A Dissertation for the Degree of Doctor of Philosophy

Coffee phenolic phytochemicals and kaempferol protect against oxidative damage of neuronal cells and memory impairments

커피 페놀성 파이토케미컬과 캠페롤의 신경세포의 산화적 손상과 기억장애에 대한 보호 효능

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Abstract

Oxidative stress and inflammation are strongly associated with neurodegenerative disorder such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS). Oxidative damage is induced by reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) and amyloid beta plays a critical role in the development and progression of AD via the generation of ROS such as H_2O_2. Cytokines such as TNF-α also play a key role in inflammatory processes in AD. High levels of pro-inflammatory cytokines are shown in the brains of dementia. Oxidative stress and inflammation mediate cellular apoptosis in damaged neurons, which might impair brain function. Therefore, natural compounds to target oxidative stress and neuroinflammation are attractive for prevention of neurodegenerative disease.

Recent studies suggest that moderate coffee consumption may reduce the risk of neurodegenerative diseases such as AD or PD. Caffeine is considered to be primarily responsible for the neuropharmacological effects of coffee, and its neuroprotective effects are demonstrated by several studies. However, coffee is a major dietary source of phenolic compounds. Chlorogenic acid (5-*O*-caffeoylquinic acid) is a major phenolic compound in coffee. However, neuroprotective effect of decaffeinated coffee or coffee

phytochemical such as chlorogenic acid and its molecular mechanisms remain to be clarified.

In this study, I confirmed that coffee, decaffeinated coffee and chlorogenic acid attenuated H_2O_2-induced PC12 and cortical neuronal cell death and apoptosis. These are characterized by nuclear condensation and DNA fragmentation, through inhibition of caspase-3 activation, poly(ADP-ribose)polymerase cleavage and the downregulation of anti-apoptotic proteins including Bcl-2 or Bcl-X_L. The accumulation of intracellular ROS and activation of c-Jun N-terminal protein kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) in H_2O_2-treated PC12 cells were diminished by decaffeinated coffee and chlorogenic acid. Coffee, both normal and decaffeinated, protect neurons against H_2O_2-induced apoptosis with similar effect, suggesting that chlorogenic acid might contribute to neuroprotective effects of coffee.

In this study, I used animal model of scopolamine-induced memory impairment to prove the effect of decaffeinated coffee on memory. Oral gavage administration of decaffeinated coffee inhibited scopolamine-induced memory impairment, which was measured by Morris water maze test and passive avoidance test. Decaffeinated coffee suppressed scopolamine-mediated elevation of tumor necrosis factor-α (TNF-α) and stimulation of

nuclear factor-κB (NF-κB) pathway (i.e., phosphorylation of IκBα and p65) in the rat hippocampus. These findings suggest that decaffeinated coffee may prevent memory impairment in human through the inhibition of NF-κB activation and subsequent TNF-α production.

Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one) is a natural flavonoid isolated from tea, mushrooms, kale, broccoli, and other plant sources. Kaempferol inhibited 4-HNE-mediated apoptosis, characterized by nuclear condensation, down-regulation of antiapoptotic protein Bcl-2, and activation of proapoptotic caspase-3. Kaempferol inhibited 4-HNE-induced phosphorylation of c-Jun N-terminal protein kinase (JNK). More importantly, kaempferol directly bound p47*phox*, a cytosolic subunit of NADPH oxidase (NOX), and significantly inhibited 4-HNE-induced activation of NOX. The antiapoptotic effects of kaempferol were replicated by the NOX inhibitor apocynin, suggesting that NOX is an important enzyme in its effects. My results suggest that kaempferol attenuates 4-HNE-induced activation of JNK and apoptosis by binding p47*phox* of NOX and potently inhibiting activation of the NOX-JNK signaling pathway in neuron-like cells. Altogether, these results suggest that kaempferol may be a potent prophylactic against NOX-mediated neurodegeneration.

Taken together, these results indicate that coffee and decaffeinated coffee prevent apoptotic neuronal death by oxidative stress and that chlorogenic acid might be largely responsible for these effects. Decaffeinated coffee might protect brain against memory impairment by attenuating NF-κB-TNF-α-mediated tissue injury in the hippocampus. Kaempferol may act through direct binding to p47*phox* and inhibit 4-HNE-induced NOX activation and JNK-mediated apoptosis in neuron-like cells. These results suggest that regular consumption of coffee, decaffeinated coffee, chlorogenic acid and kaempferol might be beneficial on brain health.

Key words: *coffee; decaffeinated coffee; chlorogenic acid; oxidative stress; neurodegeneration; scopolamine; memory; TNF-α; kaempferol; 4-hydroxynonenal; NADPH oxidase*

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Contents

Chapter 1.

