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Reactive oxygen species (ROS) are natural byproducts of normal cellular reactions. They are oxygen ions, free (non)radicals, and peroxides that are highly reactive with normal macromolecules, such as lipids, DNA, and proteins. Cells are normally able to defend against the damages of ROS via enzymes that neutralize them into water. However, when cells are not able to cope with the accumulation of ROS, disruptions in signaling pathways and gene transcription will occur, which will ultimately lead to cell death. It is now widely accepted that increased oxidative stress-induced damage in the brain is a major cause of neurodegenerative diseases, such as Alzheimer's disease (AD). Nuclear factor kappa-B (NFκB) is not only a ubiquitously expressed transcription factor but also a signaling protein that is activated by ROS-induced oxidative stress. Our laboratory has demonstrated the neuroprotective effects of 17β-estradiol (E2) are elicited via an anti-oxidant effect. The purpose of this project was to determine the role of NFκB activation in E2-mediated neuroprotection against hydrogen peroxide (H_2O_2)-induced oxidative stress. HT-22, a murine immortalized hippocampal neuronal cell line, was utilized to determine whether NFκB is activated by hydrogen peroxide-induced oxidative stress and whether E2 suppresses H_2O_2-induced NFκB activation. We observed that H_2O_2 activated NFκB by phosphorylation of IκBα (pIκBα), one of the NFκB inhibitor proteins, reduction of total IκBα, and

induction of NFκB (p65) nuclear translocation. In contrast, E2 suppressed H_2O_2-induced NFκB activation by dramatic reducing pIκBα, increasing total IκBα, and inhibiting p65 nuclear translocation. Our results show that one of the mechanisms by which estrogens are neuroprotective against oxidative stress is through the attenuation of H_2O_2-induced NFκB activation.

17BETA-ESTRADIOL SUPPRESSES HYDROGEN PEROXIDE-INDUCED NUCLEAR FACTOR KAPPA B ACTIVATION IN HT22 CELLS

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

CHAPTER I

INTRODUCTION TO THE STUDY

A. Oxidative Stress and its Role in Neurodegenerative Diseases

What is the metabolic function of oxygen molecules? How do oxygen molecules make energy for aerobic organisms? Many scientists worked on these questions between 1930's and 1960's. Finally, they found the critical function of oxygen molecules during the process of citrate cycle in the cytoplasm and during the respiration in the mitochondria (Halliwell, 2006). Thus, the utilization of oxygen molecules is deemed essential for all aerobic organisms. Further, Harman proposed the "free radical theory of aging" in the 1950's (Harman, 1956). According to this proposal, the pathogenesis of neurodegenerative diseases and aging was attributed to free radicals produced by various cell components, which was consistent with Pearl's hypothesis of the "rate of living" introduced in 1928 (Pearl, 1928). He suggested the direct relationship between the rate of metabolism and the longevity of an individual organism. However, these hypotheses were not taken seriously by the scientific community until the discovery of superoxide dismutase (SOD), an enzymatic antioxidant, in 1969 (Balaban et al., 2005). Since discovery of this enzyme, extensive evidence has demonstrated that cells produce free radicals during oxidative phosphorylation in the mitochondria. It is now widely accepted that free radical-induced oxidative stress is involved not only in neurodegenerative diseases and non-

neurodegenerative diseases, but also in normal aging (Halliwell, 2006, Coyle and Puttfarcken, 1993).

Most of ROS are made from mitochondria, which have several potential targets. ROS affect mitochondrial DNA exacerbating oxidative stress through a "vicious cycle", directly damage macromolecules such as DNA, RNA, proteins, and fatty acids, activate cytosolic stress signaling pathway, and control the expression levelsof oxidative stress-inducible genes by activation transcription regulators (Balaban et al., 2005, Storz et al., 1990).

The reactive oxygen species (ROS) are generated from the plasma membrane, peroxisomes, and cytosolic compartments (Balaban et al., 2005). However, the majority of ROS are produced from the mitochondria during oxidative phosphorylation (Balaban et al., 2005). During the oxidative phosphorylation, the electrons from NADH, FADH2 are transferred to series of electron carriers, such as coenzyme Q and cytochrome c, which may directly react with an oxygen molecule and produce active oxygen molecules, such as singlet oxygens or superoxides (Halliwell, 2006). Hydrogen peroxide (H_2O_2) itself is one of the nonradical ROS and also produces very reactive hydroxyl radicals, which contribute to oxidative stress (Halliwell, 2006). Moreover, superoxide may react with nitric oxide generating highly reactive nitrogen species (RNS) called peroxynitrite (Halliwell, 2006). Both of these reactive species (RS), ROS and RNS, are highly reactive so that they may further oxidize target molecules through its chain reactions (Halliwell, 2006). As a result of RS-induced oxidative stress, macromolecules, such as lipid, protein, and DNA/RNA, are damaged, which may lead

to the disruption of normal cellular function and ultimately to cell death (Coyle and Puttfarcken, 1993). Therefore, it is paramount for the brain as well as other organs to cope with excessive RS generation, which is the basis for antioxidant treatments (Balaban et al., 2005). The problem that the scientific community still faces is a fundamental question of the mechanism of RS generation as well as interventions that will lead to prevention or handling of RS generation.

However, we have several defense systems against ROS-induced oxidative stress, such as detoxifying enzymes and uncoupling proteins which decreases ROS generation by reducing mitochondrial membrane potential (Halliwell, 2006, Balaban et al., 2005, Zimmerman et al., 1989, St-Pierre et al., 2006). When the RS generation is excessive to the capacity of the cellular defense systems, the RS-induced oxidative stress will be initiated and eventually damages normal cellular functions (Zimmerman et al., 1989). Therefore, cells need to keep balance between oxidant- and antioxidant-system (Zimmerman et al., 1989). Particularly, there are several conditions that make a brain more susceptible to oxidative stress (Halliwell, 2006).

The brain needs high concentration of metals for its normal function, but elevated metal concentration promotes ROS generation (Halliwell, 2006). In fact, the high levels of iron and copper are found in normal aged brain, and moreover, the interaction between high concentration of metals and amyloid deposition has been reported in AD-affected brain (Smith et al., 1997). Further, isolated hemoglobin can release iron ions which react with H_2O_2 to produce more ROS in the brain (Halliwell, 2006). Also, the brain has excitotoxic neurotransmitters such as glutamate that increase intracellular

calcium concentration, which leads to mitochondrial dysfunction and further increase in ROS generation. There are also neurotransmitters such as dopamine that may serve as ROS precursor in the brain (Halliwell, 2006). In addition, neuronal plasma membranes are rich in the polyunsaturated fatty acids which supply abundant targets of RS (Halliwell, 2006). Moreover, there are microglia in the brain, which interact with neurons. During inflammation, microglia are activated and release cytokines and ROS, such as interleukin-1/6, and H_2O_2, to neurons. These released cytokines and ROS not only damage neurons, but also cause neurons to release of microglial activators to microglia (Halliwell, 2006, Block et al., 2007). This cycle enhances interaction between microglia and neurons causing more oxidative stress in the brain (Block et al., 2007).

To cope with ROS-induced oxidative stress in the brain, many compounds have been tested for their antioxidant effects by reducing ROS formation, inhibiting metal-protein interaction, and/or blocking excitotoxicity (Halliwell, 2006). Vitamin C, Vitamin E (alpha-tocopherol), coenzyme Q, carotenoids, and flavonoids have been tested as well as the combination of these antioxidants in coping with the RS generation in the brain (Halliwell, 2006). However, these antioxidants were not very effective in removing RS in a brain, and further not effective in all types of neurodegenerative diseases (Halliwell, 2006). Thus, there are limitations of antioxidant usages in the effectiveness, and in the clinical application to the neurodegenerative diseases (Halliwell, 2006). Therefore, it is important to develop effective antioxidants for normal aging and age-related neurodegenerative diseases (Halliwell, 2006).

As an antioxidant agent, our laboratory has focused on the effects of estrogens, a family of steroid hormones. Many research articles have reported that estrogens protect neurons from many kinds of neurotoxins such as H_2O_2, glutamate, and amyloid beta, which suggests the antioxidant effects of estrogens (Perez et al., 2005). Recently we found that estrogens preserve mitochondrial functions during H_2O_2 insults (Wang et al., 2003). Taken together, these findings provide strong evidence for the role of estrogens against the oxidative stress. Therefore, it is of interest to investigate not only the role of NFκB signaling in a pro-oxidant induced oxidative stress, but also the effects of estrogens on NFκB signaling after oxidative insults.

B. NFκB and its Role in Oxidative Stress

In the late of 1980's, Baltimore et al reported on a specific nuclear protein, which they named nuclear factor κ-B (NFκB), bound to B site of the enhancer sequences of the immunoglobulin κ light chain (Sen and Baltimore, 1986 a). In a subsequent report, they demonstrated that NFκB could be induced by specific stimuli even in non-immune cells (Sen and Baltimore, 1986 b). This finding implied that NFκB could exist ubiquitously and the NFκB may be translocated into a nucleus upon stimuli (Sen and Baltimore, 1986 b). Further, they showed that the NFκB was retained as a hetero- or homo-dimer in the cytosol with the binding of its inhibitor protein, IκB, in resting cells, and the dimer was translocated into a nucleus upon stimuli-induced removal of the phosphorylated IκB (pIκB) (Baeuerle and Baltimore, 1988). The translocated dimer bound to its consensus sequences of target gene to control its gene expression. This

IκB-dependent activation pathway is called a "classical or canonical pathway" (Memet, 2006). However, recently, an IκB-independent NFκB activation pathway has been discovered in which NFκB is activated by direct phosphorylation of NFκB rather than through the phosphorylation and subsequent degradation of pIκB (Schmitz et al., 2001). Moreover, there are several reports suggesting that stimuli-induced p100 processing is important for NFκB activation (Xiao et al., 2006). There are five NFκB proteins discovered so far, which are Rel A/p65, Rel B, c-Rel, p50, p52 (Xiao et al., 2006). The subunit of p52 is spliced from p100, and abnormal activation of this process has been found in autoimmune diseases (Xiao et al., 2006). Thus the NFκB activation pathways are getting complex.

A variety of stimuli activate NFκB including oxidative and physical stresses, injury, virus, bacteria, and pro-inflammatory cytokines. These stimuli activate IκB kinases (IKKs), and other kinases to phosphorylate IκB, and this phosphorylated IκB is recognized by ubiquitins and subsequently degraded by the 26S proteasome. Then, the released NFκB translocates to a nucleus and plays a role as a transcription factor (Schmitz et al., 2001, Memet, 2006). Since NFκB is involved in a variety area of physiological activities, such as inflammation, immune responses, host defense, cell differentiation, cell proliferation, apoptosis, and cell survival, it is important to identify the stimuli-specific NFκB signaling pathways and then to adjust the NFκB pathway for further clinical application (Meffert and Baltimore, 2005, Yamamoto and Gaynor, 2001).

Activated NFκB has been observed in several inflammatory human diseases such as asthma, rheumatoid arthritis, inflammatory bowel disease, and as well as non-inflammatory human diseases, such as cancer, diabetes, and Alzheimer's disease (AD) (Yamamoto and Gaynor, 2001). Therefore, NFκB inhibitors have been tested to block its activation in those diseases as a therapeutic approach (Yamamoto and Gaynor, 2001). Further, it has been proposed that the NFκB exists in the central nervous system (CNS). In fact, NFκB is highly expressed in neurons and glia in most brain areas (Meffert and Baltimore, 2005). The major composition of NFκB is p65/p50 or p50/p50 which is similar to those in peripheral tissues. NFκB is activated by several stimuli including beta-amyloid, neurofibrillary tangles, neurotransmitters, and nerve growth factor (Meffert and Baltimore, 2005, Yamamoto and Gaynor, 2001). The existence of NFκB in the CNS may suggest a role in the signaling pathway in the synaptic transmission, and also suggest a role of NFκB as a transcription factor through retrograde transport in neurons (Meffert and Baltimore, 2005). Much in vitro data have suggested that NFκB is particularly involved in the synaptic depolarization, excitatory neurotransmitters, and calcium responses (Meffert and Baltimore, 2005). However, mechanisms and consequences of the NFκB activation are not well understood so far (Meffert and Baltimore, 2005). For example, it is still debated if the NFκB plays as pro-apoptotic or anti-apoptotic in neuronal survival (Meffert and Baltimore, 2005).

The role of NFκB in neuronal survival or death has been recently explored by many laboratories. Interestingly, experimental results have shown conflicting results in which both anti-apoptotic and pro-apoptotic function of NFκB have been demonstrated. In a

review published in 1997, Lipton summarized the current experimental data that show the opposite functions of NFκB in regulating neuronal survival, and suggested his views regarding these dual effects of NFκB in CNS (Lipton, 1997). The function of NFκB in CNS might be dependent on the different components of NFκB, the kinds of stimuli, the stimuli-induced differential activation of NFκB subunits, the cell types such as neurons or glia, the interaction between neurons and glia, and the cross-talk with other signaling pathways (Kaltschmidt et al., 2005, Pizzi et al., 2002, Kaltschmidt et al., 2002). Also, the "optimal activation hypothesis" was proposed to explain the duel effects of NFκB (Pizzi et al., 2002). According to this hypothesis, NFκB is regulated by its inhibitory proteins, IκBs, but too low or too high NFκB promotes neuronal cell death due to a disrupted auto-feed back system (Meffert and Baltimore, 2005). In addition to the role of NFκB in neuronal survival, the role of NFκB in the ROS signaling has been proposed (Staal et al., 1990).

While the direct addition of H_2O_2 into the media activated the NFκB, the direct addition of antioxidants, such as N-acetyl-L cysteine (NAC), a precursor of glutathione, L-cysteine, 2-mercaptoethanol, dithiocarbamates, butylated hydroxyanisol, vitamin E and its derivatives, and chelators of iron and copper ions suppressed NFκB activation (Baeuerle and Henkel, 1994). This may imply that the NFκB activation is involved in the ROS signaling (Schreck et al., 1991). If antioxidants block or reduce NFκB activation, the role of NFκB in the ROS-induced oxidative stress is more likely to pro-apoptotic. In fact, the NFκB was highly activated in animal models of stroke, and p50 knockout mice showed significantly reduced ischemic damages suggesting pro-

apoptotic function of NFκB in the oxidative stress (Schneider et al., 1999, Wen et al., 2004).

C. Neuroprotective Effects of 17Beta-Estradiol and its Role in Oxidative Stress

The external and internal signals are transmitted into the central nervous system (CNS), which are further transmitted into the hypothalamus to releases a peptide hormone, gonadotropin-releasing hormone (GnRH) (Devlin, 2000). The GnRH stimulates gonadotrope in the anterior pituitary to releases the follicle-stimulating hormone (FSH), which travels through the blood streams and reaches peripheral target organs, such as ovaries and stimulates the organ to make estrogen (Devlin, 2000). Thus, the major function of estrogen was restricted to a reproductive function in the female (Meffert and Baltimore, 2005). However, much research has shown the involvement of estrogens in a variety of physiological functions in the CNS as well as the cardiovascular system (Meffert and Baltimore, 2005). However, it seems likely that the modes of E2 actions are getting complex.

E2 acts through its intracellular receptors (ER), such as estrogen receptor-α and -β (ERα and ERβ). After dimerization of 17β-estradiol (E2)-bound ER, they translocate into the nucleus and bind to its consensus sequences of target genes promoter to regulate the gene transcription. This genomic action of E2 is characterized by delayed onset and prolonged effects of E2 through the intracellular nuclear receptors (McEwen and Alves, 1999). On the other hand, the treatment with E2 has been shown to activate second messengers, signaling pathways, neuronal excitability, and/or calcium currents,

which were all fast but short term effects of E2 (McEwen and Alves, 1999). Therefore, this is thought to be nongenomic since E2 actions are evident within minutes (McEwen and Alves, 1999). Further, several ERβ subtypes and ERβ variants have been found in the CNS as well as a possibility of membrane bound ER. Also, a heterodimer with ERβ1 and ERα was identified in the late 1990's (Petersen et al., 1998, Moore et al., 1998).

