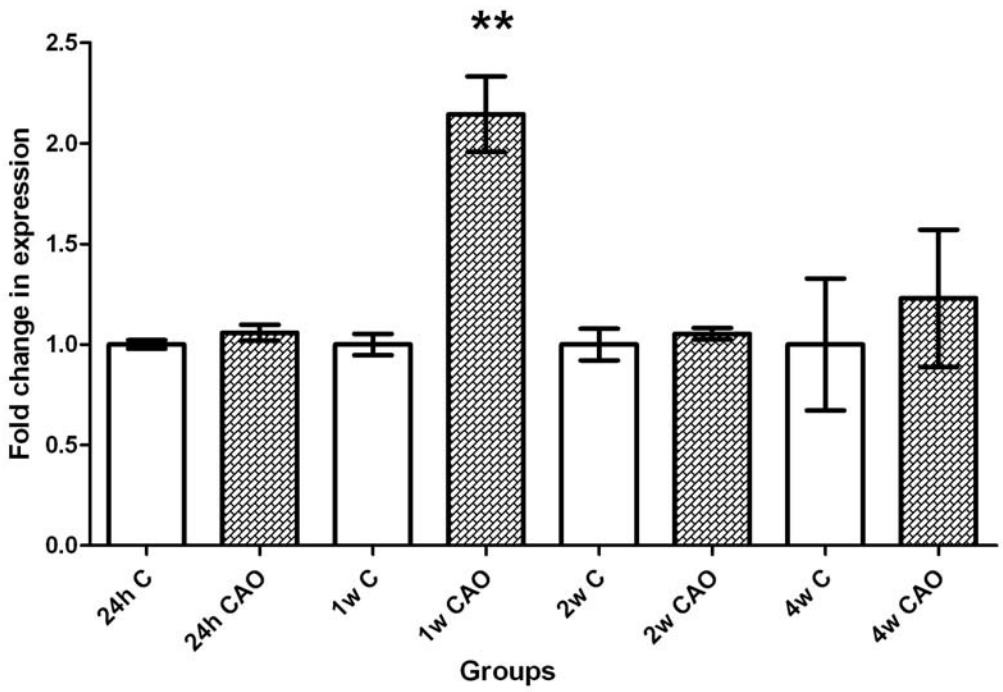

Figure 3.3: Variations in expression of SREBP-1 of rat cerebellum as determined by western blotting in S9 fractions prepared separately from rat brains at different recovery periods. Each bar represents average of 6 rat brains. ** Significantly different ($p < 0.005$) than 1w C group (Student t-test)

Table 3.3: Fold change in SREBP-1 expression in rat cerebellum S9 fraction

	24h C	24h CAO	1w C	1w CAO	2w C	2w CAO	4w C	4w CAO
Mean	1.00	1.06	1.00	2.15	1.00	1.05	1.00	1.23
± SD	0.05	0.10	0.13	0.46	0.19	0.07	0.80	0.84

There was a statistically significant ($p < 0.05$) increase of 1.79 fold in the expression level of active (42 – 47 kDa) form of SREBP-1 measured from nuclear fractions of cerebellum, only in 1 week CAO group when compared to its control (Fig. 3.4 and Table 3.4) (Appendix F). The expression levels of SREBP-1 remained almost similar to control levels in all the other reperfusion CAO groups (Fig 3.4 and Table 3.4) (Appendix F).

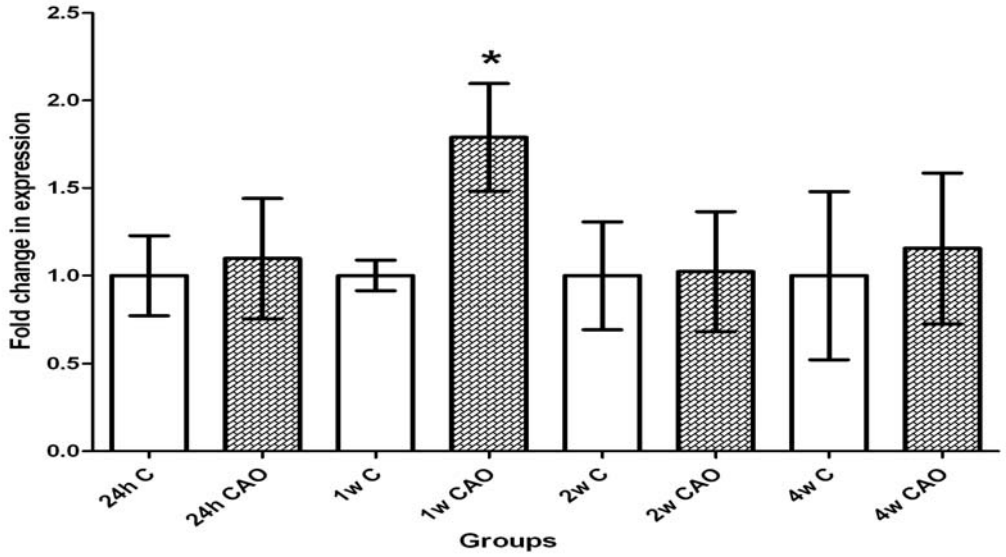

Figure 3.4: Variations in expression of SREBP-1 of rat cerebellum as determined by western blotting in nuclear fractions prepared separately from rat brains at different recovery periods. Each bar represents average of 6 rat brains.
* Significantly different ($p < 0.05$) than 1 week of Control (Student t-test)

Table 3.4: Fold change in SREBP-1 expression in rat cerebellum nuclear fraction

	24h C	24h CAO	1w C	1w CAO	2w C	2w CAO	4w C	4w CAO
Mean	1.00	1.10	1.00	1.79	1.00	1.02	1.00	1.16
± SD	0.56	0.84	0.21	0.75	0.75	0.84	0.87	0.90

3.2. Effects of CAO on C/EBPβ Expression in Cortex and Cerebellum

The effects of carotid artery occlusion on C/EBPβ (LAP* active form, ~55 kDa) expression in the rat brain cortex and cerebellum parts were examined by Western blot similar to SREBP-1, in two subcellular fractions, as cytosolic (S9) and nuclear fractions. C/EBPβ (C-19), an affinity purified rabbit polyclonal antibody, reacts with C/EBPβ of mouse, rat and human origin as examined by Western blotting, and is non cross-reactive with C/EBPα, δ, or ε as stated by producer company. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard in cytosolic samples. C/EBPβ and GAPDH antibody stained bands were observed (as shown in Appendices) and quantified with Scion image program. The results were expressed as fold differences in comparison to their control groups after normalizing with respect to internal standard of each group.

For rat brain cortex in S9 fraction, there was an increase in C/EBPβ expressions after CAO application at each recovery period. However, these increases were not significant for 2 and 4 weeks of reperfusion. The expressions of active form of C/EBPβ were significantly increased 1.19 fold and 1.58 fold after 24 hours and 1 week of reperfusion, respectively (Fig 3.5 and Table 3.5) (Appendix G). The expressions of active form of C/EBPβ in nuclear fractions of

cortex were significantly increased 1.73 fold and 1.81 fold after 24 hours and 1 week of reperfusion, respectively (Fig 3.6 and Table 3.6) (Appendix H).