Regulation of Neurodegenerative Disease by Dietary Phytochemicals: A Review

Abstract

Oxidative stress and inflammation are damaging processes and major contributors to apoptosis in cells of the neurosystem. Excessive levels of reactive oxygen species (ROS) can arise within neuronal cells as a result of mitochondrial dysfunction, as well as hyperactivated NADPH oxidase (NOX) and xanthine oxidase. Plaque formation through amyloid beta (Aβ) has also been directly linked to the development and progression of Alzheimer's disease (AD), through a mechanism thought to involve the generation of ROS. Aldehyde products of lipid peroxidation including 4-hydroxynonenal are additional key mediators of neuronal apoptosis. Sources of oxidative stress such as NOX are therefore potential targets of novel therapeutic strategies for the treatment or prevention of neurodegenerative diseases. Inflammation is another mechanism shown to have close associations with neurodegenerative disease. Activated microglia produce high levels of inflammatory cytokines, and these contribute to neuronal apoptosis primarily through MAPK pathway activation. In parallel, both oxidative stress and inflammation can result in neuronal apoptosis through JNK and p38 activation, which influence several key regulators including mitochondria-related proteins (Bcl-2, Bax), p53, tau and β-secretase. In contrast, ERK activation can attenuate neuronal cell apoptosis by regulating the expression of brain-derived neurotrophic factor (BDNF) and Bcl-2

protein levels. Phytochemicals are bioactive compounds found in plants, and many have been shown to exert influential effects on these pathways. Red wine, cocoa and coffee are widely-consumed beverages and thus major dietary sources of phytochemicals. Resveratrol, quercetin, myricetin and kaempferol in red wine have been shown to protect neurons against oxidative stress and inflammation-mediated neurodegeneration. Human and animal studies have confirmed that cocoa consumption modestly improves cognitive performance, while neuroprotective effects have been reported for epicatechin, catechin and procyanidin B2, major cocoa phytochemicals. Recent epidemiological and animal studies focused on coffee consumption have demonstrated the existence of an inverse association with the risk of neurodegenerative disease. Coffee contains high levels of chlorogenic acid, which can suppress oxidative stress-mediated neuronal cell death. Collectively, these studies suggest that phytochemicals in red wine, cocoa and coffee can target the processes of oxidative stress and inflammation, leading to the inhibition of neuronal apoptosis and neurodegeneration. Therefore, the regular consumption of proper dietary phytochemicals could be a convenient lifestyle change that confers benefits for brain health. Further studies will be needed to determine both the direct molecular mechanisms involved, as well as the pharmacokinetic and pharmacodynamic properties of these phytochemicals.

1.1. Introduction

In many of the world's developed and aging societies, neurodegenerative diseases have rapidly come to the fore as a major medical challenge. Alzheimer's disease (AD) is the most common neurodegenerative disease, affecting approximately 16 million people worldwide [1]. In Korea, 8.4 % of persons aged 65 and over were afflicted with the disease in 2008, with that percentage projected to increase to 13% by 2050 [2]. The relative duration of AD after diagnosis is approximately 8-20 years, while cancer and acquired immune deficiency syndrome (AIDS) are 5-6 years, and heart disease is 3-4 years. Therefore, the social and financial costs of labor for caregivers of AD patients present a significant social issue. AD is the sixth-leading cause of death in the United States, and the only cause of death among the top 10 that cannot be cured [3]. Between 2000 and 2008, deaths attributed to AD increased 66 percent, while those attributed to the number one cause of death, heart disease, decreased by 13 percent [3]. These statistics highlight the need to focus renewed attention on novel strategies for the treatment of this deadly disease.

Neurodegenerative disease refers to the progressive loss of structure and function of neurons, often leading to irreversible cell death [4]. They can be divided into two groups: conditions that cause problems with motor function, and conditions related to dementia. Alzheimer's disease (AD),

Parkinson's disease (PD), Huntington's disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis and Creutzfeldt-Jakob disease are all prominent examples. Neurodegeneration is the common pathway shared by these diseases, and a deeper understanding of this process may provide rationale for neuroprotective strategies using dietary phytochemicals.

This review aims to summarize the broad mechanisms involved in neurodegeneration, and focuses on the important role of oxidative stress and inflammation. I also discuss new research into the neuroprotective effects of phytochemicals in widely-consumed beverages such as red wine, cocoa and coffee, inferring that simple dietary changes could have repercussions on the risk of later neurodegenerative disease.

1.2. Mechanisms of neurodegeneration

1.2.1. Oxidative stress in neurodegenerative disease.

Oxygen is essential for the maintenance of membrane potential, as well as the release and storage of neurotransmitters [5]. However, brain tissue is highly complex and particularly susceptible to the damaging effects of oxidative stress. Neurons also consume high levels of adenosine triphosphate (ATP) which entails a high demand for oxygen [5]. Adding further complexity to this situation is the fact that neurons are known to express low levels of endogenous antioxidant enzymes and high levels of

iron and copper [6]. These properties render the brain relatively more susceptible to oxidative stress than other organs.

Amyloid beta (Aβ) plays a critical role in the development and progression of AD via the generation of reactive oxygen species (ROS) such as H_2O_2 [7]. ROS activity increases Aβ production and accumulation by stimulating β- and γ-secretases [8]. While the oxidative activity of H_2O_2 is relatively low compared to other ROS types, its higher stability and diffusion properties enhance its toxicity [5]. In neurodegenerative disorders, ROS mediates cellular apoptosis in damaged neurons, leading to the impairment of brain function.