E2 has been shown to exhibit numerous effects throughout the CNS by interacting with catecholaminergic-, serotonergic-, and cholinergic-neurons, spinal cord, glial cells, endothelial cells, and blood-brain barrier (McEwen and Alves, 1999). Therefore, E2 can influence neuronal development and differentiation, the formation of the synapse, structural plasticity, learning and memory, cognition, coordination of movement, mood, analgesia, neuronal excitability and ion channels, calcium homeostasis and neuroprotection (McEwen and Alves, 1999). Because of these various effects of E2, we need E2 not only for the maintenance of a brain function, but also for protection of a brain against aging and age-related neurodegenerative diseases, such as AD (McEwen and Alves, 1999). Since the oxidative stress-induced neuronal damages have been considered as one of the important neuropathology, we particularly are interested in the E2-mediated neuroprotection against the oxidative stress.

As we reviewed in the first section, oxidative stress may damage the normal cellular functions through peroxidation of macromolecules, affect normal signaling pathways, regulates kinase cascades leading to alteration in gene expression, and directly damage the mitochondrial functions (Balaban et al., 2005). It has been

demonstrated that E2 has antioxidant effects against ROS-induced or ROS-mediated oxidative stress. In the plasma membrane, E2 may exert its neuroprotective effects by affecting membrane fluidity or by participating cellular redox cycles through its phenolic hydroxyl group on the steroid A ring (Simpkins et al., 2005). In intracellular sites, E2 may prevent or reduce the lipid peroxidation, protein carbonylation, and DNA damages, affect signaling pathways, modulate kinase cascades, increase the activities of antioxidant enzymes, reduce inflammatory responses, and preserve mitochondrial function (Singh et al., 2006). The mitochondria have important regulatory functions, such as energy production, cell death or survival, ROS production, and aging. Therefore, the protective effects of E2 against the mitochondrial damages should be taken seriously.

It was reported that E2 is neuroprotective against glutamate-induced excitotoxicity (Yi et al., 2005). The glutamate-induced excitotoxicity is accompanied by ROS generation and calcium overload in the mitochondria (Yi et al., 2005). This overloaded calcium disrupts mitochondrial membrane potential, which further leads to disruption of ATP generation, generation of ROS, and releases the cytochrome *c* that triggers the process of apoptosis (Singh et al., 2006). However, E2 preserves mitochondrial membrane potential by preventing excessive calcium influx into the cytosol and into mitochondria as well (Singh et al., 2006, Wang et al., 2003). Because of these protective effect of E2 on the mitochondrial membrane potential, E2 also may prevent the disruption of ATP generation, production of ROS, release of cytochrome *c* into the cytoplasm, and thus, may prevent apoptosis (Singh et al., 2006). In fact, mitochondrial

failure has been seen in several neurodegenerative diseases, such as AD as well as non-neurodegenerative disease, such as glaucoma (Simpkins et al., 2005). Therefore it should be important to investigate the molecular mechanism of E2 underlying its cyto- and mito-protective effects, and further, to develop E2 as a potential therapeutic agent for oxidative-related aging or neurodegenerative diseases (Simpkins et al., 2005).

D. Neuroprotective Effects of Estrogens Mediated by Suppression of NFκB Activation in Oxidative Stress

Inflammatory and immune responses are critical for the host defense in response to a variety of external stimuli (Mckay and Cidlowski, 1999). However, consistent or inappropriate activation of the defense systems may cause damage to tissues and cells (Mckay and Cidlowski, 1999). Therefore, the balance between endogenous inflammatory and anti-inflammatory systems must be maintained (Mckay and Cidlowski, 1999). NFκB is a key factor in inflammatory and immune responses, and glucocorticoids have been shown to have strong anti-inflammatory effects against the NFκB-induced inflammation (Mckay and Cidlowski, 1999McKay et al., 1999).

In addition to the glucocorticoids, 17β-estradiol (E2) has shown the protective anti-inflammatory effects against the NFκB-mediated inflammation by blocking the NFκB nuclear translocation, inhibiting IκB degradation or reducing its downstream gene expression in peripheral and brain tissues (Ghisletti et al., 2005, Sribnick et al., 2005, Ospina et al, 2004, Wen et al., 2004). Bruce-Keller et al. reported the anti-inflammatory effects of E2 on microglia, and Galea et al. showed anti-inflammatory

effects of E2 on NFκB translocation without modifying IκB degradation suggesting the presence of specific mechanism of E2 action in the cerebral endothelial cells (Bruce-Keller et al., 2000, Galea et al., 2002). It seems likely that E2 antagonizes the NFκB-mediated inflammation with complex modes of actions. However, there are few reports that investigated the role of NFκB in oxidative stress, and the role of E2 against NFκB activation in the oxidative stress in the central nervous system (CNS).

In peripheral tissues, estrogens protect cells from oxidative stress through attenuating NFκB activation or through increasing the expression of antioxidant enzymes, such as glutathione peroxidase and manganese-superoxide dismutase (Speir et al., 2000, Omoya et al., 2001, Borras et al., 2005). We previously reported the H_2O_2-induced oxidative stress and protective effects of estrogens against the H_2O_2-induced damage of mitochondrial membrane potential, ATP production, lipid peroxidation, cell viability, and calcium homeostasis in cultured human lens epithelial cells and human neuroblastoma SK-N-SH cells (Wang et al., 2003, Wang et al., 2006). We have also observed that E2 attenuated post-ischemic inflammatory induction of rapid NFκB activation, phosphorylation of IκB, and an overexpression of inducible nitric oxide synthase (iNOS), which is one of NFκB-activated downstream targets (Wen et al., 2004). Thus, we were interested in the E2-mediated suppression of NFκB in the oxidative stress in neurons.

E. Hypothesis

In 1991, Schreck and associates showed direct activation of NFκB following H_2O_2. NFκB translocation into the nucleus was seen within fifteen minutes following 150μM of H_2O_2 treatment, and 20mM of N-acetyl-L cysteine (NAC), an antioxidant, suppressed H_2O_2-induced NFκB nuclear translocation. Our laboratory has also shown that NFκB nuclear translocation was induced by H_2O_2, and E2 reduced the DNA binding activities of NFκB in a dose-dependent manner (Green, 1999). Moreover, we have also demonstrated E2 neuroprotection against H_2O_2-induced oxidative stress via attenuation of intracellular calcium overloading, maintaining mitochondrial membrane potential, maintaining ATP production, and increasing GSH levels (Wang et al., 2003). It has also been shown that middle cerebral artery occlusion induced NFκB activation and apoptosis, and that E2 treatment attenuated these effects (Wen et al., 2004).

Based on these observations, we hypothesized that H_2O_2 induced-oxidative stress will activate NFκB while the presence of E2 will suppress the H_2O_2-induced NFκB activation. To test this hypothesis, we measured NFκB activation by the levels of phosphorylated IκBα (pIκBα), total IκBα, and p65 in each treatment group.

Specific Aim # 1: To determine whether H_2O_2 induces NFκB activation.

Specific Aim # 2: To determine whether E2 suppresses the H_2O_2- induced NFκB activation.

The first specific aim was to determine whether H_2O_2 induces NFκB activation. H_2O_2 is itself a kind of ROS, and a precursor to a strong hydroxyl radical. Thus, the H_2O_2 treatment will produce excessive ROS, which further cause damages of oxidative

stress. Previously it was shown that NFκB was activated by H_2O_2, and was suppressed by antioxidants, NAC, and E2 (Schreck et al., 1991, Green, 1999). Therefore, we hypothesized that NFκB will be activated in oxidative stress. We found that H_2O_2 activated NFκB via activation of its classical signaling pathways, such as phosphorylation of its inhibitor protein, IκBα, (pIκBα) and subsequent degradation of pIκBα, which caused the reduction of total IκBα expression, and NFκB (p65 subunit) nuclear translocation. Next we investigated if E2 suppresses the H_2O_2-induced NFκB activation. The simultaneous treatment of E2 with H_2O_2 dramatically reduced the induced pIκBα by H_2O_2, upregulated the total IκBα expressions, and inhibited the p65 nuclear translocation. Therefore, we concluded that the H_2O_2-induced NFκB activation is suppressed in the E2-mediated neuronal protection against oxidative stress.

CHAPTER II

MATERIALS AND METHODS

A. Reagents

H_2O_2 was purchased from Mallinckrodt Baker, Inc. (Paris, KY), and used with concentrations ranging from 100μM to 1mM for the cell viability assay and then, only 700μM was used for further study. Calcein acetoxymethyl (AM) was obtained from Molecular Probes, Inc. (Eugene, OR) and used for cell viability assay. 17β-estradiol (E2) was purchased from Steraloids, Inc. (Newport, RI) and HT22 cells were treated at the pharmacological concentration of E2 10μM. The ZYC compounds (ZYC-3, ZYC-26) were prepared as previously described (Perez et al., 2005), and were used at the same concentration as E2 (10μM). TNFα was obtained from Chemicon, Inc. (Temecula, CA) and was applied to both HT22 cells and HeLa cells at concentrations of 20ng/ml for western blot analysis or 30ng/ml for immunocytochemistry. Anti-pIκBα at ser 32, anti-full length of IκBα, and anti-p65 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The secondary antibody (goat anti rabbit) conjugated with Alexa Flour 488 was purchased from Invitrogen, Corp. (Carlsbad, CA).

B. Cell Culture

HT22 cells were provided by Dr. David R. Shubert (Salk Institute, San Diego, CA), and cultured in Dulbecco's modified Eagle's (DMEM) media supplemented with 10% charcoal-stripped fetal bovine serum (FBS) and 20μg/ml gentamicin at 37 °C in 5% CO_2 and 95% air. The DMEM (glucose 4.5g/L) was purchased from GIBCO, Inc. (Gaithersburg, PA) and the charcoal-stripped FBS was obtained from HyClone, Inc. (Logan, UT) and gentamicin was obtained from Sigma, Inc. (St. Louis, MO). HeLa cells were obtained previously from ATCC, American Type Culture Collection, and cultured in DMEM (glucose 4.5g/L) media supplemented with 10% regular FBS, and 20μg/ml gentamicin. Regular FBS was purchased from HyClone, Inc. (Logan, UT). Both cell types were maintained up to 70% confluency and then trypsinized for seeding into 96-well plates for measuring cell viability, into tissue culture dishes (30, 60, 100mm dishes) for western blot analysis, and into 24-well plates for immunocytochemistry. Cells were observed and pictures of dishes were obtained by a phase-contrast microscope (Nikon Diaphot-300).

C. Cell Viability Assay

HT22 cells were seeded into 96-well plates and incubated for 24 hours. Then cells were treated with H_2O_2 (100μM to 1mM) and incubated for 24 hours. Each plate was taken out of an incubator and the media was discarded. Phosphate-buffered salin (PBS) 100μl was added into each well to wash out media. After discarding the PBS, 2.5μM of calcein-AM in PBS was added into each well, and incubated at room temperature for

15 minutes. Calcein-AM is permeable through the cell membrane and a substrate for intracellular estrases which hydrolyzes the calcein-AM to calcein. This calcein emits a greenish fluorescence that is detected at excitation 485 nm and emission 530 nm in Bio-Tek FL600 microplate reader (Winooski, VT). Thus, more live cells present emit more fluorescence (Figure 10). The fluorescence was measured in the unit of relative fluorescent (RFU), and the RFU was converted to percentage of untreated control values.

We counted surviving cells by using trypan blue dye to measure cell viability. HT22 cells were seeded into Petri dishes, and treated with H_2O_2 and E2. After overnight incubation with reagents, cells were trypsinized, centrifuged, mixed with trypan blue (Cellgro, VA), and intact cells were counted using a hemacytometer (Horsham, PA). Cell counting was repeated three times at least and an average was obtained between each treated group (Figure 13). The counted numbers of survived cells were converted to percentage of untreated control values.

D. Treatments

Cells ($2x10^6$ cells per each 100mm dish) were seeded into tissue culture dishes and incubated for 48 hours. Then, all reagents, such as H_2O_2 700μM, E2 10μM, TNFα 20ng/ml, and ZYC compounds (ZYC-3, ZYC-26) 10 μM, were directly added into the media and incubated during each time point (from 1 minutes to 1 hour). Next, cells were scraped on ice with direct addition of lysis buffer for making whole cell lysates or with cold PBS for making cytoplasmic extractions.

E. Whole Cell Lysates

After treatment in each time point, the media in a dish was vacuumed out on ice, 150µl – 200µL of lysis buffer (Tris-HCl 1M, NaCl 4M, 50% NP40, 10% sodium deoxycholate, 10% SDS, protease cocktail 1 to 1000 dilution) was directly added into the dish, and cells were scraped out quickly. Each sample was transferred into a cold tube, and incubated for 10 to 30 minutes on ice after 10 times of pipetting, and 10 seconds of vortexing. Then centrifugation was performed for each sample at 12000 x g for 10 minutes, and supernatant was transferred into a cold new tube. This supernatant was used for protein assay or quickly frozen at -80°C for later assay.

F. Nuclear Extraction

The cell pellet was resuspended in cold PBS buffer, and centrifuged at 163 x g for 3 minutes, and the PBS was removed. The pellet was gently vortexed for 5 seconds, and 250µl of buffer A (Hepes buffer 10mM, pH 7.4, KCl 10mM, EDTA 0.1mM, EGTA 0.1mM, DTT 1mM, PMSF 0.5mM, NaF 0.5mM) was added into the cell pellet, and incubated them for 10 minutes on ice after gently mixing the solution with a pipet. Then, 15.6µl of 10% NP40 was added into the solution, which was mixed gently by pipetting for 5 times and vortexing for 5 seconds. Then, the solution was centrifuged at 163 x g for 2 minutes, and the supernatant was taken into a new cold tube. Then, 25µl of buffer A was added into the pellet again to wash the cytoplasmic parts out from the nuclear part and mixed by gentle pipetting for 5 times. Immediately, the solution was centrifuged again at 163 x g for 2 minutes, and the supernatant was taken. Then, 50µl

of buffer C (Hepes buffer 20mM, pH 7.4, NaCl 0.4M, EDTA 1mM, EGTA 1mM, DTT 1mM, PMSF 1mM, NaF 1mM) was added into the pellet followed by gentle vortex. The solution was mixed vigorously by 10 times pipetting and 10 seconds vortex and incubated for 15 minutes on ice. The mild vortex was performed every 5 minutes during 15 minutes incubation. Then the solution was centrifuged at 12000 x g for 15 minutes, and the supernatant was taken.

G. Western Blot Analysis

For IκBα phosphorylation, 20μg protein was separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred to Immunobilon-P polyvinylidene difluoride (Millipore Corp., Bedford, MA) overnight at 4 °C. Membranes were gently washed in Tris-buffered saline (10mM Tris-base, pH 8.0, 100mM NaCl) with 0.1% Tween 20, and blocked with 5% non-fat dry milk in PBS for 1 hour at room temperature. Membranes were incubated with primary antibody, p65 NFκB subunit (Santa Cruz, 1:200), IκBα phosphorylation (Santa Cruz, 1:200), and total IκBα (Santa Cruz, 1:200) at 4 °C overnight. After washing the membranes three times in Tris-buffered saline, membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Chemicon international Inc., Temecula, CA) for 1 hour at room temperature. After washing, the membranes were detected using a chemiluminesence system after 5 minutes incubation with ECL solution (ECL, Pierce Biotechnology, Inc., Rockford, IL).

H. Immunocytochemistry

The HeLa and HT22 cells were seeded on each cover glass in the 24-well plate with $5X10^4$ cells in 1.5mL media, and incubated for 48 hours. Then, the cells were treated with TNFα 30ng/mL, H_2O_2 700μM, and E2 10μM in 30 minutes and 1 hour time point.