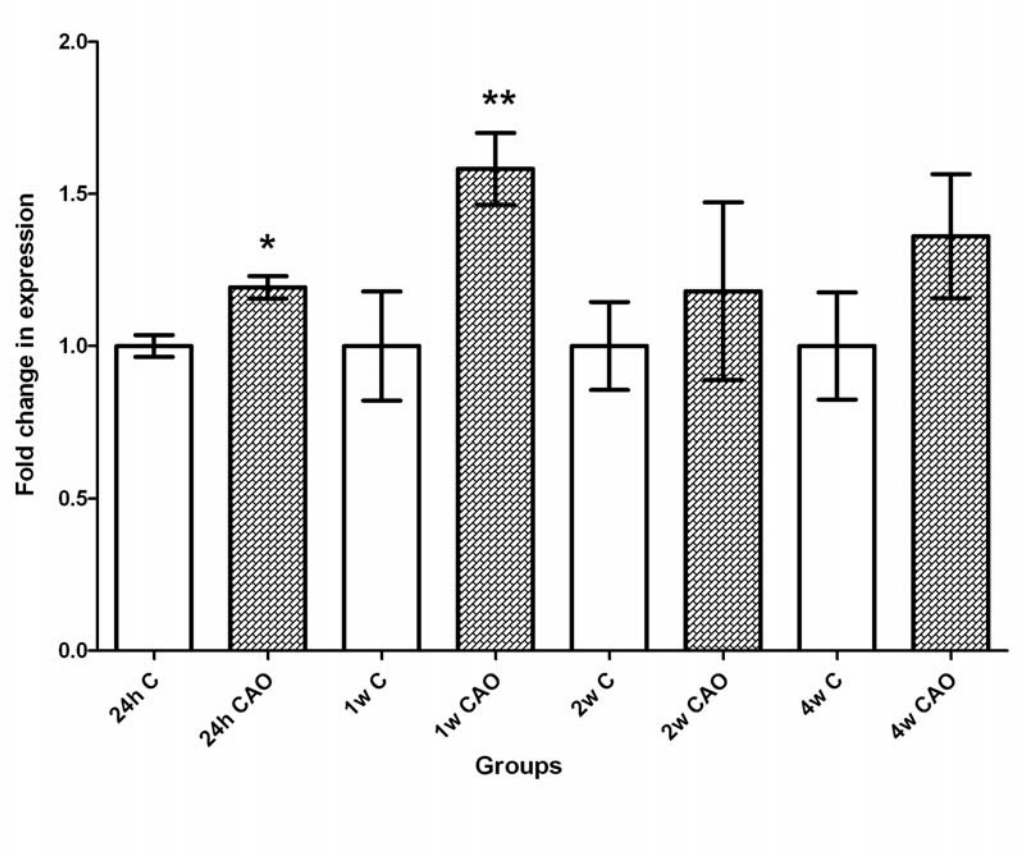

Figure 3.5: Variations in expression of C/EBPβ of rat brain cortex as determined by western blotting in S9 fractions prepared separately from rat brains at different recovery periods. Each bar represents average of 6 rat brains.
* Significantly different ($p < 0.005$) than 24h C group (Student t-test)
** Significantly different ($p < 0.05$) than 1w C group (Student t-test)

Table 3.5: Fold change in C/EBPβ expression in rat brain cortex S9 fraction

	24h C	24h CAO	1w C	1w CAO	2w C	2w CAO	4w C	4w CAO
Mean	1.00	1.19	1.00	1.58	1.00	1.18	1.00	1.36
± SD	0.09	0.09	0.44	0.29	0.35	0.71	0.43	0.50

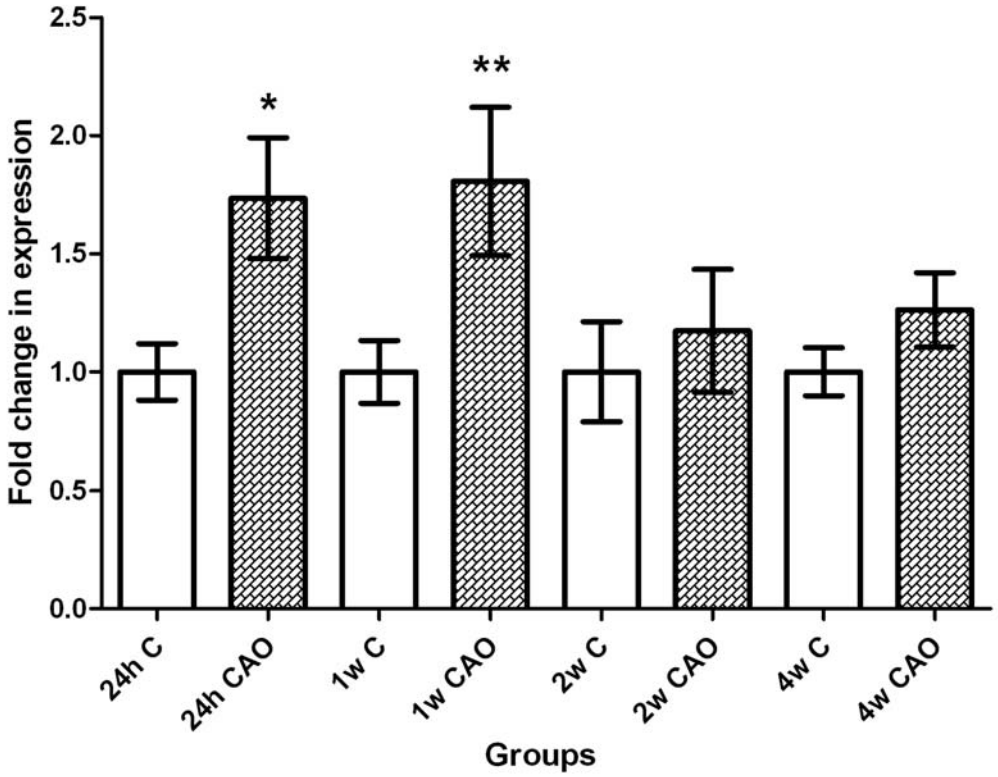

Figure 3.6: Variations in expression of C/EBPβ of rat brain cortex as determined by western blotting in nuclear fractions prepared separately from rat brains at different recovery periods. Each bar represents average of 6 rat brains.
* Significantly different ($p < 0.05$) than 24h C group (Student t-test)
** Significantly different ($p < 0.05$) than 1w C group (Student t-test)

Table 3.6: Fold change in C/EBPβ expression in rat brain cortex nuclear fraction

	24h C	24h CAO	1w C	1w CAO	2w C	2w CAO	4w C	4w CAO
Mean	1.00	1.73	1.00	1.81	1.00	1.17	1.00	1.26
± SD	0.29	0.63	0.32	0.77	0.52	0.64	0.25	0.39

For cerebellum, C/EBPβ active form expressions demonstrated a different profile when compared to cortex both in S9 and nuclear fractions. In 1 week CAO group only, there were a statistically significant increase of 1.63 fold for S9 fractions, and 1.35 fold for nuclear fractions when compared to their controls. C/EBPβ expressions of nuclear and cytosolic fractions prepared from cerebellum decreased insignificantly at all the other reperfusion time points with respect to their controls (Figs 3.7 and 3.8, Tables 3.7 and 3.8) (Appendix I and J).

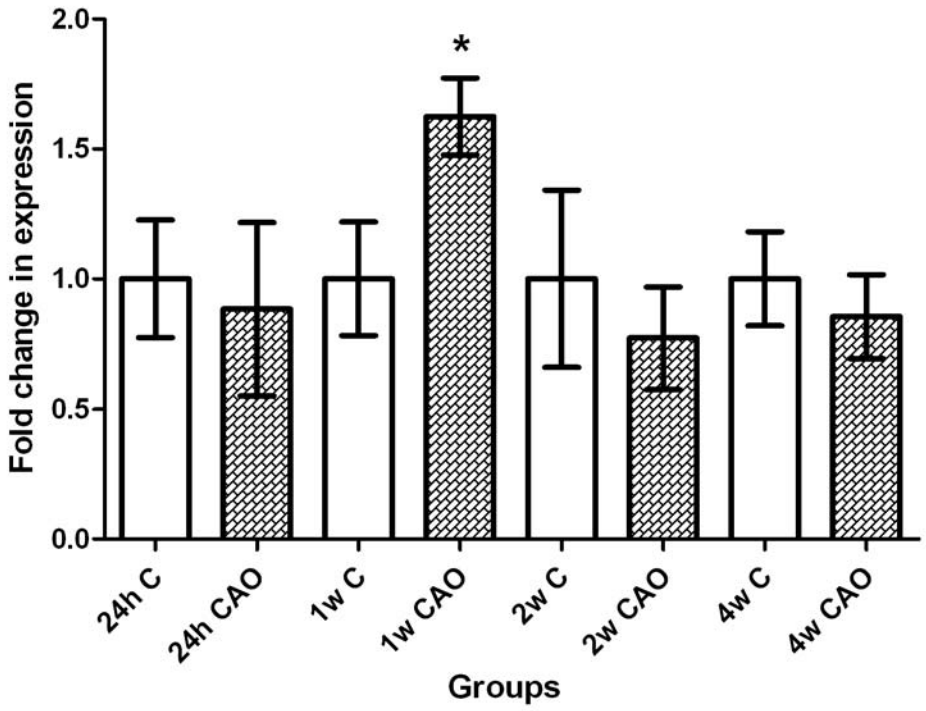

Figure 3.7: Variations in expression of C/EBPβ of rat cerebellum as determined by western blotting in S9 fractions prepared separately from rat brains at different recovery periods. Each bar represents average of 6 rat brains.
* Significantly different ($p < 0.05$) than 1w C group (Student t-test)