The oxidative modification of lipids plays an important role in cell signaling mechanisms including neuronal apoptosis [6]. 4-Hydroxynonenal (HNE) is a major aldehyde product generated by lipid peroxidation of cellular membranes [9]. 4-HNE is highly elevated in the brains of patients with AD, PD and ALS [10, 11]. Increased alkenal concentrations in AD patients have been shown to correspond to regions of the brain showing the most striking histopathologic alterations [12], and 4-HNE has been shown to have oxidative effects, increasing intracellular ROS and eliciting apoptosis in neuronal cells [13].

NADPH oxidase (NOX) is a transmembrane/cytosolic multi-subunit enzyme that transfers electrons from NADPH to molecular oxygen,

producing the highly damaging superoxide radical [13]. NOX in both neurons and astroglia is a major source of oxidative stress in the mammalian brain [16], and hyperactivated NOX has been observed in the brains of AD and PD patients [17, 18]. Therefore, the manipulation of NOX activity may represent a novel therapeutic strategy for the control of neurodegenerative diseases [15].

The significance of oxidative stress in neurodegeneration has caused has generated recent research to focus on free radical-scavenging antioxidants for the inhibition of neurodegenerative disease development. However, antioxidants that target ROS such as vitamin C, vitamin E and coenzyme Q10 have not yet shown suitable efficacy in clinical trials [14], showing that increasing antioxidants alone may not be sufficient for clinical effects. However, it is highly likely that the identification and effective inhibition of major sources of oxidative stress will lead to new progress in the treatment of neurodegenerative diseases [15].

1.2.2. Inflammation in neurodegenerative diseases.

Inflammation is related to many major neurodegenerative diseases including AD, PD, ALS and multiple sclerosis [19]. Microglia are immune cells in the brain that are activated through Toll-like receptors and the receptor for advanced glycoxidation end-products (RAGE) by Aβ [19, 20].

Activated microglia produce high levels of inflammatory cytokines including TNF-α, IL-6 and IL-1 [21, 22]. Studies involving dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have shown that the inhibition and ablation of cyclooxygenase-2 (COX-2) markedly reduces the deleterious effects of this toxin on the nigrostriatal pathway [23]. It has also been found that elevated levels of COX-2 directly contribute to neuronal vulnerability [24]. These inflammatory factors are heavily involved in the process of neuronal apoptosis and the stimulation of astrocytes, which in turn amplify proinflammatory signals [19]. Therefore, the inhibition of cytokine production and neuroinflammation could form part of a promising strategy to prevent memory impairment. This idea has generated a phase 2 clinical trial with a novel RAGE inhibitor (NCT00566397) [14]. Fig. 1 briefly outlines the role of oxidative stress and inflammation in the neurodegenerative process leading to AD.