As soon as the treatment was finished, the media was carefully removed by aspiration, and warm PBS buffer (1mL) was slowly added into the each well to wash out the media. The PBS buffer was aspirated after gentle shaking. Then, 1mL of 4% paraformaldehyde (PFA) resolved to warm PBS buffer was added slowly into the each well to fix the cells, and the cells were incubated with the 4% PFA for 20 minutes at room temperature. The 4% PFA was removed by aspiration and PBS buffer was added twice to wash out the fixation solution for 10 minutes. Each PBS buffer was aspirated after gentle shaking. The 200μl of 5% normal goat serum solution (NGS) prepared to PBS buffer was added slowly into the each well to block the cells, and the cells were incubated for 1 hour at room temperature. Then the cells were washed once with the PBS buffer, and 200μl of the primary antibody solution (5% NGS, 0.1% Triton-X100, antibody against p65 with 1 to 100 dilution) were added slowly into each well, and the cells were incubated at 4°C for 24 hours. The next day, the cells were washed out for 30 minutes with PBS buffer, and then 200μl of the secondary antibody solution (5% NGS, 0.1% Triton-X100, secondary antibody (goat anti rabbit) conjugated with green fluorescence, Alexa Flour 488, 1 to 500 dilution) were added slowly into the each well, and the cells were incubated in a dark room at room temperature for 1 hour. Then the

cells were washed for 30 minutes with PBS buffer and the each cover glass was mounted on the cover slide on which 10μl of mounting solution was already applied. The mounting solution includes DAPI staining. Each cover glass was allowed to be dried at room temperature overnight in a dark room, and then was sealed with nail polish and kept in a dark room until pictures were obtained by a confocal microscopy.

After turning on the confocal microscopy, we needed to select the kinds of laser, objective, configuration, and modes such as frame size, pinhole, and scan speed. Then we needed to place each cover glass under a confocal microscopy and to scan cells using a 40X objective.

I. Intensity Analysis

To perform intensity analysis, it is required to retake pictures of all samples with exact same settings in a confocal microscopy. Thus we looked over the all settings by which the pictures were taken to find out the lowest level of detector gain. Finally, we reset the all conditions with the exact same levels throughout all the samples. For example, the detector gain was set 404 for dapi staining, and 460 for Alexa Flour 488, the green fluorescent signals, and the number of slices was set as 14 for all the samples. All the pictures should be below the saturation for intensity analysis.

After retaking the pictures, each saved picture was opened by the "Image J" program for intensity analysis. Two cytoplasmic areas, two nuclear areas, and one background area were selected from one cell by the "Image J" program, and the fluorescent intensity of each selected area was read by the numbers throughout the 14

slices in each cell. Then, the program showed which slice had the strongest signal, and three slices (one below, one above the strongest slice, and the strongest slice) were selected. We copied the numbers of the three slices into an excel file. Next we obtained an average of cytoplasmic, nuclear, and background intensity, and subtracted the average of background intensity from the average of cytoplasmic and nuclear intensity.

J. Statistical Analysis

The T-test was used to determine whether two groups, control versus each treated group, are statistically different from each other. $p < 0.05$ or $p < 0.01$ was considered significant between two groups.

CHAPTER III

RESULTS

We measured cell viability via calcein-AM assay and cell counting to confirm the neurotoxic effects of H_2O_2 and the neuroprotective effects of E2. Western blot analysis was performed to determine if short-time (up to 1 hour) treatment of H_2O_2 and E2 induce or suppress NFκB activation. Immunocytochemistry was performed to determine NFκB (p65 subunit) localization.

A. Neurotoxicity of Hydrogen Peroxide and Neuroprotection of 17β-estradiol against Hydrogen Peroxide-induced Neurotoxicity

To determine the neurotoxicity of H_2O_2 on HT-22 cells, HT22 cells were seeded into 96 well plates at varying cell densities (K: 1,000 cells/well in Figure 1) for 24 hrs. Then, cells were treated with varying concentrations of H_2O_2 ranging from 100μM to 1mM. Twenty-four hours following treatment, cell viability was measured using calcein-AM assay. We repeatedly observed the dose-dependent H_2O_2 neurotoxicity on HT22 cells in various cell numbers (Figure 1). H_2O_2 500μM caused ~ 50% cell death, while H_2O_2 700μM caused ~ 80% cell death.

For confirmation the H_2O_2-induced neurotoxicity and as well as western blot analysis, HT-22 cells were seeded into different sized culture dishes, 35, 60, or 100 mm. The toxic effects of 1mM H_2O_2 (100% cell death) was seen within 30 minutes of

treatment (Figure 2). Thus, 1mM H_2O_2 was used as a positive control for H_2O_2-induced neuronal insults. Treatment with 700μM H_2O_2 caused approximately 70-90% cell death but was more delayed (1 hour) as compared to 1mM H_2O_2 (Figure 2). Therefore, we confirmed that neurotoxic effects of H_2O_2 on HT22 cells in these tissue culture dishes were similar to that seen in 96-well plates (Figure 1), and found the optimal time (1 hour) point of H_2O_2 700μM.

Our laboratory has shown that non-feminizing estrogen analogues are as potent if not more potent than E2 in neuroprotection (Perez et al., 2005). Chronic treatment with feminizing estrogens alone or its combination with a synthetic progestin has been shown to have detrimental side effects, such as uterotrophic or pro-thrombotic effects in post-menopause women (Perez et al., 2005). Theses side effects are commonly attributed to ERα-mediated effects in estrogen responsive tissues (Perez et al., 2005). Therefore, developing a non-estrogenic compound with the neuroprotective effects of E2 would be ideal especially in treating postmenopausal women. ZYC compounds are non-feminizing estrogen analogues, such as 2-(1-adamantyl)-3-hydroxy-4-methylestra-1,3,5(10)-trien-17-one (ZYC-26) which has a bulky group flanking the 3-hydroxyl group in the A ring of the estrone (Perez et al., 2005). We have shown that ZYC-3 is as neuroprotective as E2; while ZYC-26 is more potent than E2 (Perez et al., 2005, Figure 6). This project confirmed that E2 and its non-feminizing analogues protect HT22 cells against the oxidative stress caused by H_2O_2 (Figure 3, 4, 5, and 6). Simultaneous treatment of 10μM E2 protected HT22 cells against the cytotoxic effects of 700μM H_2O_2 by 30-40% as compared to H_2O_2 treated group; however, E2

pretreatment (30 minutes, 1 and 3 hours) did not show any further significant neuroprotective effects as compared to simultaneous treatment (Figure 5). Live cells were stained by trypan blue dye and manually counted under a microscope using a hemacytometer (Figure 5).

In summary, we observed the dose-dependent neurotoxic effects of H_2O_2 on HT22 cells. H_2O_2 700μM showed strong and fast toxic effects, which insulted cells within 1 hour. In contrast, E2 10μM protected cells from the strong H_2O_2-induced neurotoxicity by 30%-40%. Next, we performed western blot analysis to determine if NFκB activation is induced by H_2O_2 or suppressed by E2 treatment in 1 hour.

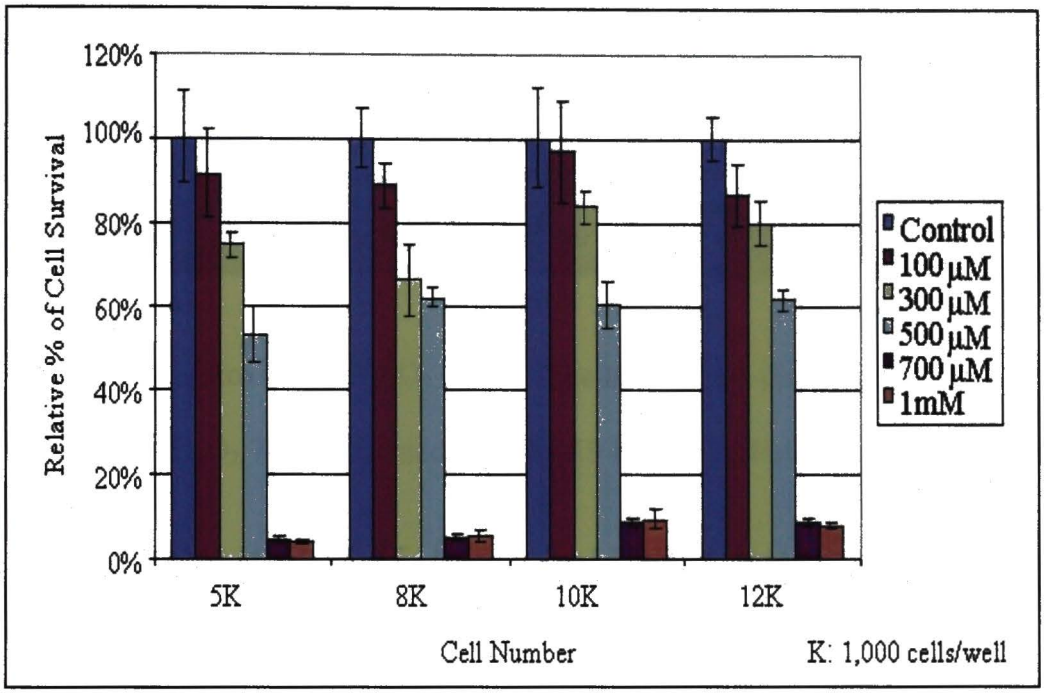
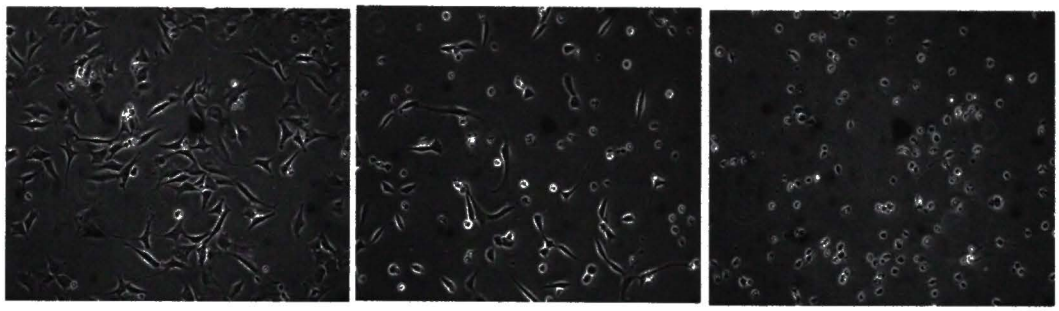


Figure 1. Dose-dependent neurotoxicity of H_2O_2 on HT22 cells in 96 well plates. HT22 cells were seeded into a 96-well plate with different numbers of cells from 5,000 cells/well to 12,000 cells/well. After 24 hours incubation, cells were treated with various concentrations of H_2O_2 ranging from 100μM to 1mM. After 24 hours incubation with H_2O_2, calcein-AM assay was performed to measure cell viability.



(a) (b) (c)

Figure 2. Neurotoxicity of H_2O_2 on HT22 cells in 60mm dishes.

Control (a), H_2O_2 700μM treated cells for 19 hours (b), H_2O_2 1mM treated cells (c) for 19 hours.

(a) (b) (c)

Figure 3. Neuroprotection of E2 against H_2O_2-induced neurotoxicity.

(a) Control, (b) H_2O_2 700μM treated for 21 hours, (c) Cotreatment of H_2O_2 700μM with E2 10μM for 21 hours.

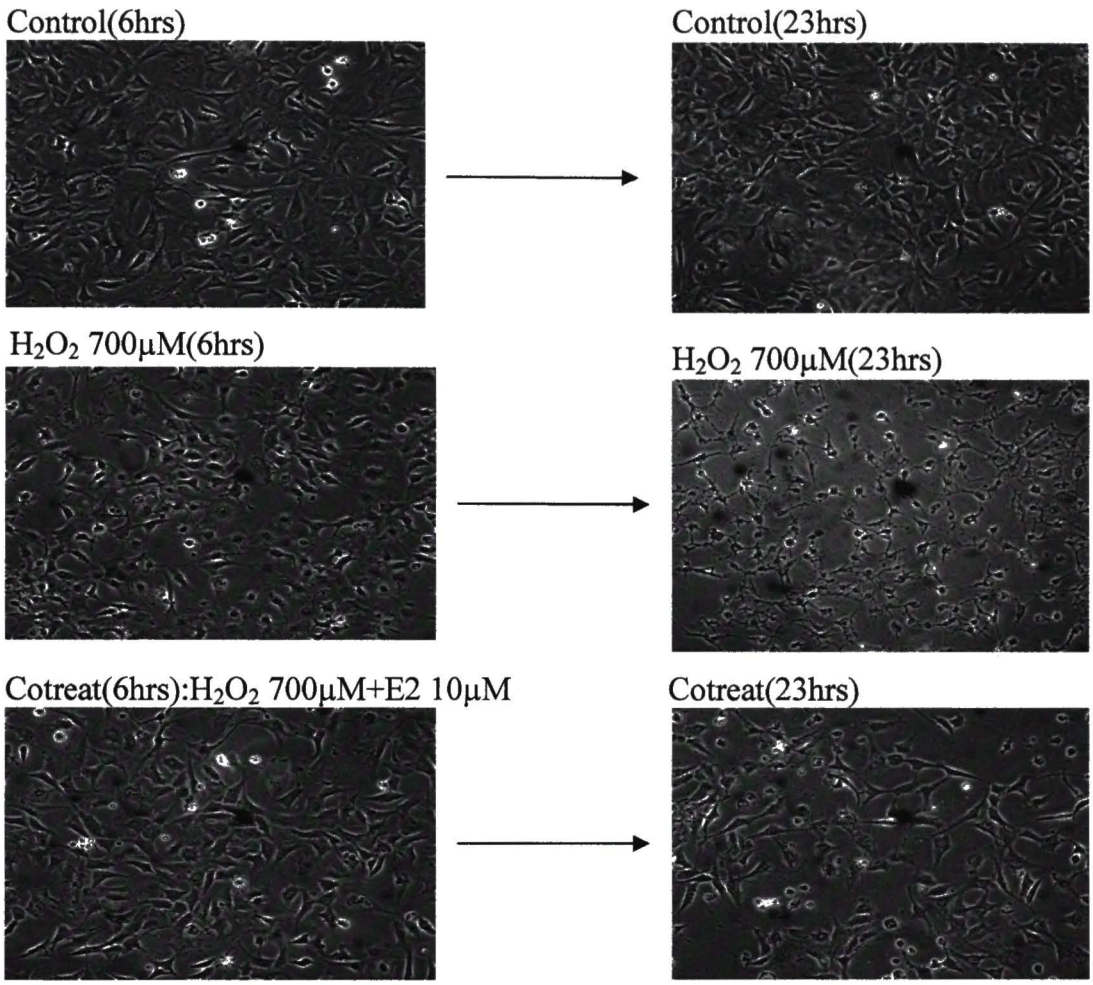


Figure 4. Neuroprotection of E2 against H_2O_2-induced neurotoxicity in an early time point. There are three groups: control, H_2O_2 700μM treated, and cotreated group in 6 hours and 23 hours.