Table 3.7: Fold change in C/EBPβ expression in rat cerebellum S9 fraction

	24h C	24h CAO	1w C	1w CAO	2w C	2w CAO	4w C	4w CAO
Mean	1.00	0.88	1.00	1.63	1.00	0.77	1.00	0.85
± SD	0.55	0.81	0.54	0.36	0.83	0.48	0.44	0.40

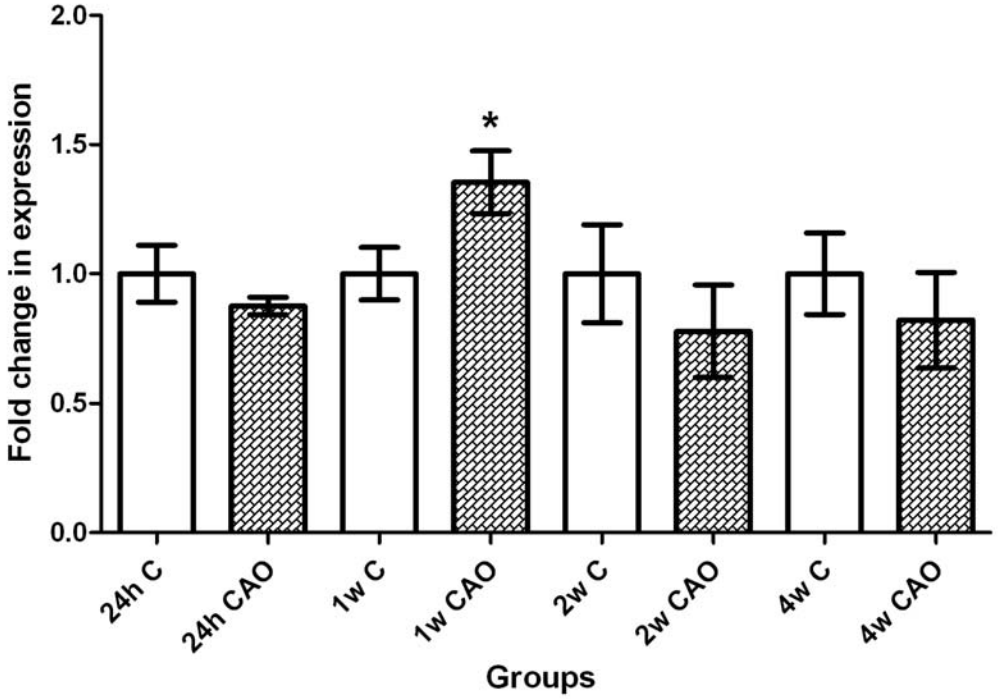

Figure 3.8: Variations in expression of C/EBPβ of rat cerebellum as determined by western blotting in nuclear fractions prepared separately from rat brains at different recovery periods. Each bar represents average of 6 rat brains.
* Significantly different ($p < 0.05$) than 1w C group (Student t-test)

Table 3.8: Fold change in C/EBPβ expression in rat cerebellum nuclear fraction

	24h C	24h CAO	1w C	1w CAO	2w C	2w CAO	4w C	4w CAO
Mean	1.00	0.87	1.00	1.35	1.00	0.78	1.00	0.82
± SD	0.27	0.08	0.25	0.30	0.46	0.44	0.39	0.45

The effects of CAO on transcription factors SREBP-1 and C/EBPβ in rat brain cortex and cerebellum were summarized in Table 3.9. Only the statistically significant effects were shown in the table.

Table 3.9: Summary of the significant changes observed in the expressions of transcription factors during reperfusion time course.

	24 hours	1 week	2weeks	4weeks
C/EBPβ Cortex Cytosolic	Increase	Increase	-	-
C/EBPβ Cortex Nuclear	Increase	Increase	-	-
C/EBPβ Cerebellum Cytosolic	-	Increase	-	-
C/EBPβ Cerebellum Nuclear	-	Increase	-	-
SREBP-1 Cortex Cytosolic	-	Increase	-	-
SREBP-1 Cortex Nuclear	-	Increase	-	-
SREBP-1Cerebellum Cytosolic	-	Increase	-	-
SREBP-1 Cerebellum Nuclear	-	Increase	-	-

3.3. Effects of CAO on Lipid Peroxidation in Rat Brain Cortex

Effects of CAO on lipid peroxidation in rat brain were examined in crude cytosolic extracts of brain cortex by TBARS assay. As seen in Figure 3.9, lipid peroxidation significantly ($p < 0.005$) increased (43.0 %) in 24 hours of reperfusion and decreased back significantly ($p < 0.05$) to the control levels after 1 week and levels were stayed stable during 2 – 4 weeks of reperfusion when compared to 24 hours (Fig 3.9).

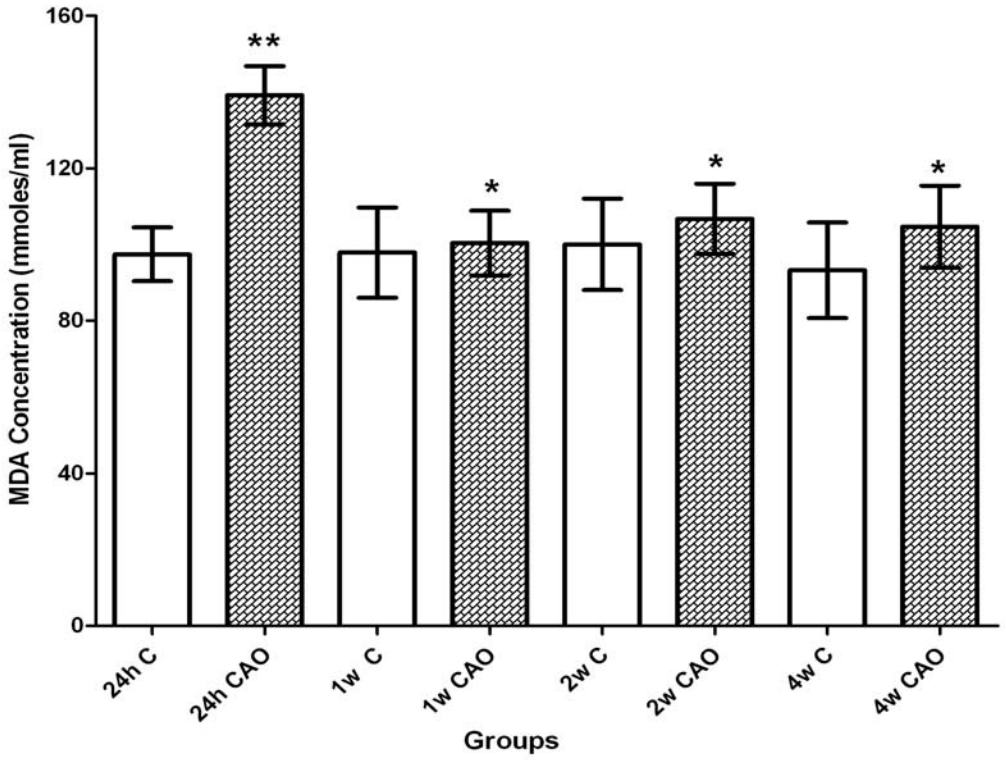

Figure 3.9: Comparison of TBARS Levels in CAO and sham operated control rat brain cortex crude cytosolic extracts prepared separately from 6 individuals per group.
* Significantly different ($p < 0.05$) than 24 hours CAO (ANOVA)
** Significantly different ($p < 0.005$) than 24 hours Control (ANOVA)

3.4. Effects of CAO on Total Superoxide Dismutase (SOD) Activity in Rat Brain Cortex

Effects of CAO on total superoxide dismutase (SOD) activity was determined in S9 fraction prepared from rat brain cortex by measuring the inhibition of autoxidation of pyrogallol. There was a significant ($p < 0.005$) increase in SOD (47.3 %) in 24h CAO group in comparison to that of control as seen in Fig. 3.10). There was not any significant alteration between CAO and control groups for 1 – 2 and 4 weeks of reperfusion periods (Fig 3.10).