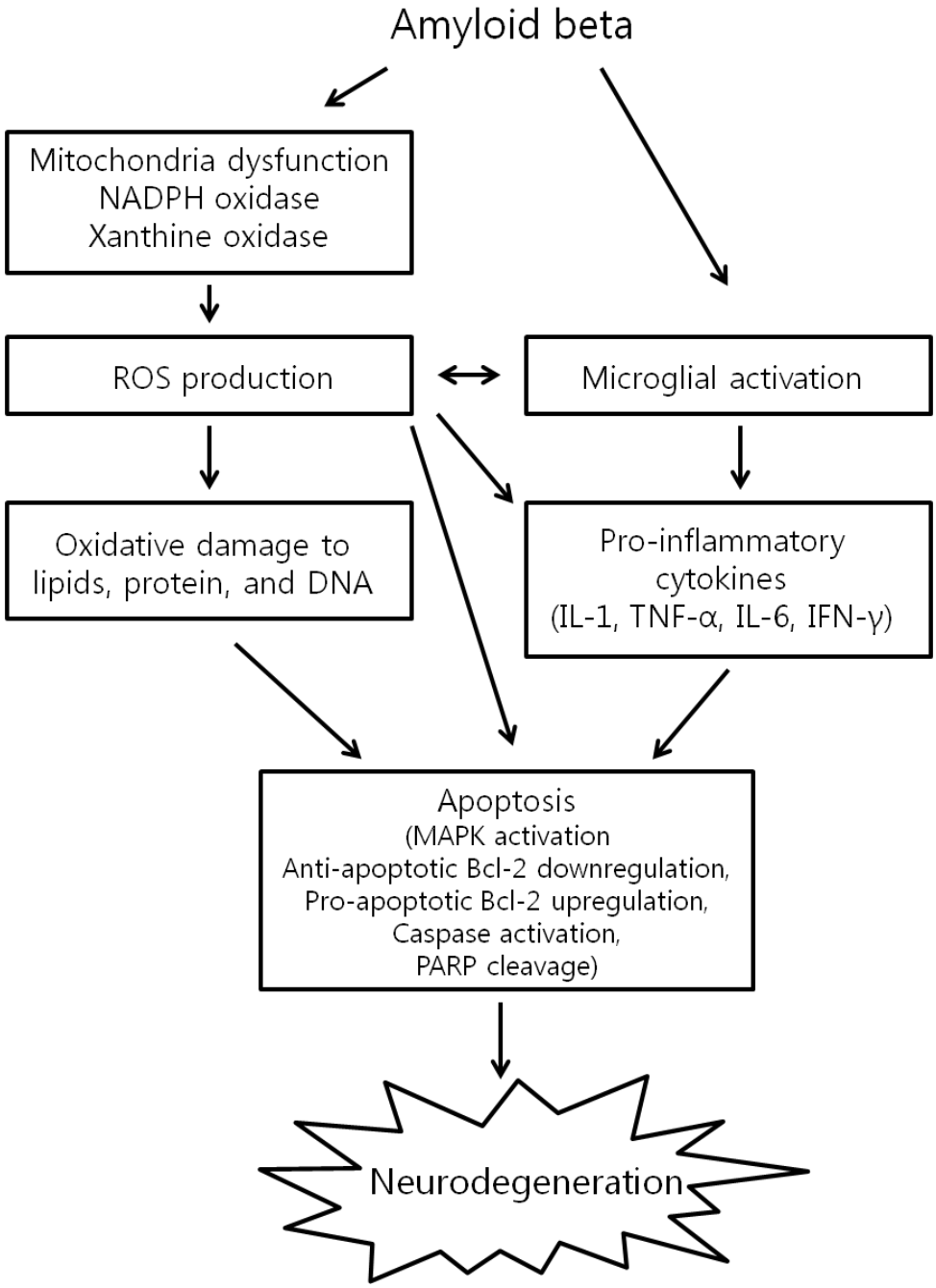

Figure 1. The role of oxidative stress and inflammation in the neurodegenerative process leading to Alzheimer's disease.

1.2.3. Signal transduction pathways and neuronal apoptosis

The Mitogen-Activated Protein Kinase (MAPK) family including ERK1/2, JNK/SAPK and p38, is involved in diverse processes regulating cells of the nervous system including survival, proliferation, differentiation and apoptosis [25]. The activation of MAPK signaling intermediates can also contribute to AD pathogenesis through various mechanisms [26]. Oxidative stress is often induced by ROS such as H_2O_2, the hydroxyl radical and the superoxide anion, which are potent activators of JNK and p38 signaling [26, 27].

JNK-mediated phosphorylation sets off a signal cascade that can lead to Bax activation, mitochondrial translocation and eventually apoptosis [28]. JNK further tips cell fate toward apoptosis by suppressing anti-apoptotic Bcl-2 [29] and stabilizing the tumor suppressor p53, which in turn further suppresses Bcl-2 and enhances Bax induction [30, 31]. In addition, the hyperphosphorylation of tau is a hallmark of AD, which JNK has also been reported to enhance [32].

Studies have shown that p38 mediates Bax translocation in neurons undergoing apoptosis after nitric oxide induction [33]. p38-mediates apoptosis through the phosphorylation and downregulation of Bcl-X_L [34], and is also involved in the hypoxic regulation of Mdm2 and p53 [35]. Dimerization of the amyloid precursor protein, which is mediated by the Aβ-

42 portion of the protein, induces activation of the ASK1-MKK6-p38 signaling pathway, leading to tau phosphorylation [26, 36]. As a result, signaling by p38 MAPK mediates Fas-dependent apoptosis through the upregulation of NO production in motor neurons [26, 37]. Both JNK and p38 are activated by oxidative stress and increase the expression of β-secretase.

ERK plays an opposing role to the JNK and p38 pathways [38]. Activation of JNK and p38, as well as the concurrent inhibition of ERK are all critical for the full induction of apoptosis in neurons [39]. The ERK signaling pathway can mediate phosphorylation of CREB, resulting in transcriptional activation of the brain-derived neurotrophic factor (BDNF) [40], while phosphorylated CREB induces Bcl-2 expression and inhibits apoptosis [41]. Fig. 2. provides an overview of the role of MAPK signaling in neuronal apoptosis.