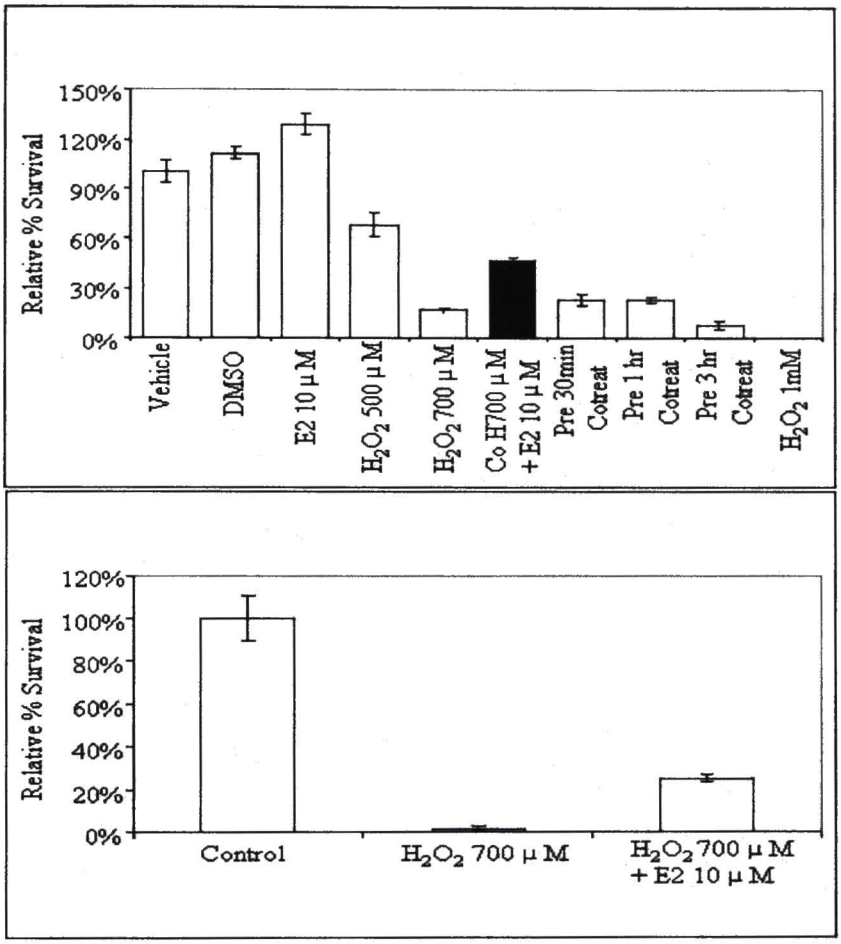


Figure 5. Neuroprotection of E2 against H_2O_2-induced neurotoxicity (cell counting). After 24 hours incubation with H_2O_2 and E2, cells were trypsinized and resuspended into media. Then, 18μl of the cell suspension was taken out and mixed with trypan blue dye 2μl. Survived cells (purple stained cells) were counted four times in each group under a microscope using a hemacytometer. Then, the counted numbers were averaged and the average was normalized by that of control group.

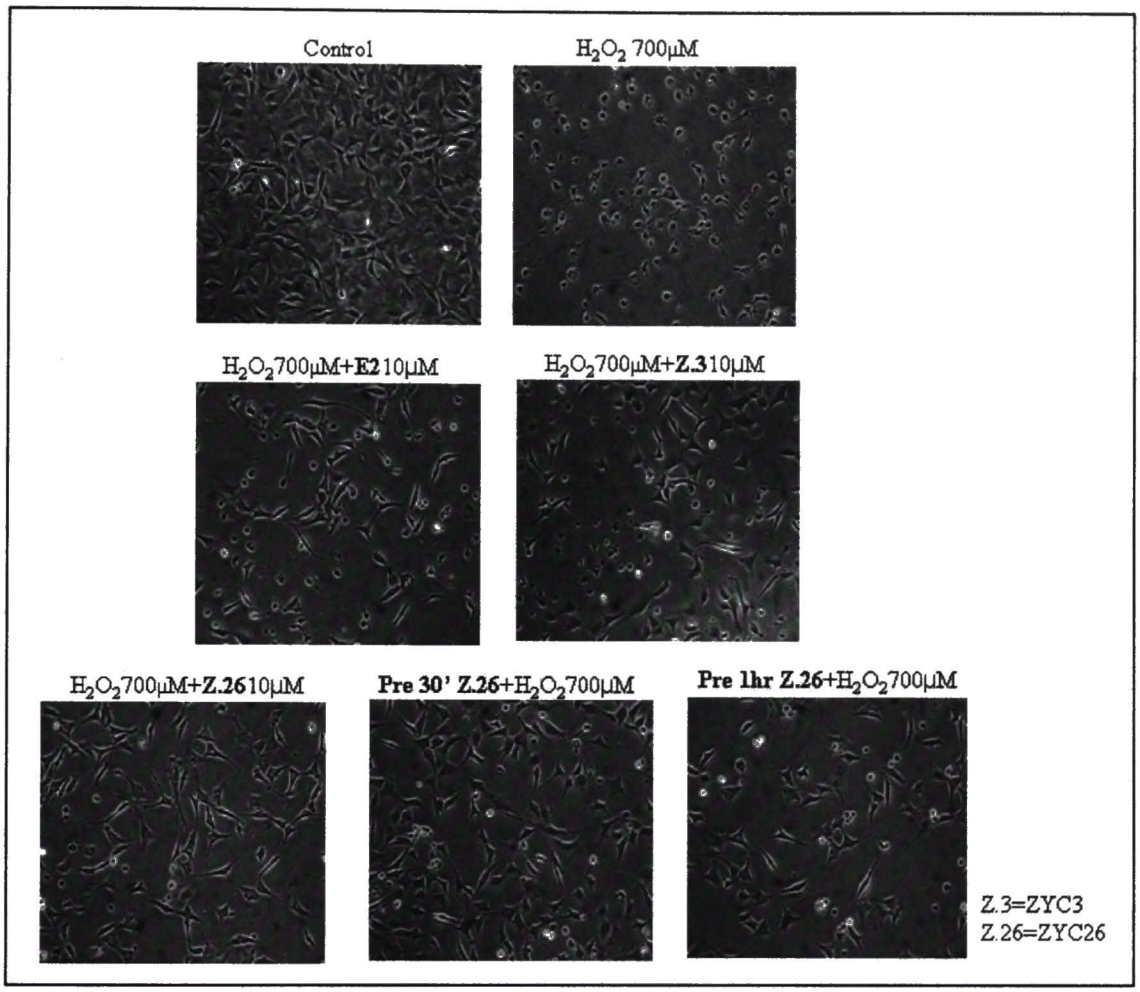


Figure 6. Neuroprotection of E2 and estrogen analogues against H_2O_2-induced neuronal toxicity. Cotreatment of E2 and ZYC3 are effective in neuroprotection. However, both cotreatment and pretreatment (30 minutes, and 1 hour ZYC26 treatment before H_2O_2 pulse) of ZYC26 showed neuroprotective effects. Moreover, ZYC26 seems to be more potent in neuroprotection against H_2O_2 toxicity than E2.

B. Western Blot Analysis

1. E2 Reduced H_2O_2-induced IκBα Phosphorylation in Whole Cell Lysates

In the resting state of a cell, NFκB activation is inhibited by binding of inhibitory proteins, IκBs, to the nuclear localization signal (NLS) of NFκB. Various stimuli, such as cytokines, injury, inflammation, oxidative stress, nerve growth factor, and neurotransmission, activate protein kinases that phosphorylate IκBs, leading to the activation of NFκB. While phosphorylated IκB (pIκBα) is ubiquitinated and subsequently degraded, the released NFκB from IκBs binding translocates into the nucleus, binds to target gene promoter, and controls the gene expressions.

According to this classic NFκB activation pathway, IκB phosphorylation has been considered as a critical step for NFκB activation. Therefore, we wanted to determine whether H_2O_2 induced phosphorylation of IκBα, and whether E2 blocked H_2O_2-induced IκBα phosphorylation. Treatment with 700 μM H_2O_2 induced IκBα phosphorylation (Figure 7), and the presence of E2 significantly reduced the phosphorylation of IκBα induced by H_2O_2 (Figure 8). Since pIκBα are ubiquitinated and degraded, total IκBα including phosphorylated and non-phosphorylated IκBα should be decreased. Figure 9 shows H_2O_2 treatment decreased total IκBα in a time-dependent manner, and E2 attenuated the H_2O_2-induced total IκBα reduction (Figure 15).

Then, we repeatedly performed western blot analysis with the same sample to confirm the pIκBα data shown in Figure 7 and 8, and analyzed data by densitometry (Figure 10 and Figure 11).

In summary of results from western blot analysis with whole cell lysates, H_2O_2 induces IκBα phosphorylation, and reduces total IκBα due to the degradation of pIκBα, and E2 antagonizes the action of H_2O_2 by reducing the phosphorylation level of IκBα. In whole cell lysates, basic expression level of phosphorylated IκBα was consistently detected in control group. Thus, the result of H_2O_2-induced pIkBa did not seem to be consistent due to the relatively high level of pIκBα in control group. In figure 7 and 10, there is increased pIκBα induced by H_2O_2, but there is no big difference in the level of pIκBα between control and H_2O_2 treated groups in Figure 8 and 11. However, in the presence of E2, pIκBα was significantly reduced in cotreatment groups, which was consistently observed throughout this project. How can E2 reduce pIκBα, and what is the meaning of E2-mediated reduced pIkBa in this project? These questions should be answered for better understanding of E2 action in neuroprotection against oxidative stress. Next, we made cytoplasmic extracts to determine the level of pIκBα, total IκBα and p65 induced by H_2O_2 and E2 more exactly than whole cell lysate in the absence of nuclear proteins.

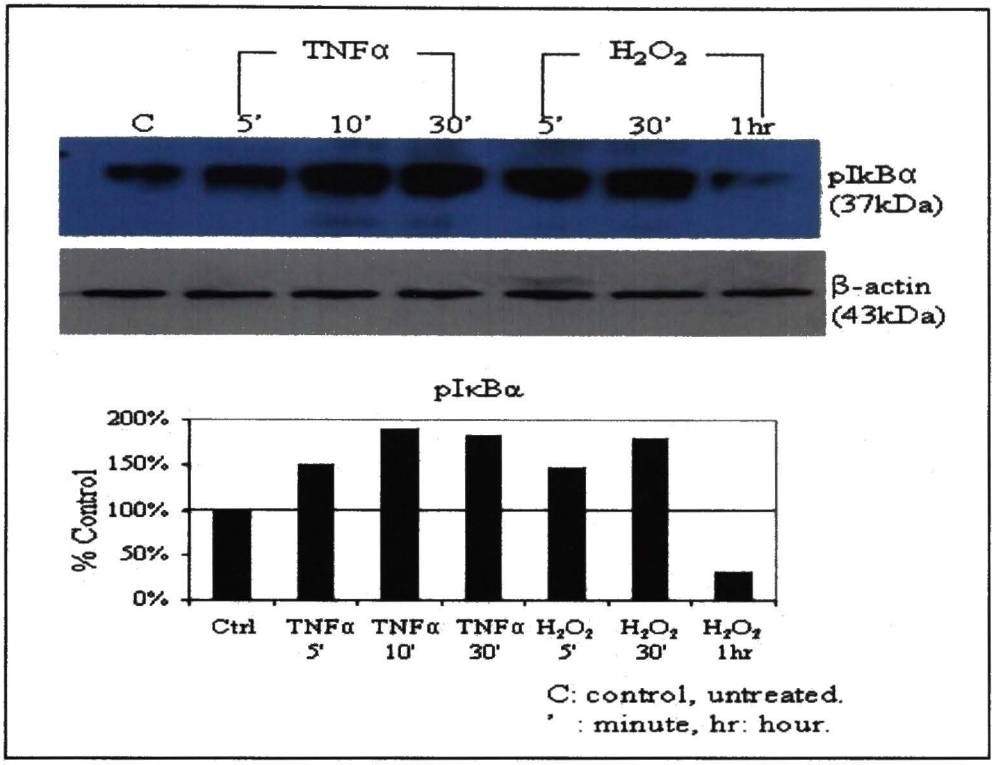


Figure 7. H_2O_2-induced pIκBα in whole cell lysates.

H_2O_2 700μM, and TNFα 20ng/ml were directly added into media in which HT22 cells had been grown for 48 hours after seeding on tissue culture dishes. Then, cells were harvested with lysis buffer for protein extraction. Then proteins were quantified, separated on 10% SDS-PAGE gel, and transferred onto PVDF. The blots were blocked with 5% skim milk for 1 hour, and incubated with primary antibody against phosphorylation of IκBα at Ser 32. Each band was normalized by β-actin, and band intensity was analyzed by densitometry. TNFα was treated for positive control for NFκB activation.

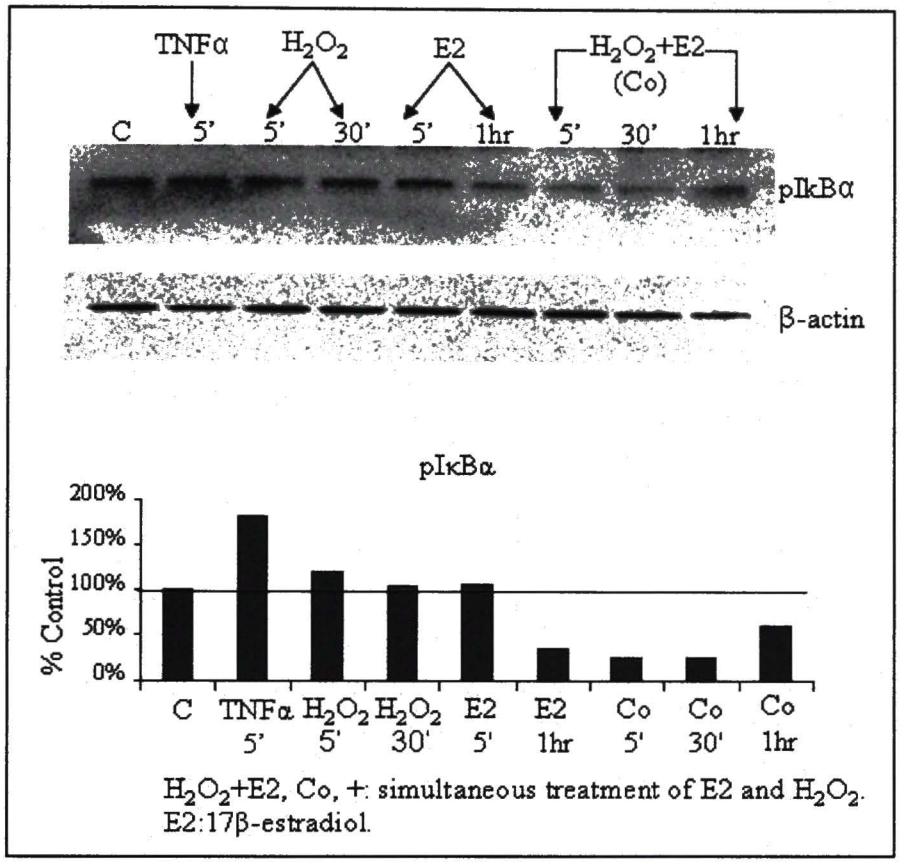


Figure 8. E2-mediated pIκBα reduction in whole cell lysates.

The procedure for making whole cell lysates and western blot analysis are described in Figure 7.

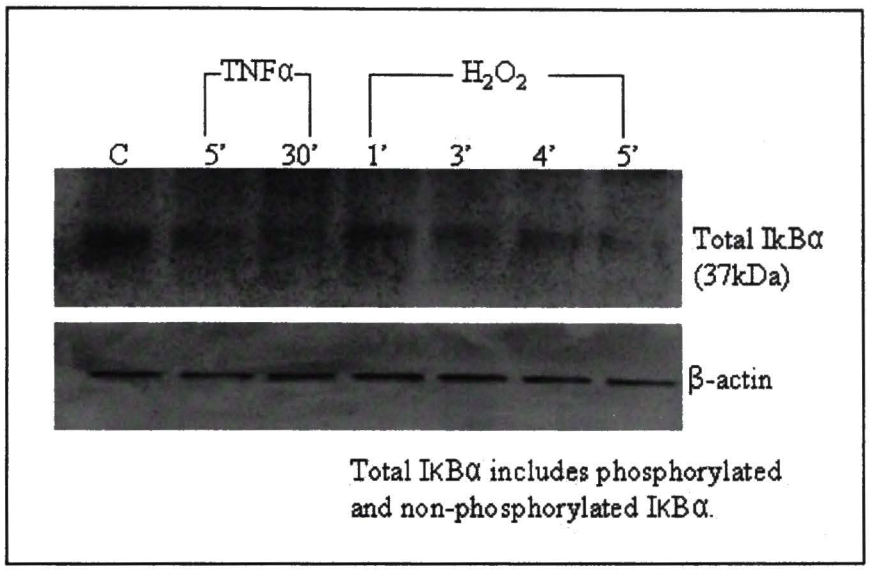


Figure 9. H_2O_2-induced total IκBα reduction in whole cell lysates.