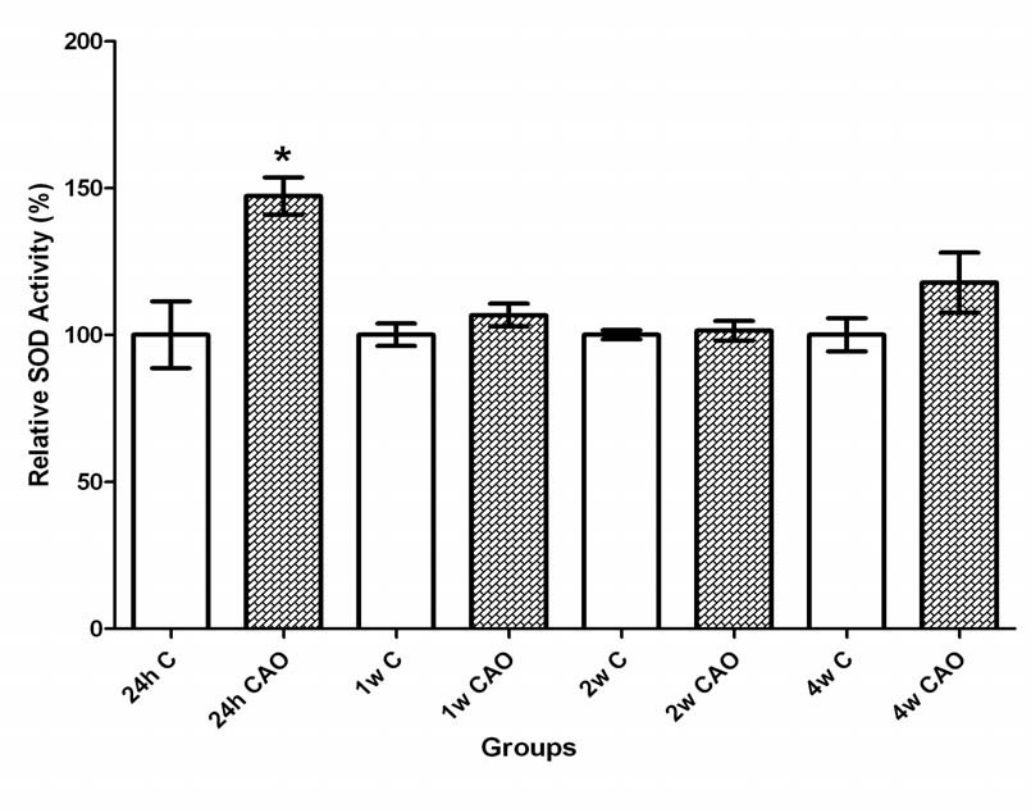

Figure 3.10: The relative SOD activity values for rat brain cortical S9 fraction obtained from 6 individuals per group. Each bar represents the average SOD activity of six rats determined in triplicates.
* Significantly different ($p < 0.005$) then 24h C (ANOVA)

3.5. Effects of CAO on Total Glutathione S-Transferase (GST) Activity in Rat Brain Cortex

Effects of CAO on total GST activity measured using CDNB as substrate was determined in rat brain cortex S9 fraction. There was not any significant alteration of total GST activity between CAO groups and sham operated control groups. Results are shown in Fig 3.11.

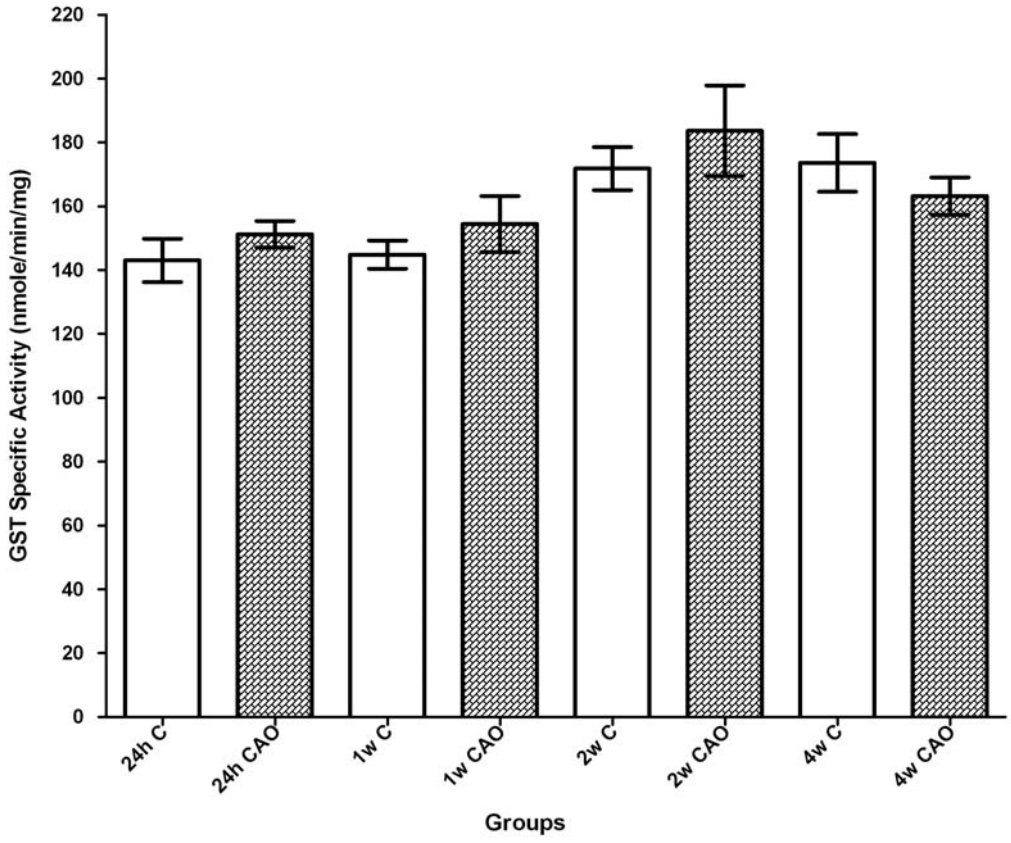

Figure 3.11: The average specific activity values for GSTs measured against the common substrate CDNB of rat brain cortical S9 fractions obtained individually from 6 rats per group. Each bar represents the average GST activity of six rats determined in triplicates. (ANOVA)

3.6. Effects of CAO on Total Thiol Group Levels in Rat Brain Cortex

Effects of CAO on total thiol group levels of rat brain cortex was determined in S9 fraction prepared as explained in Materials and Methods. There was not any significant difference in total thiol levels between CAO and sham operated control groups (Fig 3.12).