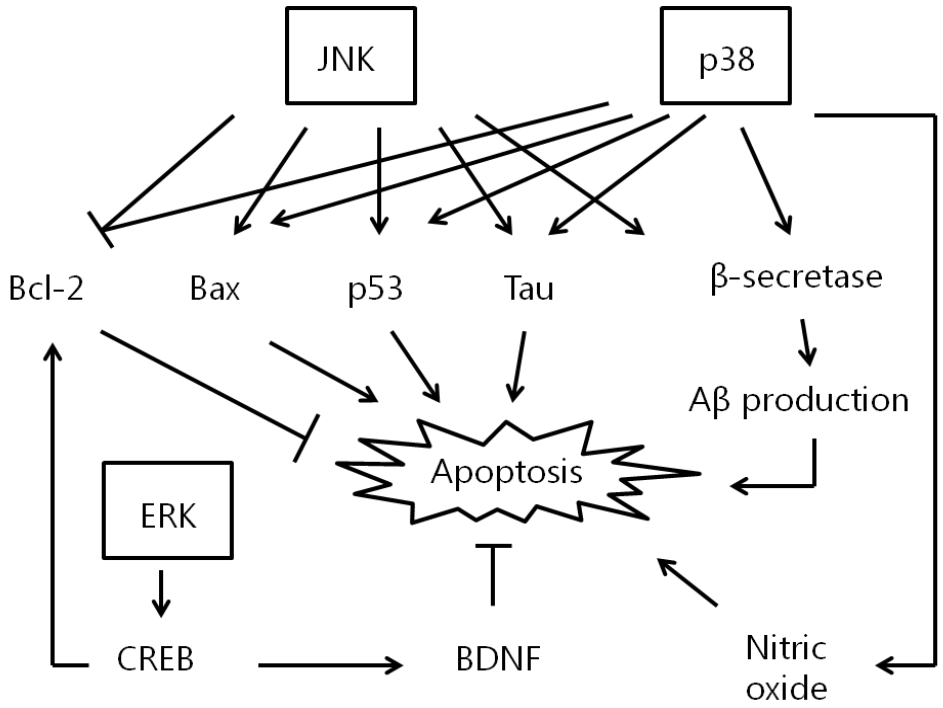

Figure 2. The multiple roles of MAPK pathway in neuronal apoptosis.

1.3. Molecular mechanisms of neuroprotection by dietary phytochemicals.

1.3.1. Red wine phytochemicals

Red wine has been reported to possess a wide range of biological and pharmacological activities including antioxidant, anti-inflammatory and anticarcinogenic effects, as well as conferring protective effects against cardiovascular disease [42-44]. Such health benefits of red wine are largely thought to be attributed to the presence of various polyphenols such as phenolic acids, stilbenes, tannins, flavanols, flavonols and anthocyanins, with red wines containing relatively higher concentrations than white wines [45]. Resveratrol is perhaps the most well-studied phytochemical among the red wine polyphenols and exhibits potent neuroprotective effects in certain scenarios [46]. Zhang et al. showed that resveratrol can protect cortical neurons against microglia-mediated neuroinflammation [47], and it also has been reported to protect rats from Aβ-induced neurotoxicity through the inhibition of iNOS expression and lipid peroxidation [48]. 4-HNE induces dysregulation of the mitochondria-mediated apoptotic markers (Bax, Bcl-2 and Caspase-3), which can be significantly restored by resveratrol pre-treatment [49].

However, it is important to note that red wine generally contains

approximately 1mg/L of resveratrol, which is markedly lower than that of other polyphenols [50]. For example, kaempferol concentrations approximate 18 mg/L, while quercetin and myricetin approach 18.8 mg/L, and 16.2 mg/L, respectively [50]. Therefore, the individual neuroprotective effects of each phytochemical needs to be considered together with known concentrations and bioavailability to fully understand the neuro-pharmacological potential of red wine.

A study by Suematsu et al. revealed that quercetin protected human neuronal SH-SH5Y cells from H_2O_2-induced apoptosis [51]. Other reports show that quercetin exerts neuroprotective effects through inhibition of the iNOS/NO system and pro-inflammatory gene expression in PC12 cells and in zebrafish [52]. Pretreatment with quercetin can significantly suppress Aβ-induced cytotoxicity, protein oxidation, lipid peroxidation and apoptosis in primary hippocampal cultures [53].

Meanwhile, Shimmyo et al. determined that the protective effects of myricetin against glutamate-induced neuronal cell death occurs via the regulation of ROS production and caspase-3 activation [54]. Similarly, kaempferol was shown to have protective effects against oxidative stress in PC12 cells and Aβ-induced cytotoxicity in ICR mice [55]. Kaempferol can attenuate 4-HNE-induced neuronal apoptosis by directly inhibiting NADPH oxidase [13]. More recently, studies involving kaempferol treatment have

revealed that the phytochemical exhibits neuroprotective effects in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced mouse model of PD [56]. It is currently unknown whether combinations of these phytochemicals may synergistically enhance their neuroprotective effects.

1.3.2. Cocoa phytochemicals

Cocoa is a source of abundant polyphenols including catechin, epicatechin and procyanidin oligomers (comprising catechin and epicatechin subunits) [57]. Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than both tea and red wine [58]. Clinical studies have suggested that potential advantages may be gained by consuming flavanol-rich cocoa when performing cognitive tasks and during brain perfusion [59, 60].

Epicatechin and catechin are flavonoids in cocoa that can protect PC12 cells from Aβ-induced neurotoxicity, and therefore may have anti-neurodegenerative effects [61]. Catechins can protect neurons from mitochondrial toxins and HIV proteins through their activation of the BDNF pathway [62]. The most potent detectable antioxidants in cocoa are dimers and trimers of catechin and epicatechin, including the procyanidin B2 complex, followed by catechin and epicatechin [63]. It has been reported that long-term administration of a cocoa polyphenolic extract improves cognitive

performances and can increase the life span of aged rats [64]. Other reports suggest that procyanidin B2 and cocoa procyanidin fractions can convey protection against H_2O_2-induced apoptosis by inhibiting Bcl-X_L and Bcl-2 downregulation, a process that occurs through blocking of JNK and p38 MAPK activation [65]. Procyanidin B2 and cocoa procyanidin fractions have also been reported to protect PC12 cells against HNE-induced apoptosis by directly blocking MKK4 activity and ROS accumulation [57].