The procedure for making whole cell lysates and western blot analysis are described in Figure 7. The blots were incubated with primary antibody against total IκBα.

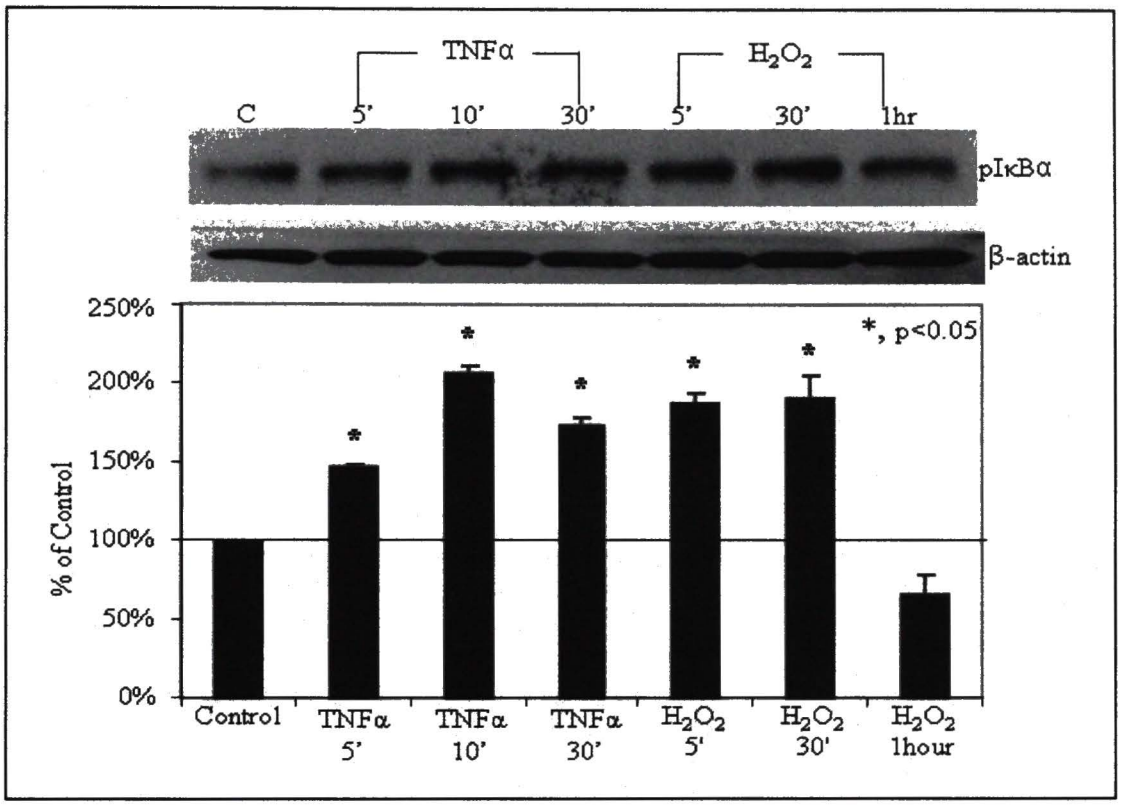


Figure 10. H_2O_2-induced pIκBα in whole cell lysates.

The procedure for making whole cell lysates and western blot analysis are described in Figure 7. Error bars are based on variances between three independent runs of one sample. *, represents statistical significance between control and each group.

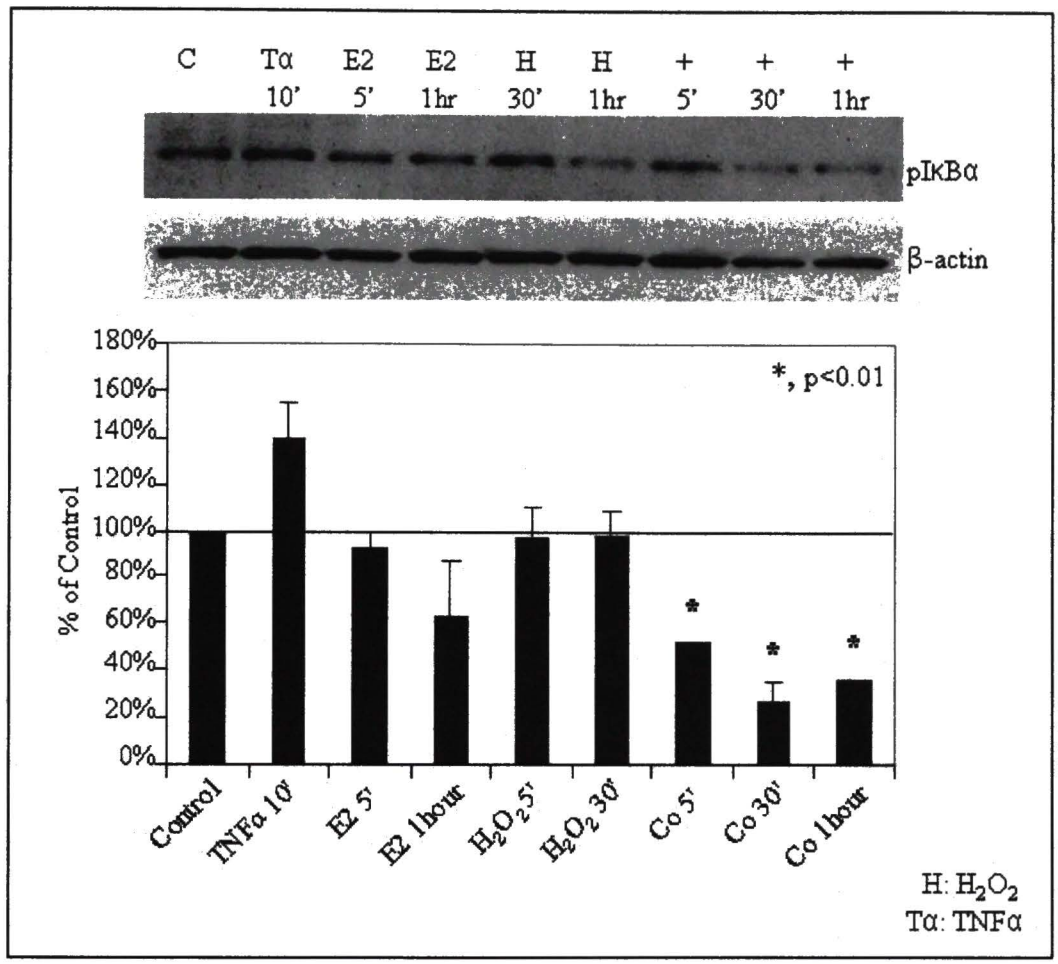


Figure 11. E2-mediated pIκBα reduction in whole cell lysates.

The procedure for making whole cell lysates and western blot analysis are described in Figure 7. Error bars are based on variances between three independent runs of one sample. *, represents statistical significance between control and each group.

2. E2-mediated Reduced IκBα Phosphorylation, Increased total IκBα, and Inhibited p65 Nuclear Translocation in Cytoplasmic Extracts

Once IκBα is phosphorylated upon stimuli, NFκB is released and translocated into the nucleus. As shown in whole cell lysates, H_2O_2 treatment caused increased IκBα phosphorylation and subsequent IκBα degradation (Figure 7, 9, and 10). In contrast, E2 treatment significantly suppressed the H_2O_2-induced IκBα phosphorylation (Figure 8 and 11). However, it was not clearly seen in whole cell lysates whether E2 affects the total IκBα expression, and whether H_2O_2 induces p65 nuclear translocation and E2 suppresses it (p65 is one of the NFκB subunits). Moreover, IκBα, pIκBα, and NFκB (p65) reside in the cytoplasm in the absence of stimuli and p65 is expected to translocate into the nucleus upon H_2O_2 treatment. Thus, cytoplasmic extracts were prepared to determine the levels of IκBα, pIκBα, and p65 induced by H_2O_2 and E2 more exactly than whole cell lysates.

As expected, H_2O_2 treatment induced IκBα phosphorylation and decreased total IκBα and p65 expression in cytoplasmic extracts (Figure 12). Moreover, simultaneous treatment of E2 had an attenuating the effects of H_2O_2 on pIκBα and p65 (Figure13). In the presence of E2, H_2O_2-induced IκBα phosphorylation was significantly reduced, and H_2O_2-induced p65 reduction was inhibited (Figure 13). Interestingly, control groups of the cytoplasmic extracts do not have relatively high expression level of pIκBα. Thus, the change in the pIκBα expression level was more clearly observed in cytoplasmic extracts than in whole cell lysates.

Then, we repeatedly performed western blot analysis with current samples and a new sample to test whether E2 antagonizes the action of H_2O_2 regarding all these important proteins, pIκBα, total IκBα, and p65 for NFκB activation in HT22 cells.

As shown in Figure 14 and 15, we repeatedly observed the H_2O_2-induced increased pIκBα, and reduced total IκBα expression due to degradation of pIκBα. On the other hand, E2 reduced the increased phosphorylation level of IκBα, upregulated total IκBα in cotreatment groups (Figure 14 and Figure 15). Moreover, we found that E2 inhibited p65 reduction that was dramatically induced by H_2O_2 treatments (Figure 16).

In summary, while H_2O_2 activates NFκB by inducing pIκBα, reducing both total IκBα, and cytoplasmic p65 expression, E2 suppresses H_2O_2-induced NFκB activation by reducing pIκBα, increasing total IκBα expression, and maintaining cytoplasmic p65 in cotreatment groups. However, it was not determined whether cytoplasmic p65 was reduced or translocated into the nucleus upon H_2O_2 pulse. Thus, we next performed immunocytochemistry to observe directly p65 localization induced by H_2O_2 and E2 treatments.

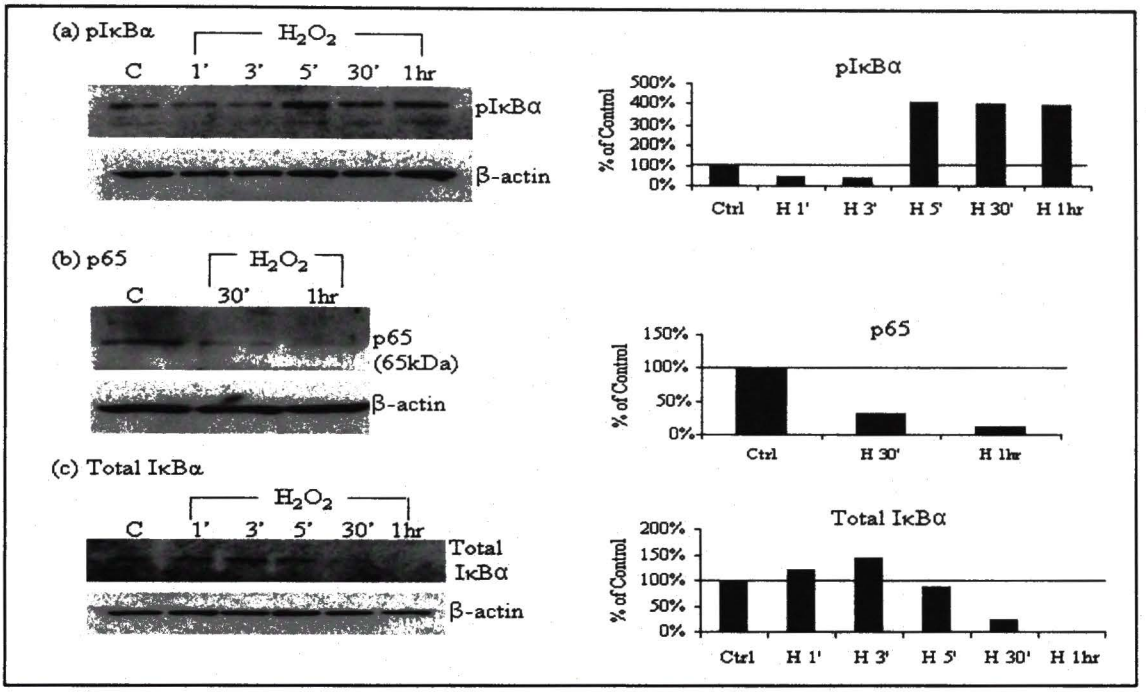


Figure 12. H_2O_2-induced pIκBα, total IκBα, and p65 in cytoplasmic extracts. H_2O_2 700μM was directly added into media in which HT22 cells had grown for 48 hours after seeding on tissue culture dishes. Then, we harvested each sample with cold PBS buffer, and added buffer A into the cell pellets. After 10 minutes incubation on ice, we centrifuged them, and took the supernatants that were supposed as cytoplasmic extracts. Then, we quantified the protein concentration, separated the cytoplasmic proteins on 10% SDS-PAGE gel, and transferred the gel to PVDF. The blot was blocked with 5% skim milk for 1 hour and incubated with primary antibody against (a) phosphorylation of IκBα at Ser 32, (b) p65, and (c) total IκBα. Each band was normalized by β-actin, and then band intensity was analyzed by densitometry.

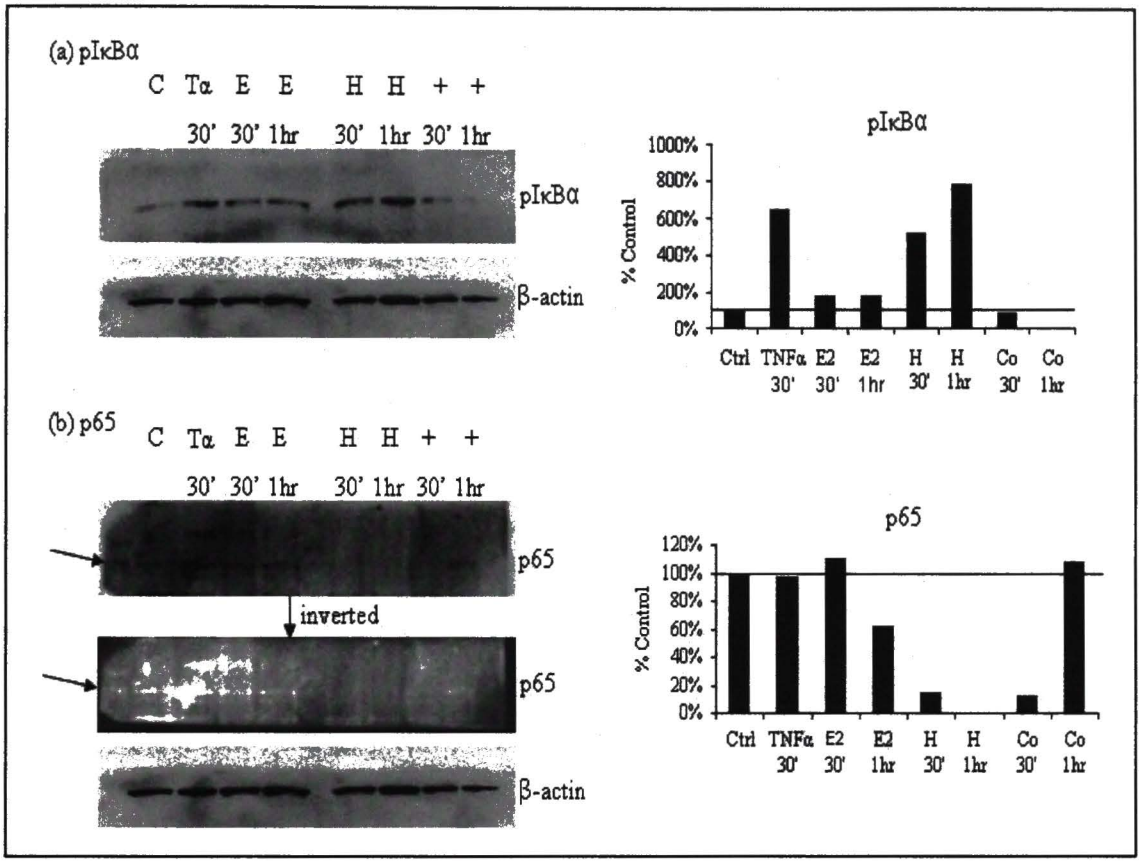


Figure 13. E2-mediated pIκBα, and p65 in cytoplasmic extracts.