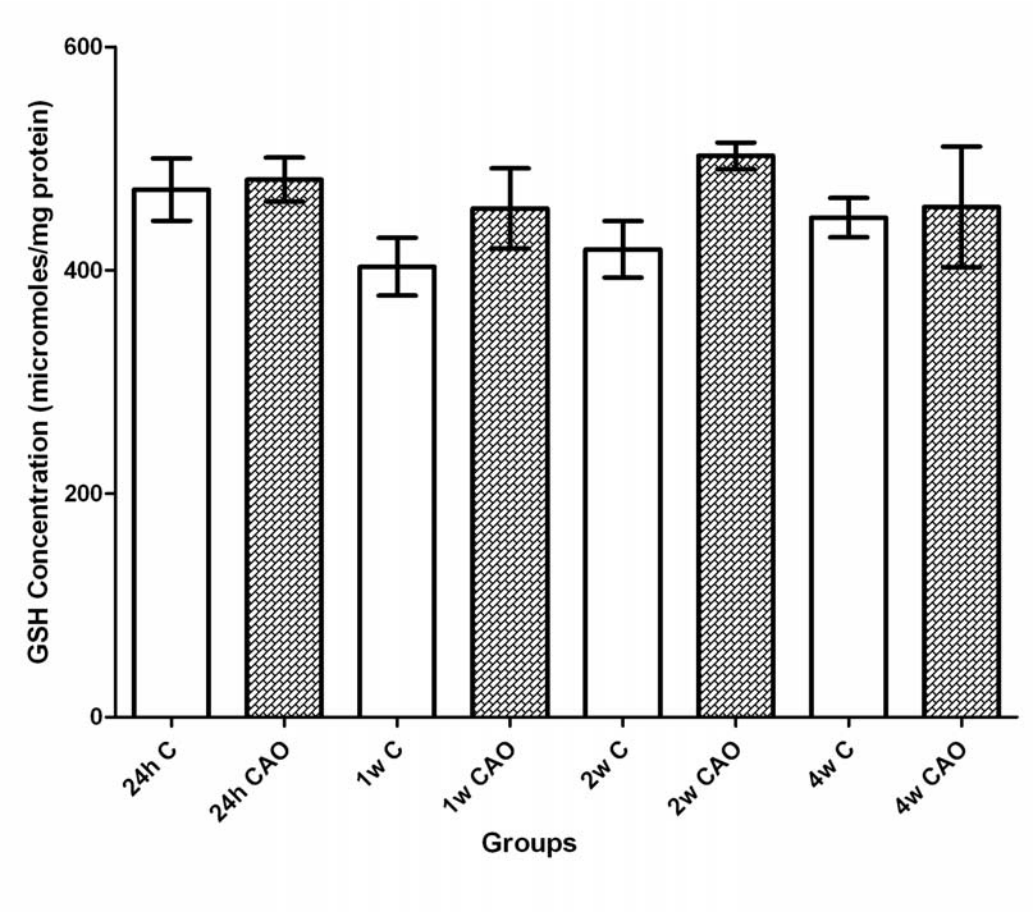

Figure 3.12: The average total thiol amount values for rat brain cortical S9 fraction obtained from 6 individuals per group. Each bar represents the average GSH amount of six rats determined in triplicates. (ANOVA)

CHAPTER IV

DISCUSSION

The mechanism of degeneration as a consequence of ischemia and reperfusion has not been clarified yet. The two of the so far proposed mechanistic causes of damage on brain cells involve the transcriptional regulation and oxidative stress. Therefore, the possible interactions between these two mechanisms are needed to be studied.

In this study, two-vessel occlusion + hypotension model was applied on rats for 10 minutes to mimic stroke or cardiac arrest effects in brain (Saito, Maier et al. 2005). The alterations in SREBP, C/EBP transcription factors and oxidative stress related molecules (SOD, GST, MDA, GSH) caused by global brain ischemia in time course after reperfusion (24 hours to 4 weeks) were examined in rat brain cortex and cerebellum at both cytosolic and nuclear fractions.

The C/EBP family is composed of basic leucine zipper DNA-binding proteins (C/EBPs α,β,δ,ε,ζ) that recognize a common DNA-binding sequence (Williams, Cantwell et al. 1991) and that are expressed in virtually all tissues, including the developing and adult brain (Sterneck and Johnson, 1998). The C/EBPs can either transactivate or repress gene expression, and within the nervous system are known to regulate neurogenesis during development (Menard, Hein et al. 2002) and neuronal plasticity in the adult brain (Alberini, Ghirardi et al. 1994). A number of intriguing findings suggest that this family of transcription factors might be involved in neuronal regeneration. First, the

C/EBPs are well-known mediators of injury and inflammation responses in other tissues (Lekstrom-Himes and Xanthopoulos, 1998) and potentially in the brain (Soga, Yamanaka et al. 2003; Cortes-Canteli, Wagner et al. 2004), where they act to couple numerous extrinsic cellular cues to the nucleus.

In the present study, the levels of crude cytosolic and nuclear C/EBPβ protein was significantly increased after transient global ischemia/reperfusion and hypotension experiments in the rat cortex after 24 hours to 1 week. After 1 week, the level of transcription factor, decreased although not significantly. However, in the cerebellum C/EBP amount was not increased at 24 hours, a delayed increase was observed at 1 week. This tissue specific difference may be caused by the cellular differences of these tissues. Besides, cortex may be more adaptive to the cellular death mechanisms than cerebellum. Cerebellum's response is similar to hippocampus which is more vulnerable than the others (Richards, Fiskum et al. 2007). These results are consistent with previously published studies that demonstrated an increased expression of C/EBP in ischemic injury (Kapadia, Tureyen et al. 2006). In the previous studies, C/EBP family protein levels during reperfusion were investigated after a single episode of sublethal forebrain ischemia in the gerbil hippocampus to determine their expression after ischemia and correlation with neuronal cell death. It has been shown that C/EBPβ expression increased 1 hour, 6 – 24 hours after brain ischemia in rat cerebrum (Buttner, Cordes et al. 2009). Also parallel results obtained for hippocampus. These results appear to correlate with neuronal degeneration and may serve as a signal that neurons are progressing to cell death and DNA fragmentation (Soga, Yamanaka et al. 2003). It has also been reported that focal cerebral ischemia induced by tMCAO significantly increases C/EBPβ gene expression in the mouse brain after 6 – 72 hours of reperfusion. To understand the functional significance of C/EBPβ in postischemic inflammation and brain damage, tMCAO was induced in cohorts of adult C/EBPβ null mice and their wild-type litter-mates. At 3 days of reperfusion following tMCAO, C/EBPβ null mice showed significantly smaller infarcts, reduced neurological deficits, decreased extravasated neutrophils and fewer

activated microglia/macrophages compared with their wild-type litter-mates. In the same study, it was also shown that postischemic induction of many transcripts known to promote inflammation and neuronal damage was less pronounced in the brains of C/EBPβ-/- mice compared with C/EBPβ+/+ mice (Kapadia, Tureyen et al. 2006).

SREBP has an essential role in sterol, lipid and cholesterol metabolism in cell (Kelicen and Nordberg, 2006). The importance of SREBP is increasing in neurodegeneration. Since in the nerve cells lipid and cholesterol metabolisms altered during neurodegeneration. Hence this transcription factor has a relation between sterol mechanism and neurodegenerative enzymes which are sterol dependent. One of the most important sterol metabolism enzymes is aromatase which plays an important role in neuroprotection. SREBP-1 levels in human neuroblastoma cells are enhanced under stress conditions (Kelicen and Nordberg, 2006).

In the literature it was shown that the SREBP-1c RNA levels weren't changed by middle carotid artery occlusion in mice brain after 8 hours, also there was a decrease but not significant when compared to control (Morales, Ballesteros et al. 2008). SREBP-1c expressed dominantly is under control of insulin, glucose and fatty acids not cholesterol. Whereas SREBP-1a activates both pathways of insulin-lipid and cholesterol (Camargo, Smit et al. 2009). In our study an insignificant decrease in SREBP-1 expression was also observed in cortex nuclear fraction in 24 hours. However in 1week SREBP-1 expressions were increased significantly in all our CAO groups. The SREBP-1 antibody used in our studies recognizes not only SREBP-1c but also SREBP-1a which activates both pathways insulin-lipid and cholesterol. Therefore the changed detected in SREBP-1 expression might be correlated with sterol, lipid and cholesterol metabolism.