1.3.3. Coffee phytochemicals

Recent research has focused on the interesting relationship between coffee intake and the risk of neurodegenerative disease. Human studies have suggested that coffee intake (at 3-5 cups daily) during middle age can decrease the later risk of AD and cognitive decline [66, 67]. A prospective analysis has also reported that coffee consumption is associated with a reduced risk of AD [68]. Meanwhile, epidemiological evidence suggests an inverse correlation exists between coffee intake and ALS risk [69]. In contrast, there exist opposing studies that have failed to show an association between coffee consumption and cognitive performance or development of AD [70, 71].

Coffee is a major dietary source of antioxidants, and like cocoa, contains more antioxidants than most tea, fruit and wine. Caffeine is a major

bioactive compound in coffee and has been reported to be negatively associated with the development of a number of neurodegenerative diseases. Data from a number of *in vitro* and animal studies suggest that caffeine intake is associated with a reduced risk of AD and PD [75-77]. A comprehensive meta-analysis of human prospective studies has also confirmed that caffeine intake is inversely associated with PD risk in both men and women [72]. Consistent with this notion, evidence from several human epidemiological studies suggests that habitual caffeine consumption may lead to better long-term memory performance [73, 74]. However, excessive caffeine intake may lead to undesirable side effects including addiction, insomnia and hallucination [78, 79].

Dietary supplementation with decaffeinated green coffee has been shown to improve diet-induced insulin resistance and brain energy metabolism in mice [80]. Decaffeinated coffee provides neuroprotection in Drosophila models of PD through an NRF2-dependent mechanism [81]. These results suggest that coffee phytochemicals with antioxidative effects may synergistically enhance the neuroprotective effects of coffee, together with caffeine.

Cho et al. reported that decaffeinated coffee and chlorogenic acid, a major phenolic compound in coffee, inhibits H_2O_2-induced PC12 cell death by regulating ROS accumulation and inhibiting the MAPK pathway [82].

Coffee, decaffeinated coffee, and the phenolic phytochemical chlorogenic acid can all upregulate NQO1 expression and prevent H_2O_2-induced apoptosis in primary cortical neurons [83]. Chlorogenic acid was reported to have neuroprotective effects against amnesia via anti-acetylcholinesterase and antioxidative effects in mice [84].

Coffee contains an abundance of other phytochemicals such as caffeic acid, cafestol and kahweol. Caffeic acid effectively inhibits ceramide-induced binding activity of NF-kappa B and subsequent apoptosis [85]. Cafestol has been shown to confer neuroprotection in Drosophila models of PD [81], while kahweol and cafestol are effective at ameliorating H_2O_2-induces oxidative stress and DNA damage, which is likely to occur through the scavenging of free oxygen radicals [86]. These studies are adding to a mounting body of evidence highlighting the neuroprotective benefits of regular coffee consumption.

1.4. Conclusion

Oxidative stress and inflammation are damaging processes that contribute to neuronal apoptosis. Once neurons die, they rarely revive to their original form or generate proper synapses with other neurons. Therefore, the prevention of neuronal cell death before the onset of neurodegenerative disease must remain a priority. The effective regulation of intermediates

involved in oxidative and inflammatory damage represents a logical protection strategy. The MAPK signaling pathway can regulate neuronal apoptosis by regulating several critical substrates involved in this process. Signaling cascades such as the JNK and p38 MAPK pathways are induced by both oxidative stress and inflammation, and are valid targets for the prevention of neurodegeneration. Various phytochemicals in red wine, cocoa, and coffee are known to effectively reduce oxidative stress and inflammation, thereby inhibiting neuronal apoptosis and neurodegeneration in several models of neurodegenerative disease. It therefore stands to reason that regular consumption of dietary phytochemicals could be beneficial for the maintenance of brain health and protection from disease. Further studies will contribute significantly to my understanding of these processes by revealing the direct targets and molecular mechanisms involved.

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Chapter 2.

Attenuation of oxidative neuronal cell death by coffee phenolic phytochemicals

Abstract

Neurodegenerative disorders such as Alzheimer's disease (AD) are strongly associated with oxidative stress, which is induced by reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2). Recent studies suggest that moderate coffee consumption may reduce the risk of neurodegenerative diseases such as AD, but the molecular mechanisms underlying this effect remain to be clarified. In this study, I investigated the protective effects of coffee, decaffeinated coffee and chlorogenic acid (5-*O*-caffeoylquinic acid), a major phenolic phytochemical found in coffee against oxidative PC12 and primary cortical neuronal cell death. Coffee, decaffeinated coffee and chlorogenic acid attenuated H_2O_2-induced PC12 and primary cortical neuronal cell death. H_2O_2-induced nuclear condensation was strongly inhibited by pretreatment with coffee, decaffeinated coffee or chlorogenic acid. DNA fragmentation is attenuated by decaffeinated coffee or chlorogenic acid in PC12 cells. Pretreatment with coffee, decaffeinated coffee or chlorogenic acid also inhibited the H_2O_2-induced cleavage of poly(ADP-ribose) polymerase (PARP), and downregulation of anti-apoptotic Bcl protein and caspase-3. The accumulation of intracellular ROS in H_2O_2-treated PC12 cells was dose-dependently diminished by decaffeinated coffee and chlorogenic acid. The activation of c-Jun N-terminal protein kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) by H_2O_2 in PC12