The procedure for making cytoplasmic extracts and western blot analysis are described in Figure 12. Blots were probed with primary antibodies to detect for (a) pIκBα and (b) p65. This second cytoplasmic sample has cotreatment groups compared to Figure 12. Tα indicates a TNFα treated group, E indicates E2 treated group, H indicates H_2O_2 treated group,and + indicates cotreatment of H_2O_2 and E2.

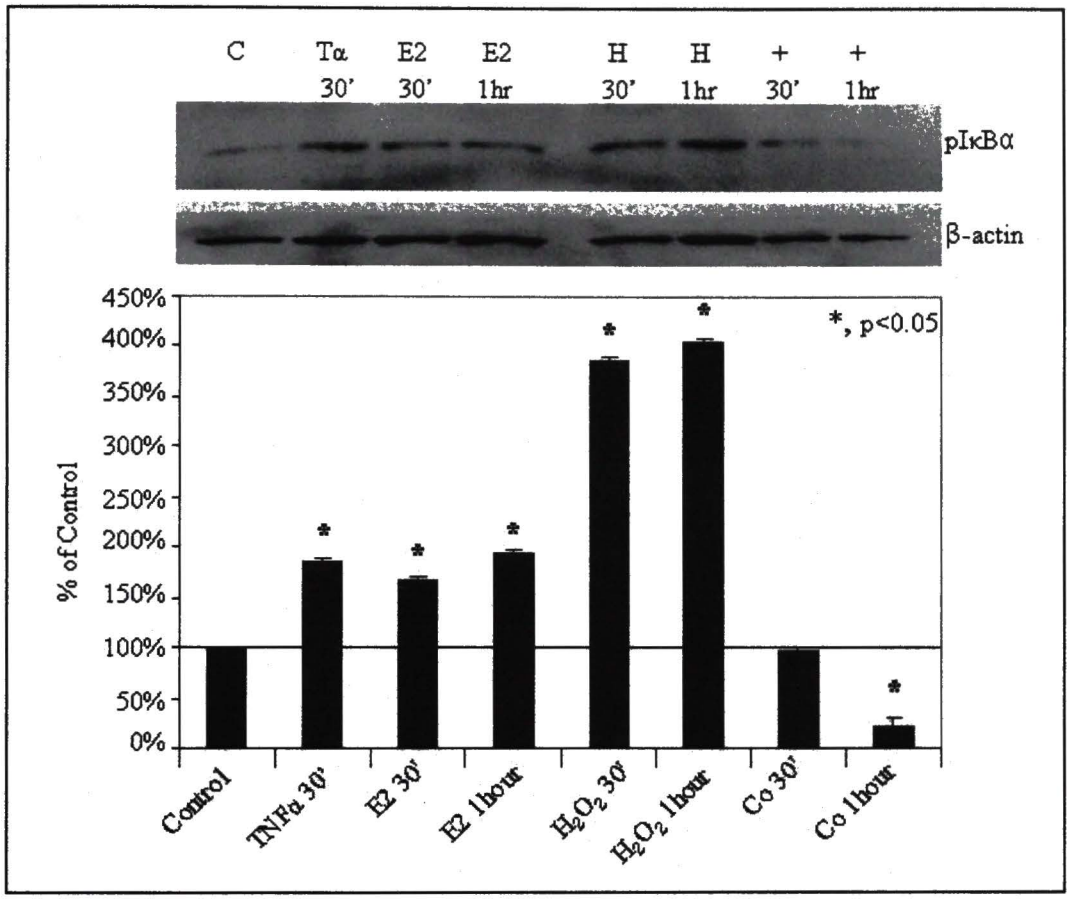


Figure 14. pIκBα in cytoplasmic extracts.

The procedure for making cytoplasmic extracts and western blot analysis are described in Figure 12. Error bars are based on variances between eight independent runs of three different samples. *, represents statistical significance between control and each group.

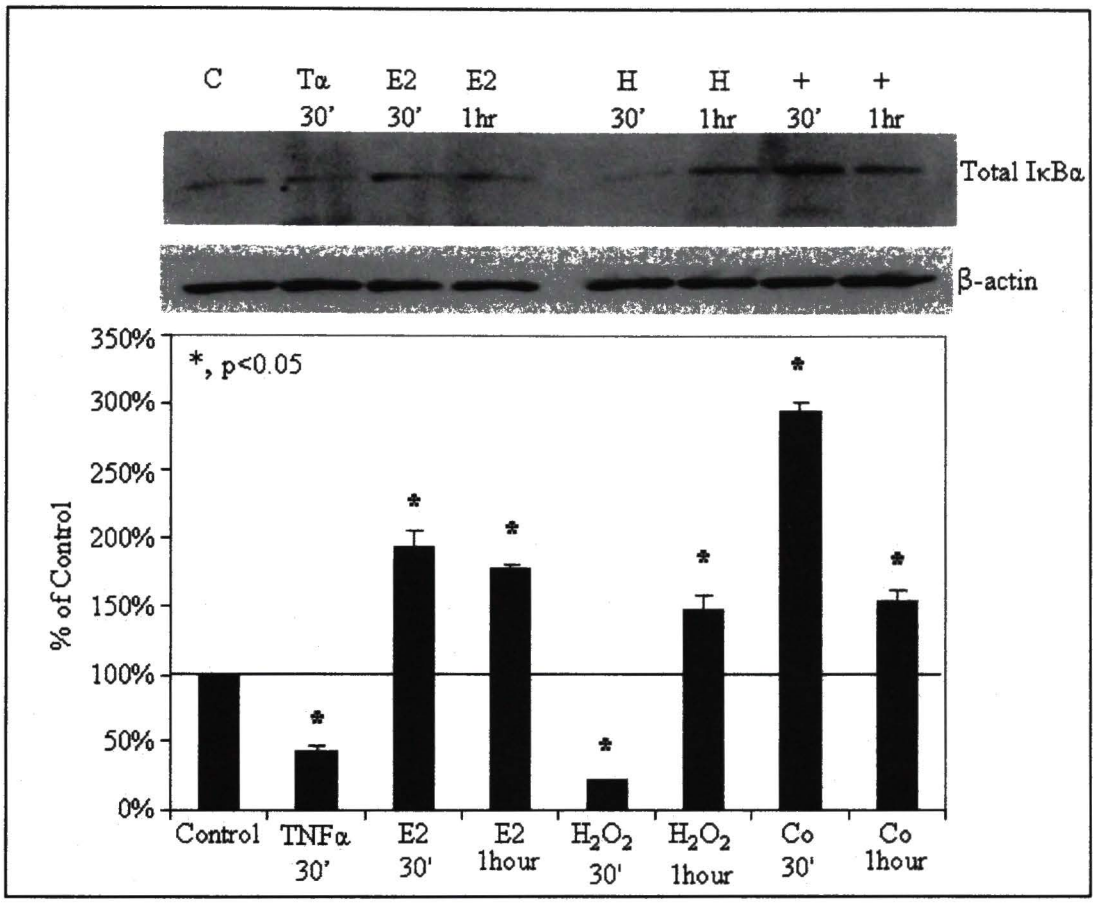


Figure 15. Total IκBα in cytoplasmic extracts.

The procedure for making cytoplasmic extracts and western blot analysis are described in Figure 12. Error bars are based on variances between three independent runs of one sample. *, represents statistical significance between control and each group.

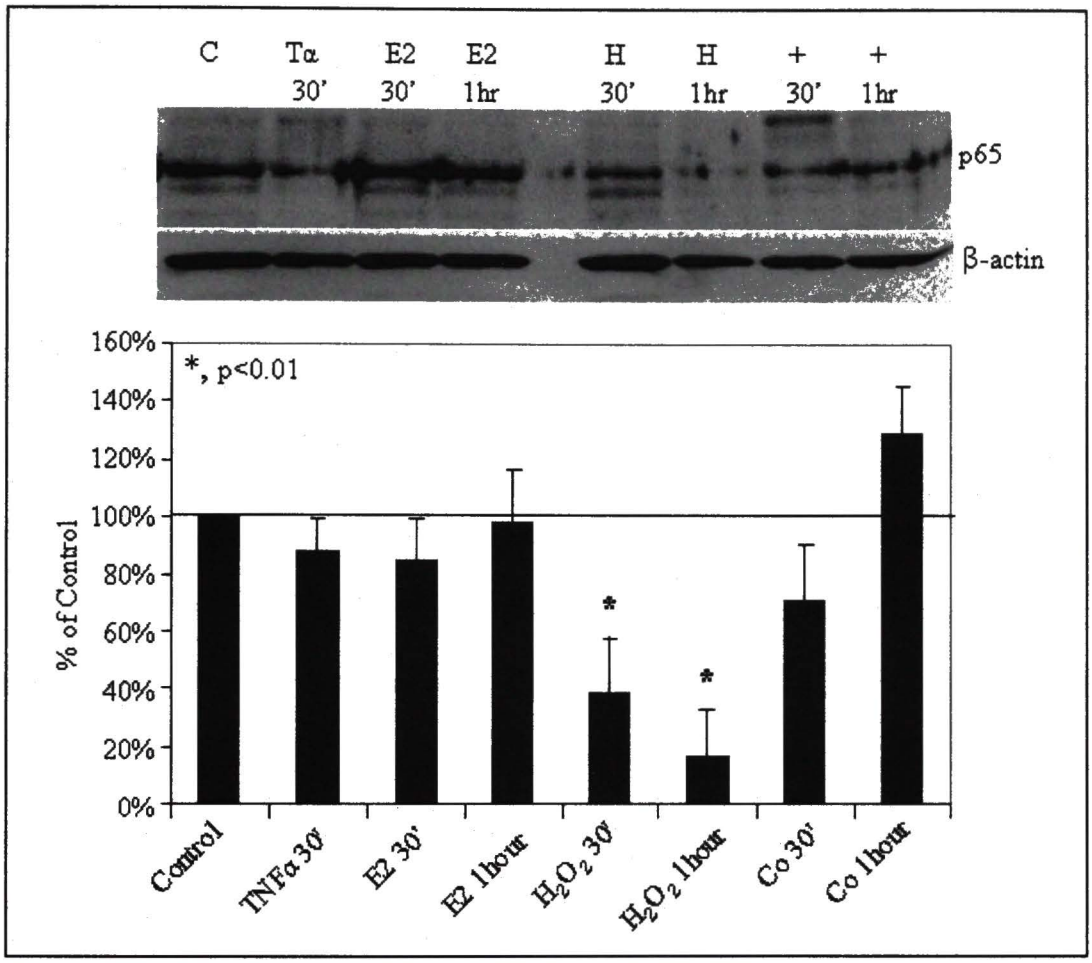


Figure 16. p65 in cytoplasmic extracts.

The procedure for making cytoplasmic extracts and western blot analysis are described in Figure 12. Error bars are based on variances between six independent runs of two different samples. *, represents statistical significance between control and each group.

C. Immunocytochemistry

1. Immunocytochemistry Pictures

The stimuli-induced pIκBα releases NFκB (p65) from cytoplasm, which in turn translocates into the nucleus and binds to a target gene promoter to control the gene expression. Thus, p65 nuclear translocation is also an important indicator for NFκB activation. Previously, we observed that cytoplasmic p65 was reduced by H_2O_2 and maintained by E2 treatment in cytoplasmic extracts (Figure 12, 13, and 16). Thus, it was not clear whether the H_2O_2-induced reduction of cytoplasmic p65 was occurred by its degradation in the cytoplasm or by its nuclear translocation. Thus, we performed immunocytochemistry (Figure 17, 18, and 19) to determine the exact p65 localization induced by H_2O_2 and E2, and further performed intensity analysis to quantify how much H_2O_2 induces p65 nuclear translocation, and how much E2 suppresses H_2O_2-induced p65 nuclear translocation (Figure 20).

We seeded HT22 cells on each cover glass in 24-well plates with $5x10^4$ cells per well. Then the cells were incubated for 48 hours, and treated with the same concentration of H_2O_2, E2, and TNFα as used in western blot analysis. Then we fixed the cells with 4% paraformaldehyde for 20 minutes, and incubated cells with primary antibody against p65 (1:100 dilution) for 24 hours. The next day, we incubated the cells with secondary antibody (1:200 dilution) which was conjugated with Alexa Flour 488, the green fluorescent signal, and mounted each cover glass on each cover slip with mounting solution that includes DAPI stainging. Then, we left all cover slips at room temperature

overnight, and put nail polish around the cover glass for sealing. Next, we took all the pictures of each treated group under a confocal microscopy. The HeLa cells were used as a positive control that showed NFκB activation by TNFα treatment.

In HeLa cells, the control group showed the cytoplasmic localization of green signal (p65), but the TNFα treated group showed the translocated p65 green signal into nuclei (Figure 17 (a)). As shown in Figure 17 (a), HeLa cells showed strong NFκB activation induced by TNFα. In other words, most of p65 were translocated into nuclei upon TNFα treatment in HeLa cells. In HT22 cells, the control group showed the cytoplasmic localization of p65 and both TNFα and H_2O_2 treated groups also showed the nuclear translocation of p65 (Figure 17 (b)). However, the p65 nuclear translocation in HT22 cells was not as strong as in HeLa cells. It showed the partial nuclear translocation of p65 signals instead of nearly complete translocation. Thus, p65 signals in HT22 cells were found both in the cytoplasm and the nucleus as well upon TNFα and H_2O_2 pulse even though the cytoplasmic signals are weaker than those in the control group. In contrast, more p65 signals were found in the cytoplasm than in the nucleus in cotreatment groups compared to TNFα and H_2O_2 treated groups (Figure 17 (b)).

Next, we needed to confirm these data shown in Figure 17, and to analyze how much of the p65 were translocated into nuclei by H_2O_2 and how much E2 suppressed H_2O_2-induced p65 nuclear translocation. Thus, we made a new sample and performed immunocytochemistry with the same experimental procedures. As we expected, HeLa cells showed strong p65 nuclear translocation upon TNFα treatment (Figure 18 (a)). In HT22 cells, both TNFα and H_2O_2 caused the partial nuclear translocation of p65 (Figure

18 (b)). However, E2 seemed to inhibit p65 nuclear translocation which was induced by H_2O_2 (Figure 18 (b)). We added several pictures stained with DAPI nuclear staining to show intact nuclei (Figure 19).

We observed the change of p65 localization induced by H_2O_2 and E2 treatment in HT22 cells through the pictures taken by a confocal microscopy. However, we could not conclude how much of the p65 signal was translocated or was not translocated into the nucleus with those pictures. Thus, we next quantified the p65 fluorescent intensity observed in the cytoplasm and the nucleus in each treated group.

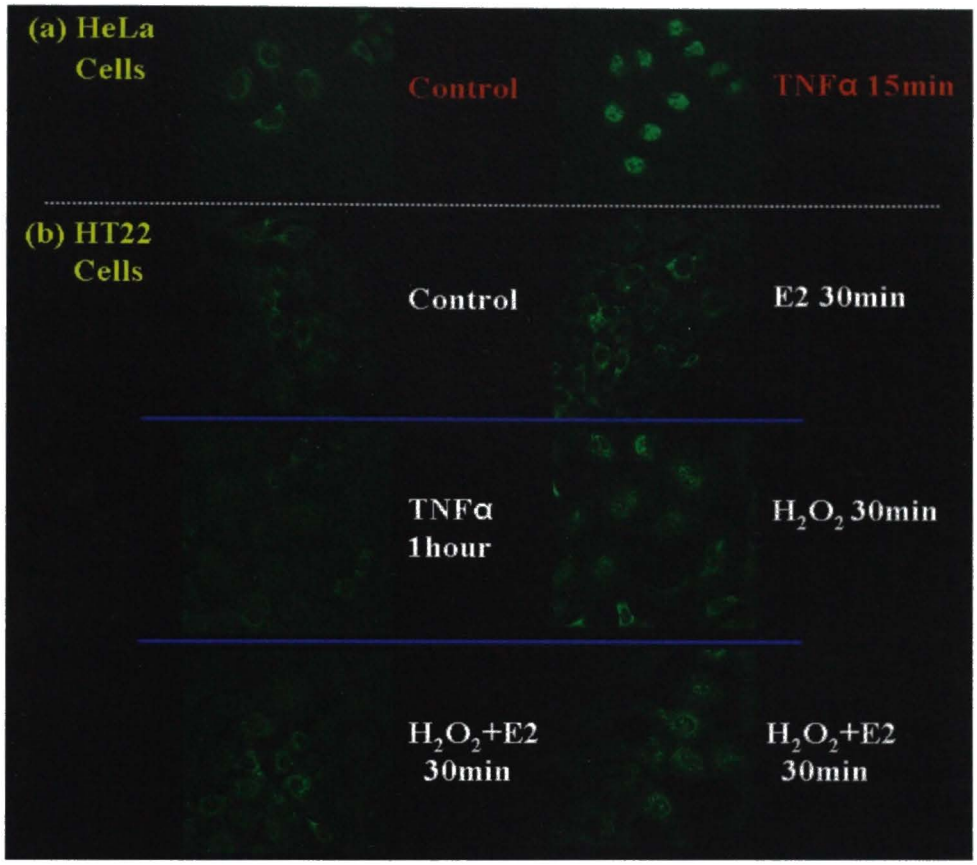


Figure 17. Immunocytochemistry.