In many studies it was shown that oxidative stress is tightly involved in reperfusion injury and inflammation caused by excitotoxicity (Saito, Maier et al. 2005). There are some biomarkers as MDA and GSH levels, SOD and GST activities to detect the oxidative stress.

TBARS assay which is still valuable for determination of lipid peroxidation was used (Del Rio, Stewart et al. 2005) to detect the oxidative stress in brain cortex caused by CAO/Reperfusion . After 24 hours of ischemia there was a 43 % increase in lipid peroxidation when compared to control, this increase settled back to the normal range for later time intervals. This shows oxidative stress causes the peroxidation of lipid which is vital for neurons. Total SOD activity is directly bound to the oxidative stress defense mechanisms in the cell. There is also a significant increase in the total SOD activity after 24 hours of reperfusion, which is then decreased to the control groups' levels for weekly periods. The increase in the SOD activity demonstrates that the ROS levels were increased, as a defense system SOD activity increased in parallel. SOD activity shows the increase in the oxidative stress also as a defense mechanism. Also it was shown in a study that SOD was active after 1 day in a preconditioned rat cortex (Danielisova, Nemethova et al. 2005). In GSH levels there was not any significant alteration at each time interval. Our study is not directly related to xenobiotics, a possible comment to explain these findings; may be extensive use of GSH against oxidative stress at an earlier stage than 24 hours. Accordingly there was not any significant alteration in GST activity. Since GST is the enzyme which utilize GSH for its activity as a cofactor, depletion of GSH at early stages of reperfusion may be responsible from these results. On the contrary, in another study it was shown that there was an increase after 24 hours of reperfusion period (Kumari Naga, Panigrahi et al. 2007).

Finally our results support the speculations proposed by some other researchers in the literature, so that there may be different mechanisms in the cell working consecutively; the oxidative stress related enzymes or molecules takes place at an early stage of the reperfusion. However expression of transcription factors generally takes place at a later stage (Block 1999; Dirnagl, Iadecola et al. 1999). In the literature, the effects of CAO on SREBP-1 have not been examined before. This work presents pioneering results in this issue. However, further investigation is necessary to decipher the role of SREBP-1 in neurodegeneration.

CHAPTER V

CONCLUSION

- It has demonstrated that 24 hours and 1 week reperfusion period after 10 minutes global brain ischemia dramatically increased expression levels of active form of C/EBPβ in the rat cortex.
- There observed an increase in the C/EBPβ expression in cerebellum both cytosolic and nuclear fractions in 1 week.
- Significant increase has been detected in the SREBP-1 expression for both cerebellum and cortex in nuclear and cytosolic fractions in 1 week.
- SOD and TBARS levels were enhanced in the ischemic rat brain cortex in 24 hours of reperfusion. Whereas GST and GSH levels were unchanged.

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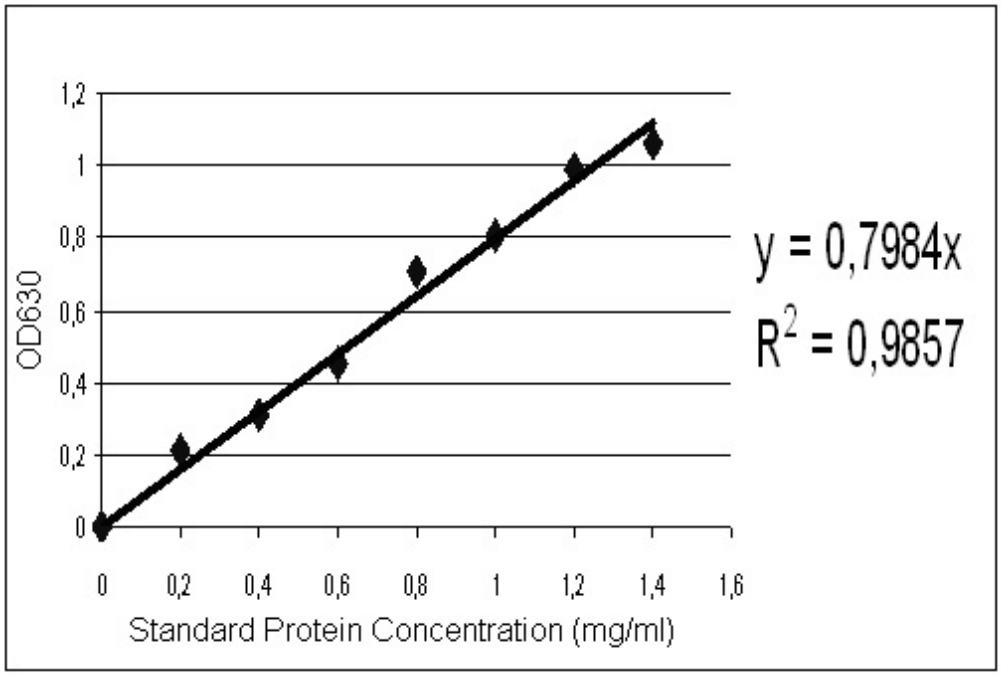
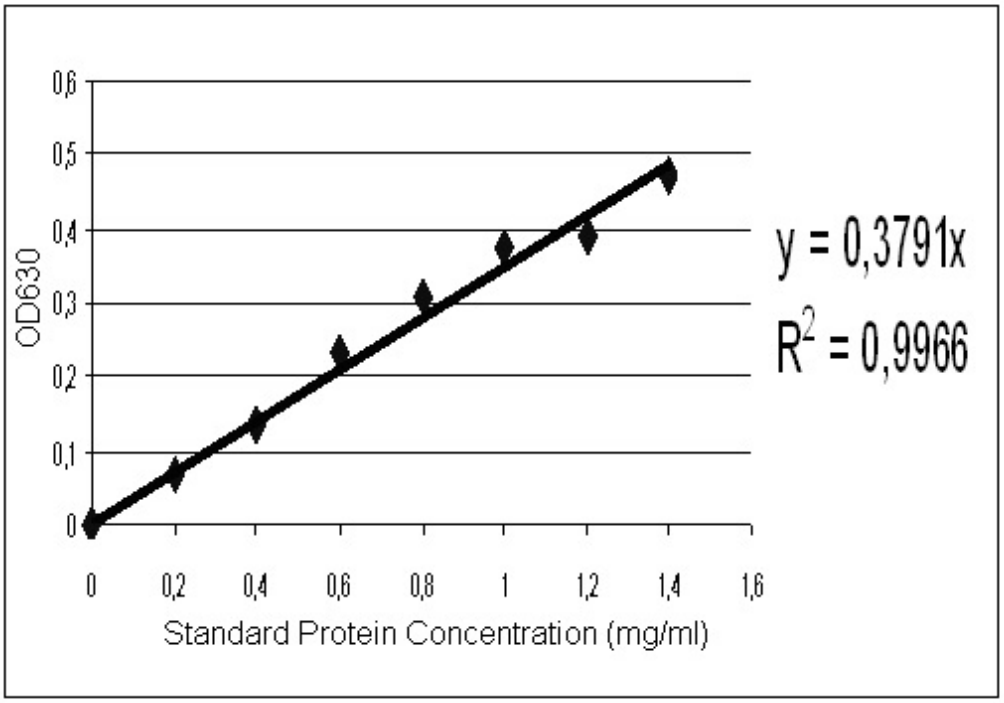
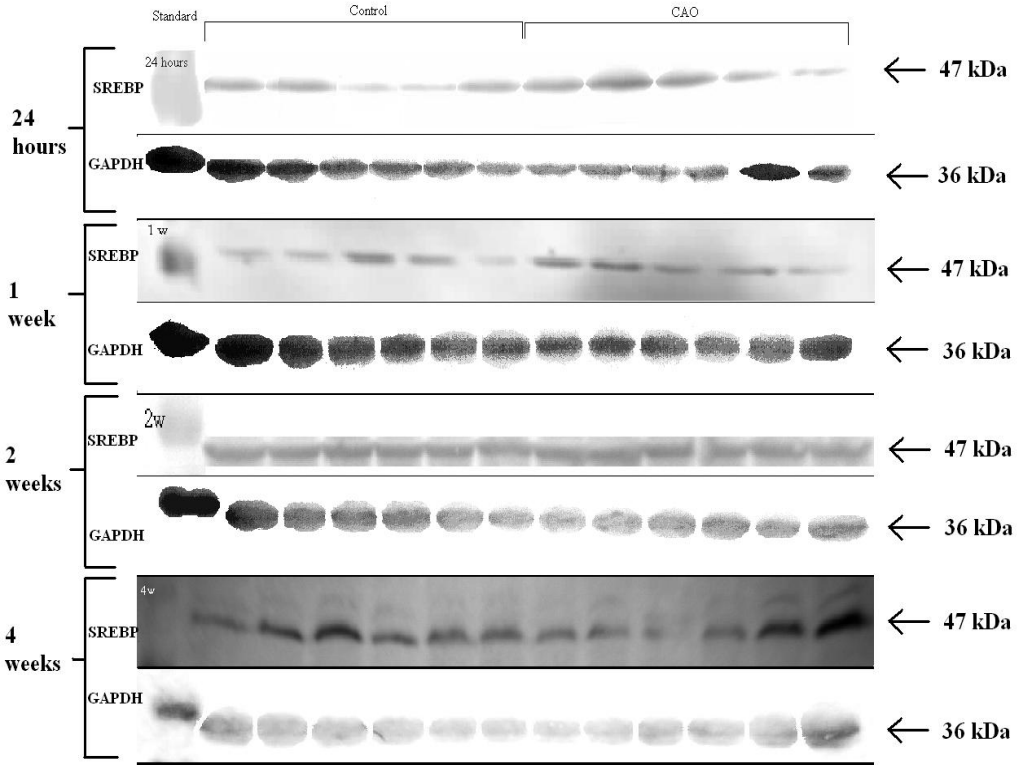
APPENDIX A. Lowry Assay Standard Curve