cells was also inhibited by decaffeinated coffee or chlorogenic acid. Both coffee and decaffeinated coffee inhibited H_2O_2-induced apoptotic neuronal death similarly, indicating that chlorogenic acid might contribute to these effects. Collectively, these results indicate that decaffeinated coffee and chlorogenic acid protect neuronal cells from H_2O_2-induced apoptosis by blocking the accumulation of intracellular ROS and the activation of MAPKs.

Key words: *Decaffeinated coffee; chlorogenic acid; coffee; ROS; mitogen-activated protein kinases*

2.1. Introduction

Alzheimer's disease (AD), a common type of dementia, is a progressive neurodegenerative disorder characterized by the accumulation of senile plaques containing amyloid β (Aβ) and neurofibrillary tangles composed of phosphorylated tau in the brain. The cause of AD is uncertain, but several recent studies have implicated reactive oxygen species (ROS)-induced oxidative stress in its pathogenesis [1, 2]. Hydrogen peroxide (H_2O_2) is a major mediator of oxidative stress [3], and Aβ plays a critical role in the development and progression of AD via the generation of ROS such as H_2O_2 [4]. H_2O_2 in turn is involved in the production of highly reactive hydroxyl radicals via Fenton's reaction, which promotes apoptosis [5]. Both these by-products and H_2O_2 itself can react with nearly all cellular macromolecules to damage proteins, lipids, mitochondria, and DNA [6].

H_2O_2-induced apoptosis is accompanied by changes in apoptosis-related factors such as the Bcl-2 family of regulatory proteins. Among its members, Bcl-X_L is a major antiapoptotic protein [7] that reportedly protects neurons against H_2O_2-induced cell death [8]. The cysteine protease caspase-3 is a key executor of apoptosis that is also reportedly activated by H_2O_2 [9]. The cleavage of poly(ADP-ribose) polymerase (PARP) by activated caspase-3 is a hallmark of apoptosis [10]. In addition, mitogen-activated protein kinase (MAPK) signaling is involved in cellular events such as gene

expression, mitosis, and apoptosis via the phosphorylation of target proteins at specific serine and/or threonine residues. In particular, the activation of c-Jun N-terminal protein kinase (JNK) and p38 MAPK plays a critical role in the induction of apoptosis in neurons [11, 12].

Coffee, as a rich source of caffeine, has been reported to have an effect on neurodegenerative disorders such as Parkinson's disease [13]. Similarly, a recent epidemiologic study reported that moderate daily consumption of caffeine was significantly associated with a reduced risk of AD [14]. In an AD murine model, caffeine was shown to have an effect on cognitive protection through the suppression of Aβ production; specifically, caffeine was found to antagonize the activity of the adenosine A receptor [15, 16].

Although caffeine is an ingredient believed to have major neuroprotective effects, coffee is a major dietary source of phenolic compounds [17]. Chlorogenic acid (5-*O*-caffeoylquinic acid) (Fig. 1) which is formed by the esterification of quinic acid with *trans*-cinnamic acid [18] is a major phenolic compound in coffee; in fact, the CGA content of a 200-ml cup of coffee is 70–350 mg [17, 19]. However, the mechanism underlying the neuroprotective effects of coffee and chlorogenic acid against oxidative neuronal cell death and injury remains to be clarified. The PC12 rat pheochromocytoma (PC12) cell line is a useful model system for

investigating neuronal cell death [20]. Several previous studies have shown that H_2O_2 triggers apoptosis in PC12 cells [3, 21]. The present study investigated the potential protective effects of coffee, decaffeinated coffee and chlorogenic acid against H_2O_2-induced apoptosis in neuronal cells. These results indicate that both coffee and decaffeinated coffee inhibited H_2O_2-induced apoptotic neuronal death similarly, suggesting that chlorogenic acid might contribute to neuroprotective effects of coffee.

2.2. Materials and Methods

2.2.1. Chemicals and reagents

H_2O_2 was purchased from Junsei Chemical (Tokyo, Japan). Poly-D-lysine (PDL), chlorogenic acid, 4,6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2',7'-dichlorofluorescin diacetate (DCFH-DA), Folin and Ciocalteu's phenol reagent, and trypan blue (0.4% solution) were purchased from Sigma Chemical (St. Louis, MO, USA). Mg^{2+}- and Ca^{2+}-free Hanks' balanced salt solution (HBSS), neurobasal medium, B27, L-glutamine (200 mM), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, horse serum, and a penicillin/streptomycin mixture were obtained from Gibco–BRL (Grand Island, NY, USA). Trypsin was obtained from In Vitrogen (Carlsbad, CA, USA). Anti-PARP, anti-caspase-3, anti-JNK, and anti-p38 MAPK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-actin antibodies were purchased from Sigma Chemical, while anti-Bcl-X_L, anti-Bcl-2 and anti-phosphorylated-JNK antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-phosphorylated-p38 MAPK antibodies were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). All other chemicals were of analytical grade.