The green signal indicates p65 localization in (a) HeLa and (b) HT22 cells.

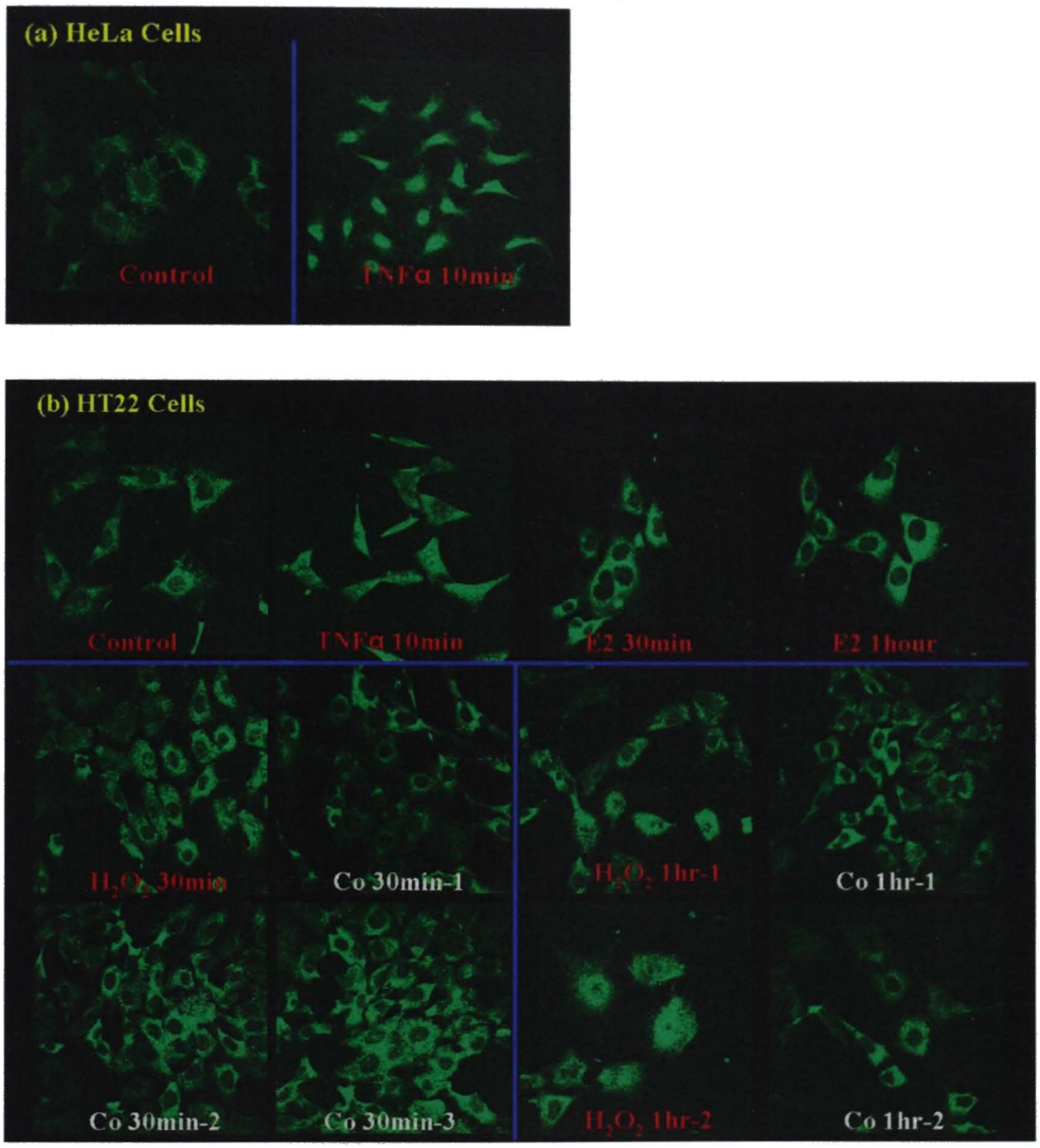
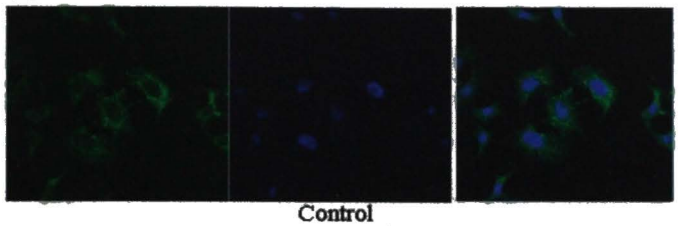


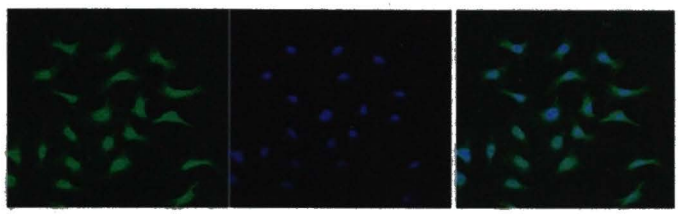
Figure 18. Immunocytochemistry.

The green signal indicates p65 localization in (a) HeLa and (b) HT22 cells.

(a) HeLa Cells

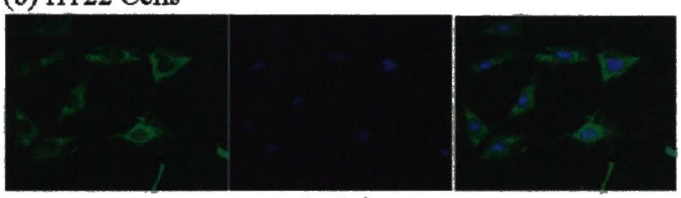


Control

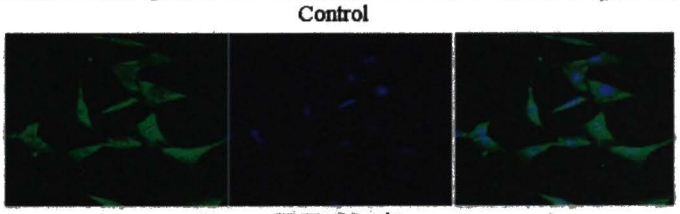


TNFα 10 min

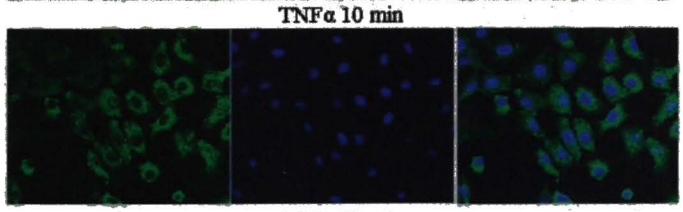
(b) HT22 Cells



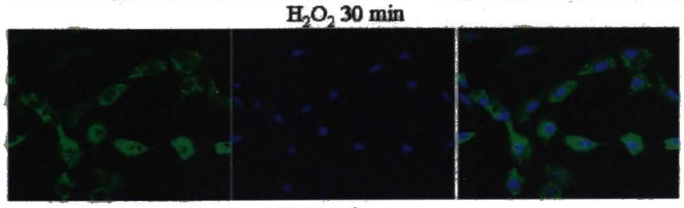
Control



TNFα 10 min



H_2O_2 30 min



H_2O_2 1 hour

Figure 19. Immunocytochemistry with DAPI nuclear staining.

The green signal indicates p65 localization shown in the fist picture, blue signal indicates an individual nucleus shown in the second picture. Merged pictures of p65 green signals and blue DAPI staining are shown in the last. The panel (a) shows HeLa cell groups and (b) shows HT22 cell groups.

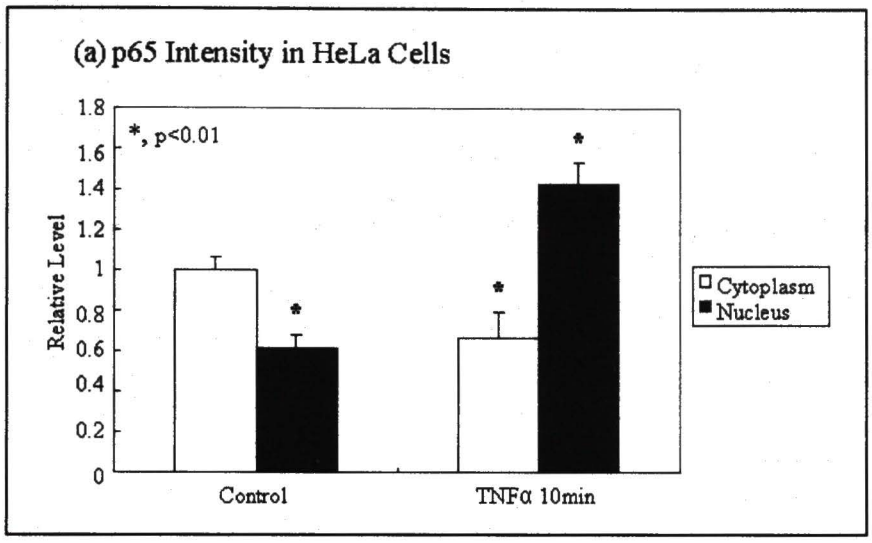
2. Intensity Analysis

To do the intensity analysis, we retook pictures of all groups with exactly same settings. Then, each picture was opened through an "Image J" program, and selected two regions of interests (ROI) in the cytoplasm and the nucleus from one cell (several background area were also selected). Then, the program reads the intensity of each ROI throughout the frames as numbers (when retook pictures for intensity analysis, we set the frame numbers as 14 throughout all pictures). Three frames were selected out of 14 for analysis, which were the highest, just above the highest, and just below the highest one. Then, mean intensity value for each cytoplasm, nucleus, and background were calculated using an excel program.

In HeLa cells, TNFα caused profound p65 translocation into nuclei (Figure 20 (a)). Also H_2O_2 caused p65 nuclear translocation in HT22 cells, but H_2O_2 didn't reduce cytoplasmic p65 a lot compared to TNFa treated group (Figure 20 (b)). Interestingly, E2 significantly inhibited the H_2O_2-induced p65 nuclear translocation in cotreatment groups (Figure 20 (b)).

In summary, all data from western blot analysis and immunocytochemistry indicate that H_2O_2 activates NFκB by inducing pIκBα, reducing total IκBα, and inducing p65 nuclear translocation. On the other hand, E2 suppresses the H_2O_2-induced NFκB activation by reducing the pIκBα, upregulating IκBα, and inhibiting p65 nuclear translocation.

Therefore, we conclude that E2 suppresses H_2O_2-induced NFκB activation in a neuronal cell line, HT22 cells.


(a) p65 Intensity in HeLa Cells
*, p<0.01
Relative Level
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
TNFα 10min
Cytoplasm
Nucleus

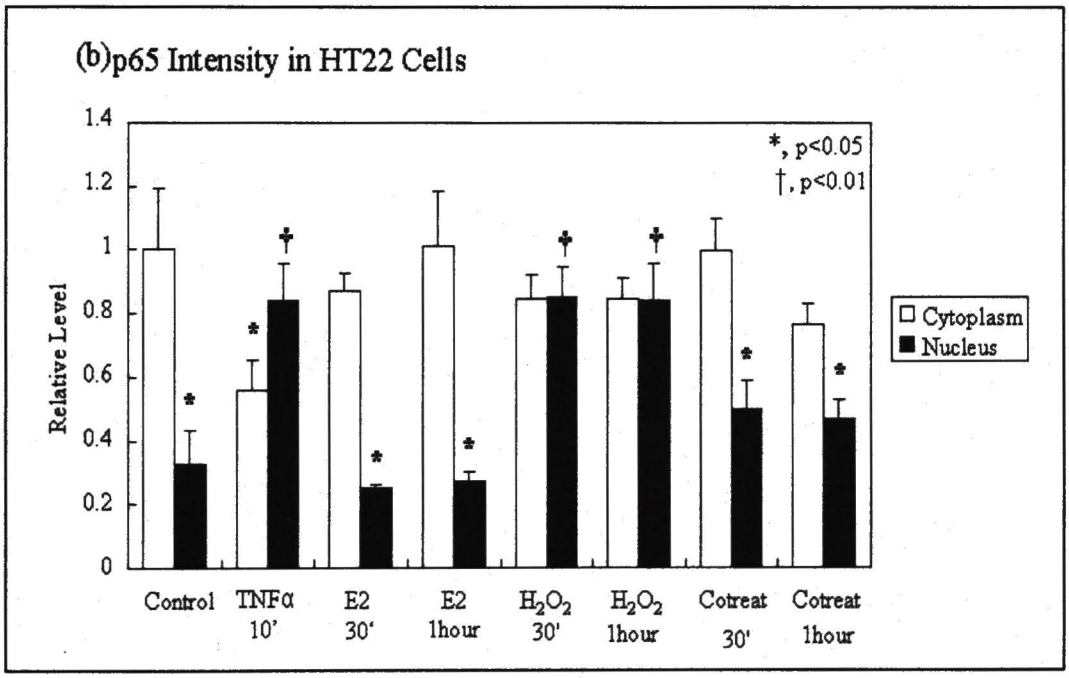

(b)p65 Intensity in HT22 Cells
*, p<0.05
†, p<0.01
Relative Level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
TNFα 10'
E2 30'
E2 1hour
H2O2 30'
H2O2 1hour
Cotreat 30'
Cotreat 1hour
Cytoplasm
Nucleus

Figure 20. Intensity analysis of p65 in HeLa and HT22 cells.

Total 15 cells (N=15) were selected for intensity analysis from each group, and two cytoplasmic- and two nuclear-regions were selected per one cell. The empty bar indicates cytoplasmic p65 intensity, and black bar indicates nuclear p65 intensity. Error bars are based on variances between 30 selected cytoplasm and 30 selected Nuclei (total 15 cells). *, represents statistical significance between control's cytoplasm and each group, and + indicates statistical significance between control's nucleus and the nucleus of each group.

CHAPTER IV

DISCUSSION

NFκB is known as a transcription factor that is activated during inflammatory and immune responses among other stimuli. Activated NFκB translocates into the nucleus where it binds to target gene promoter and controls gene expression with the help of cofactors. NFκB inhibitors, which have been tested for the treatment of inflammatory, immune diseases, and cancers, block various steps in the NFκB signaling pathway, including upstream IκB kinases (IKK), formation of IKK complex, IKK activation, phosphorylation of IκB proteins, protein degradation of pIκBs, upregulation of IκBs, phosphorylation of NFκB, p105 processing into p50, NFκB nuclear translocation, NFκB DNA binding, interaction between NFκB and its cofactors, and transactivation activities of NFκB on its target gene expression (Kalaitzidis and Gilmore, 2005).