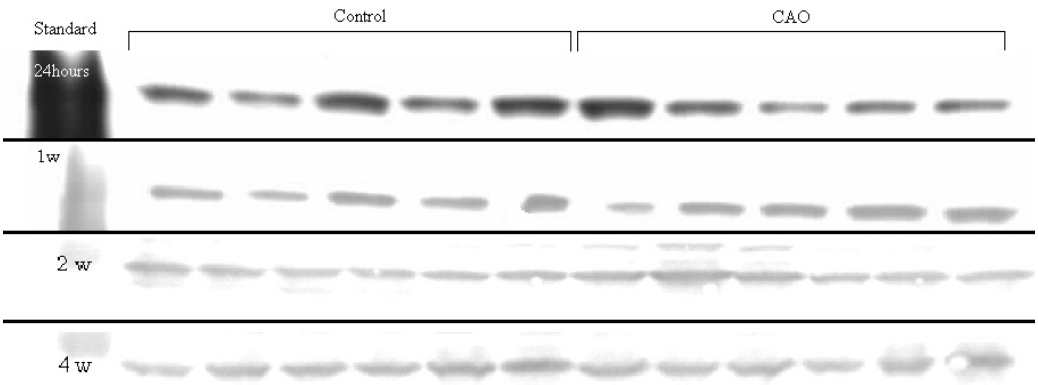
APPENDIX B. Bradford Assay Standard Curve



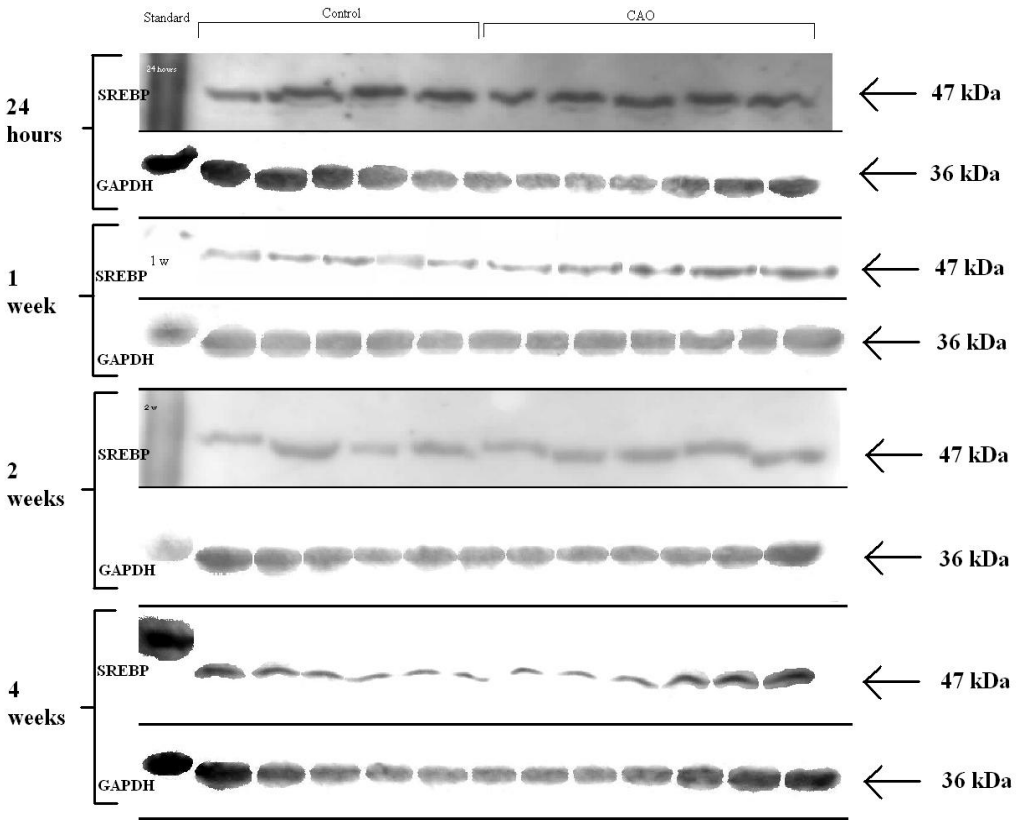
APPENDIX C. SREBP-1 and GAPDH Expression in Cortex S9 Fraction



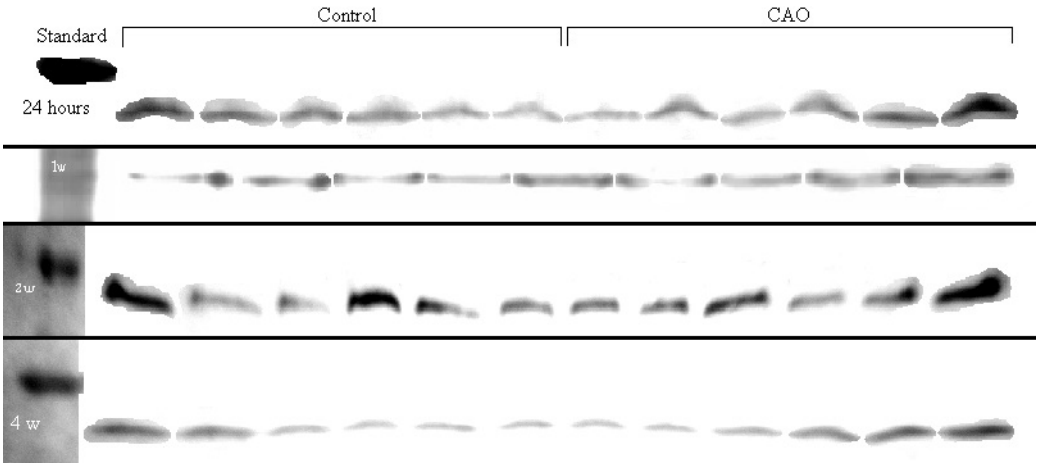
APPENDIX D. SREBP-1 Expression in Cortex Nuclear Fraction



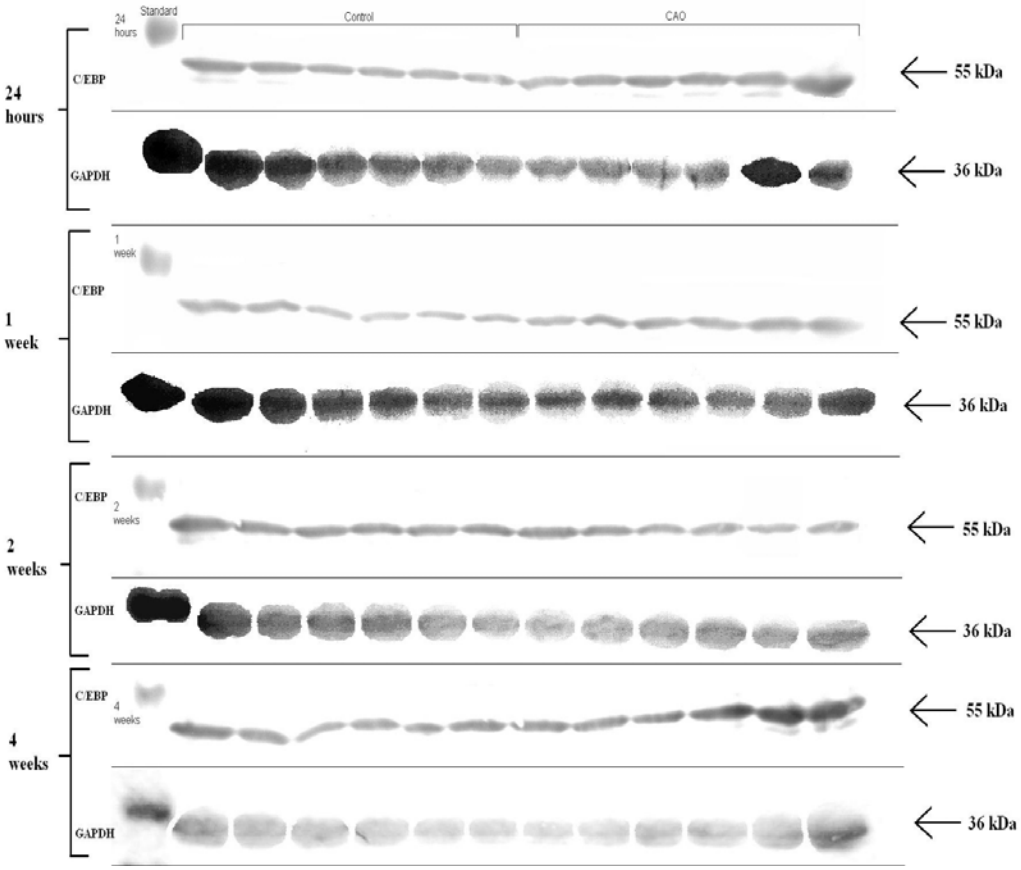
APPENDIX E. SREBP-1 and GAPDH Expression in Cerebellum S9 Fraction



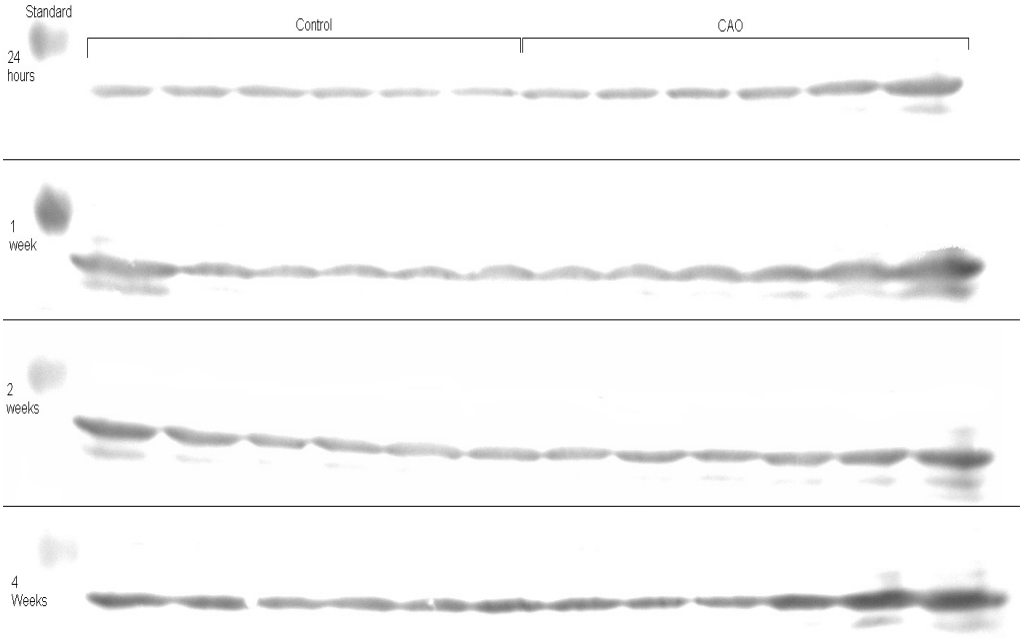
APPENDIX F. SREBP-1 Expression in Cerebellum Nuclear Fraction



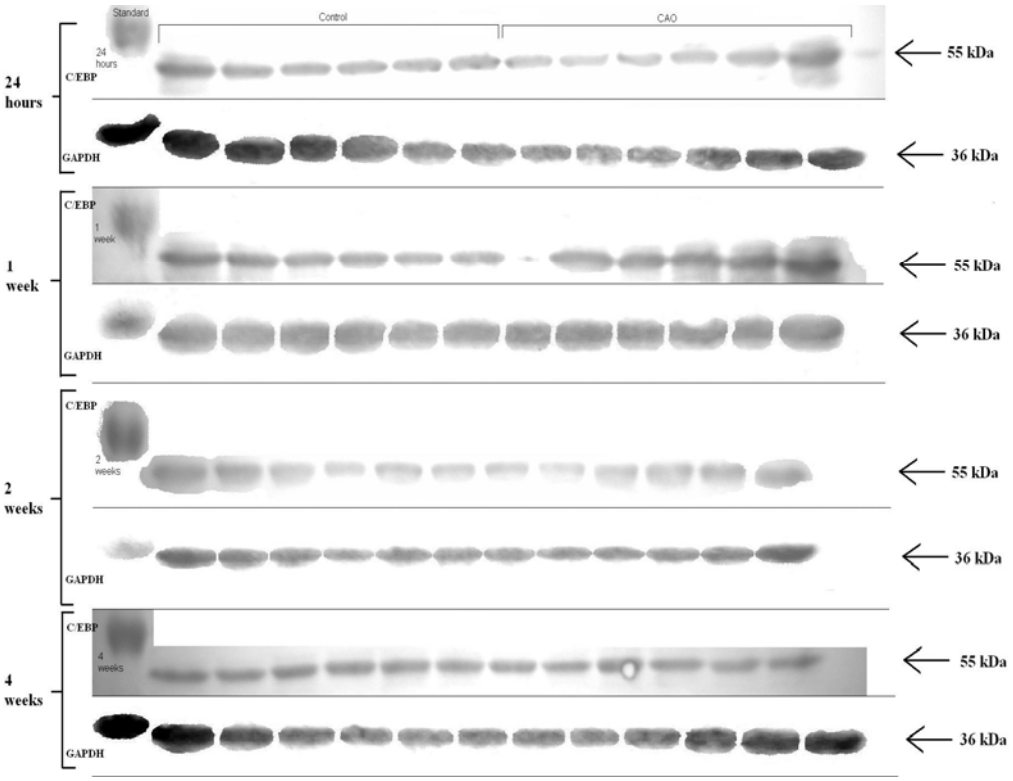
APPENDIX G. C/EBPβ and GAPDH Expression in Cortex S9 Fraction



APPENDIX H. C/EBPβ Expression in Cortex Nuclear Fraction



APPENDIX I. C/EBPβ and GAPDH Expression in Cerebellum S9 Fraction



APPENDIX J. C/EBPβ Expression in Cerebellum Nuclear Fraction