The estrogen receptor (ER) is a member of the nuclear receptor superfamily, and also a well-known transcription factor involved in the regulation of reproduction, bone metabolism, cardiovascular system, and multiple CNS functions (Kalaitzidis and Gilmore, 2005). Particularly, 17β-estradiol (E2) has been shown neuroprotective effects in various cultured neurons against various toxicities including serum deprivation, mast cell activation, H_2O_2, superoxides, amyloid β peptide (Aβ), and glutamate-induced excitotoxicity (Green, 2000, Yi et al., 2005). In addition to these *in vitro* data, E2 also has shown neuroprotective effects *in vivo*. Stroke causes oxidative

stress, free radical activity, excitotoxicity, inflammatory response, mitochondrial dysfunction, and apoptosis, which were suppressed by E2 (Yang et al., 2003). Regarding the mechanism under these E2-mediated neuroprotection, ER-dependent genomic mechanism has been centrally considered. However, ER-independent nongenomic mechanism was recently proposed because of the rapid and transient effects of E2 on signaling pathways, membrane receptors or synaptic transmission instead of delayed and persistent effects of E2 seen in the genomic mechanism (Singh et al., 2006). We and others found that non-ER binding estrogen analogues such as 17α-estradiol, the enantiomer of 17β-estradiol, and ZYC compounds (showed in Chapter III result, A) also have similar potent of E2 in neuroprotection (Perez et al., 2005, Yi et al., 2007). Therefore, it has been still debated whether the neuroprotective effects of E2 are mediated by genomic or nongenomic mechanism. Interestingly, E2 shows anti-inflammatory effects by blocking the NFκB activation at various steps (Kalaitzidis and Gilmore, 2005) in a number of experimental models, such as rat astroglial cultures, brain endothelium, microglial activation, rat cerebral blood vessels, and acute spinal cord injury in rats (Dodel et al., 1999, Galea et al., 2002, Bruce-Keller et al., 2000, Ospina et al., 2004, Ghisletti et al., 2005, Sribnick et al., 2005). Thus, it has been proposed that there might be mutual cross-talk between ER or estrogen and NFκB (Kalaitzidis and Gilmore, 2005).

However, there are few reports that investigated the potential neuroprotective mechanism of E2 mediated by suppression of NFκB in oxidative stress model. Thus, we endeavored to determine the potential mechanism of E2-mediated neuroprotection

via suppression of NFκB in the prooxidant-induced oxidative stress because we have previously shown that E2 is able to attenuate the MCAO mediated activation of NFκB and apoptosis (Wen et al., 2004).

In this project, we confirmed that H_2O_2 causes cell death in transformed neuronal cells (HT22) within an hour (Figure 1 and 2) and E2 rescues the cells from H_2O_2-induced cell death by 30-40% (Figure 3, 4, 5, and 6). Moreover, H_2O_2 activated NFκB by increasing phosphorylation level of IκBα (Figure 14), decreasing total IκBα (Figure 15), presumably due to ubiquitination and degradation of pIκBα. We also showed that H_2O_2 treatment induces translocation of p65 into the nucleus (Figure 16, 17, 18 (b) and 20 (b)). On the other hand, simultaneous treatment with E2 suppressed the H_2O_2-induced NFκB activation by decreasing in phosphorylation of IκBα (Figure 14), upregulating IκBα expression (Figure 15), and inhibiting p65 nuclear translocation (Figure 16,17, 18 and 20 (b)). Therefore, the net effect of E2 was attenuation of H_2O_2-induced NFκB activation within one hour. However, we did not establish how these early cellular responses to NFκB are associated with neuronal cell death or survival. If additional experiments showed the relevance of H_2O_2-induced NFκB activation to neuronal cell death, and the relevance of E2-mediated NFκB suppression to neuronal cell survival, we may suggest that one of the mechanisms of E2-mediated neuroprotection against oxidative stress is through the suppression of NFκB activation. Interestingly, E2-mediated NFκB suppression occurred within an hour, suggesting that E2-mediated NFκB suppression might be an ER-independent event. However, the mechanisms by which E2 suppresses H_2O_2-induced phosphorylation of IκBα, decrease

in total IκBα, and enhanced p65 nuclear translocation need to be better understood. It is not clear yet whether the E2-mediated inhibition of p65 nuclear translocation is due to an indirect effect of E2 in reducing phosphorylation of IκBα and preventing the degradation of IκBα, or a direct effect of E2.

This set of studies showed that H_2O_2 treatment caused a four-fold increase in pIκBα as compared to the untreated group; further, the presence of E2 significantly reduced the increased pIκBα level induced by H_2O_2 (Figure 14). This E2-mediated suppression of IκBα phosphorylation was comparable to control (Figure 14). What mechanisms do lead E2 to dramatically reduce phosphorylation level of IκB? There are several possible ways: E2 may reduce kinases activities that phosphorylate IκB, increase phosphatase expression and/or activities that dephosphorylate pIκBα, and enhance degradation of pIκBα.

We have recently shown that serine/threonine phosphatase activities are essential in E2-mediated neuroprotection against glutamate-induced excitotoxicity and oxidative stress (Yi et al., 2005). General phosphatase inhibition as well as glutamate is cytotoxic in neuronal and glial cells. As expected, E2 prevented the glutamate-induced cell death; however, E2 didn't protect the cell against phosphatase inhibitor-induced cell death. Further, in the presence of phosphatase inhibitors, E2-mediated neuroprotection against glutamate toxicity was not observed. We have also shown that E2 and its analogues abolished the glutamate-induced decreases in protein phosphatase expression levels (Yi et al., 2007) and activities (unpublished data). Therefore, it is possible that E2-mediated suppression of H_2O_2-induced IκBα phosphorylation may be

due to the maintenance of phosphatase activities, which would result in dephosphorylation events.

According to our data, single-treatment of E2 also induces pIκBα by a two-folded compared to control (Figure 14), even though the induced pIκBα (two-folded) is much less than that (four-folded) of H_2O_2-induced. Thus, it seems likely that E2 alone stimulates (a) potential protein kinase(s) that phosphorylate(s) IκBα. However, we did not investigate the potential E2-stimulated protein kinases and do not have answer for the cellular function of E2-meduated induced pIκBα. Moreover, we still need to figure out several questions regarding pIκBα: which protein kinases are involved in H_2O_2-induced dramatic increase in pIκBα, how E2 reduces H_2O_2-induced pIκBα in cotreatments even though E2 alone increases pIκBα, and what the differences in cellular functions are between E2-mediated (two-folded) and H_2O_2-mediated (four-folded) induced pIκBα. There is a theory called "optimal activation hypothesis" proposed in 2005 (Kaltschmidt et al., 2005).

This theory hypothesizes that too high or too low NFκB activation cause neuronal death. According to this theory, it is possible to suggest that E2-mediated induced pIκBα may not cause any damages on HT22 cells, but may help cells maintain normal cellular function better than control group. In fact, we did not observe any cytotoxic effects of E2 single-treatment on HT22 cells. Also, it is possible to suggest that H_2O_2-induced dramatic increases in pIκBα may be too high that may cause neuronal cell death. In summary, E2 alone induces pIκBα (two-folded), and H_2O_2 dramatically induces pIκBα (four-folded) more than E2 does. However, in the presence of E2,

H_2O_2-induced pIκBα was dramatically inhibited. Back to the our question regarding how E2 reduces H_2O_2-induced pIκBα, it is unlikely that E2 reduces H_2O_2-induced pIκBα through decreasing in protein kinase expression and/or activities because single-treatment of E2 also induces pIκBα,

Our data also showed the changes in total IκBα in Figure 15. As expected, H_2O_2 significantly reduces total IκBα in 30 minutes due to the degradation of pIκBα, but restores the reduced total IκBα up to the control level in an hour due to the upregulation of IκBα to prevent persistent NFκB activation as an auto-regulatory function (Sun et al., 1993). According to our data, E2 alone increases total IκBα by a two-folded, which may suggest that E2 participates in upregulation of IκBα transcriptionally or translationally. Then, IκBα will bind to NFκB dimer to block further activation. More interestingly, simultaneous treatment of E2 for 30 minutes dramatically increases total IκBα compared to H_2O_2 30 minutes group. When we consider that E2 reduces H_2O_2-induced pIκBα (Figure 14), the increased total IκBα may be resulted from IκBα upregulation. However, what is the mechanism by which E2 can increase in IκBα expression?

Since we are unaware of estrogen response elements on IκBα promoter (Ray et al., 1994), it is possible that E2 translationally up-regulates IκBα. Various laboratories have shown E2 involvement at the protein translation level but not at the transcription level (Milner et al., 2001, Akama and McEwen, 2003). Our laboratory has also shown that E2 can modify protein translation at the dendrites (unpublished data). ERα has been shown to be located in neurite spines suggesting non-genomic action of estrogen

on the protein translation, synaptic growth, and calcium flows in dendrites (Milner et al., 2001). Also, estrogen alone was found to stimulate protein kinases, such as AKT (protein kinase B), which in turn phosphorylated eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) that was involved in the rapid increase of PSD-95 protein synthesis but not in mRNA levels in dendritic spines (Akama and McEwen, 2003). Recently, our laboratory reported that glutamate reduces protein phosphatase 1 (PP1) protein expression by 50% compared to control in 15 minutes, and ZYC3 compound, an estrogen analog, increases PP1 expression level up to three-folded in 30 minutes (Yi et al., 2008). Thus, it is possible that E2 or its analog upregulates protein expression in 30 minutes. In summary, H_2O_2 dramatically reduces total IκBα in 30 minutes but upregulates total IκBα in one hour because its auto-regulatory function presumably starts to action to prevent further NFκB activation. E2 alone increases in total IκBα (two-folded) and moreover, simultaneous cotreatment of E2 increases in total IκBα (three-folded) dramatically in 30 minutes against H_2O_2-induced significant reduction of total IκBα.

In summary, E2 antagonizes the action of H_2O_2 on NFκB activation by reducing pIκBα and upregulating total IκBα. If E2 reduces pIκBα only, the total IκBα expression would not be expected to change. However, the present data shows the increased level of total IκBα induced by E2. Therefore, we may suggest that E2 may act on H_2O_2-induced NFκB activation through reducing pIκBα and increasing IκBα expression at the same time. Further, we observed that E2 inhibited H_2O_2-induced NFκB (p65) nuclear translocation through immunocytochemisty (Figure 17, 18, and

20). What is(are) a possible mechanism(s) by which E2 inhibits H_2O_2-induced p65 nuclear translocation?

It is possible that E2 directly inhibits NFκB (p65) nuclear translocation (Dodel et al., 1999, Galea et al., 2002). Dodel et al. showed the effects of E2 on p65 nuclear translocation not on the pIκBα and total IκBα. However, it is still possible the indirect effects of E2 on p65 nuclear translocation by reducing upstream activities, such as reduced IκBα phosphorylation, reduced pIκBα degradation, and increased total IκBα. Further experimentation must be performed using specific pIκBα inhibitors to determine if these upstream events are necessary for E2–mediated suppression of NFκB nuclear translocation during oxidative stress.

In summary, we observed that H_2O_2 activates NFκB signaling pathway, and E2 suppresses H_2O_2-induced NFκB activation in a short time period (up to one hour). However, what are the biological functions of activated NFκB in H_2O_2-induced neuronal cell death, and what are the beneficial effects of E2 by suppression of NFκB at early time points?

It has been debated whether NFκB is involved in pro-apoptotic or anti-apoptotic in the nervous system (Lipton S. A., 1997). There are numerous potential factors that determine the dual role of the NFκB, such as cell texture, specific stimulus, stage of neuronal development, time of stimulation, interaction between neurons and glial cells, and differential stimulation of NFκB composition (Lipton, 1997). Interestingly, our immunocytochemistry data shows that a single E2 treatment induced p65 signal around the perinuclear region where most mitochondria are localized (Figure 18). Moreover,

E2 treatment alone seems to induce pIκBα, not to the extent seen with H_2O_2 treatment, but E2 alone does not induce p65 nuclear translocation (Figure 14, 17, 18 and 20). Thus, where does E2-induced NFκB go, if E2-induced pIκBα releases NFκB?

According to our immunocytochemistry data, both TNFα and H_2O_2 treatment induces lots of p65 nulcear translocation than cytoplasmic distribution of p65 (Figure 20). Thus, it seems likely that nuclear translocation of p65 may be associated with death signals due to the cytotoxic effects of H_2O_2, while it is not sure whether cytoplasmic p65 is located just in the cytoplasm or in a particular cytoplasmic organelle. As we mentioned above, E2 single-treatment induces stronger p65 signals than control group around perinuclear region at which most mitochondria are located, suggesting the possibility of E2-induced p65 translocation into a mitochondrion (Figure 18).

Mitochondrion is an essential organelle for cellular energy production (ATPs). It has own genes associated with making enzymes involved in oxidative phosphorylation, some tRNA, and rRNA (Fukui et al., 2008). Since the lack of mitochondrial DNA repair systems, a mitochondrion is susceptible to ROS produced during respiratory chain reactions (Fukui et al., 2008). The function of NFκB on the mitochondrial gene expression has been already proposed (Cogswell et al., 2003). They reported that NFκB and IκBα are localized in the mitochondria and associated with the mRNA level changes of cytochrome c oxidase III, and cytochrome b. Moreover, we have previously reported the presence of ERβ on the mitochondria (Yang et al., 2004). Taken together, is it possible that E2 induces NFκB translocation into the mitochondria instead of

nucleus to command NFκB to turn some genes on for enhanced function of a mitochondrion in E2 single-treatment or for protection cells from H_2O_2-induced mitochondrial damages in co-treatment? Or is it possible that E2 activates ERβ on the mitochondria and then, ERβ crosstalks with NFκB as a transcription factor on the mitochondria? In summary, differential translocation of p65 into either the nucleus or the mitochondria may be relevant to the role of NFκB as a pro-apoptotic or anti-apoptotic. These all related information led us to make a hypothesis that E2-induced pIκBα could subsequently induce p65 translocation into the mitochondria where NFκB may function as a transcription factor to commit cells to turning survival signals on both under normal and cytotoxic conditions in HT22 cells.

In conclusion, we observed that E2 suppresses H_2O_2-induced NFκB activation in HT22 cells at early time period, up to one hour. However, our data set raised many questions: if these early events are necessary for inducing neuronal cell death or survival, how E2 upregulates IκBα, reduces H_2O_2-induced pIκBα, and inhibits p65 nuclear translocation, how H_2O_2 induces pIκBα, if E2 induces p65 translocation into a mitochondrion, and what the potential function of p65 is in the mitochondria. Also, we observed that ZYC26 compound is more potent than E2 in neuroprotection against H_2O_2-induced neurotoxicity (Figure 6). It would be very interesting to determine the mechanism of the non-feminizing compound in its neuroprotective effects.

Excessive ROS disrupts normal cellular functions by targeting macromolecules, proteins, DNA, signaling pathways, and gene transcriptions (Balaban et al., 2005). These ROS-induced oxidative damages are associated with normal aging,

neurodegenerative diseases, and inflammation (Balaban et al., 2005). If we develop an antioxidant, it may delay the onset or the progress of oxidative stress-induced neuronal damages. E2 has been shown antioxidant effects by reducing ROS production, lipid peroxidation, maintaining mitochondrial functions, and reducing calcium overloading (Wang et al., 2003). Thus, we induced oxidative stress in a neuronal cell line, HT22 cells, by treatment a strong pro-oxidant H_2O_2 to determine if E2 protects cells from the H_2O_2-induced neurotoxicity. E2 rescues cells from the strong H_2O_2 toxic effects by 30%-40%. Moreover, we observed that E2 suppresses H_2O_2-induced NFκB activation during cotreatment for 30 minutes and 1 hour. We didn't further investigate how much this early NFκB activation or deactivation is relevant to neuronal cell death or survival. However, if E2-mediated suppression of NFκB is proven to be a key factor in neuroprotection in ROS-induced oxidative stress, and then, we may suggest not only a potential neuroprotective mechanism of E2, but also one of the potential clinical application for the treatment of oxidative-related diseases.

CHAPTER V

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