2.2.2. Primary neuronal culture

Cerebral cortices from littermate embryos (gestation day 14) were removed, placed in ice-cold HBSS, centrifuged (300 × g, 2 min), and digested in 0.05% trypsin in HBSS at 37 ℃ for 10 min. Tissues were washed twice with HBSS and resuspended in Neurobasal medium supplemented with 2% B27, 2 mM L-glutamine, and 1% penicillin/streptomycin. Cell suspension was sieved through a cell strainer (70 μm; BD Biosciences, San Jose, CA, USA) and plated on PDL-coated plates. After 45 min of initial plating, the medium was changed to new Neurobasal medium supplemented with 2% B27, 2 mM L-glutamine, and 1% penicillin/streptomycin. Cultures were maintained at 37 ℃ in a humidified atmosphere of 5% CO_2.

2.2.3. PC12 cell culture

PC12 cells derived from a pheochromocytoma of the rat adrenal medulla were kindly supplied by Dr. Y.-J. Surh (Seoul National University, Seoul, Korea). PC12 cells were grown in DMEM supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, and 0.1% penicillin/streptomycin at 37℃ in a humidified atmosphere of 10% CO_2 and 90% air.

2.2.4. Sample preparation and measurement of the total coffee phenolic content

Instant coffee (Maxim original, carbohydrate 625mg, chlorogenic acid 43.75mg, caffeine 31.3mg per gram of coffee) and instant decaffeinated coffee (Maxim Decaffeinated, carbohydrate 625mg, chlorogenic acid 55mg, caffeine 0.939mg per gram of decaffeinated coffee) were purchased from Dongsuh Food (Seoul, Korea). Distilled water (80℃, 100 ml) was added to 10 g of a commercially prepared instant coffee and instant decaffeinated coffee and stirred for 5 min. The solution was then filtered through a membrane filter under a vacuum. The total phenolic content of the liquid was measured independently six times using the Folin–Ciocalteu method. Folin and Ciocalteu's phenol reagent (5 μl) was added to 50 μl of diluted sample (decaffeinated coffee) or standard solution (chlorogenic acid) and shaken for 6 min, and then 50 μl of 7% Na_2CO_3 was added to the reaction mixture. The mixture was immediately diluted with 100 μl of distilled water and incubated for 90 min at 23℃. After incubation, the absorbance at 750 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The total phenolic content in decaffeinated coffee was expressed as milligrams per gram of chlorogenic acid equivalents. A stock solution of 100 mg/ml coffee and decaffeinated coffee was used in this study.

2.2.5. MTT assay

MTT is metabolized to an insoluble purple formazan by mitochondrial dehydrogenases, which are active only in live cells. Thus, cell viability was measured based on the formation of a purple formazan metabolite, which was solubilized by the addition of dimethyl sulfoxide (DMSO). PC12 cells (2×10^4 cells/well in 96-well plates) and primary neuronal cultures (2×10^5 cells/well in 96-well plates) were incubated at 37℃ with 200 μM or 50 μM H_2O_2 for 24 h with or without pretreatment with decaffeinated coffee or chlorogenic acid, and then treated with 1 mg/ml MTT (final concentration) for 2 h. The dark blue formazan crystals in the intact cells were dissolved in DMSO and the absorbance at 570 nm was measured with a microplate reader. The results are expressed as the percent reduction in absorbance relative to that in the control cells.

2.2.6. Trypan blue exclusion assay

Trypan blue can be used to identify nonviable cells since it specifically interacts with damaged cell membranes. PC12 cells (10^5 cells/well in 6-well plates) were incubated at 37℃ with 200 μM H_2O_2 for 24 h with or without pretreatment with decaffeinated coffee or chlorogenic acid. After centrifugation at 600 × *g* for 6 min, the cells were resuspended in 200 μl of phosphate-buffered saline (PBS). The entire suspension was then mixed

with 200 μl of 0.4% trypan blue solution and incubated for 5 min at room temperature. The cells were then loaded into a hemocytometer, and those exhibiting dye uptake were counted under a microscope (Olympus, Tokyo, Japan). The percentage of stained cells was based on 150 cells.

2.2.7. DAPI staining

The fluorescent dye DAPI was used to detect nuclear fragmentation, which is characteristic of apoptotic cells. PC12 cells (2×10^4 cells/well in 24-well plates) and primary neuronal cultures (5×10^5 cells/well in 24-well plates) were incubated at 37℃ with 200 μM or 50 μM H_2O_2 for 24 h with or without pretreatment with caffeinated coffee, decaffeinated coffee or chlorogenic acid, and then washed with PBS and fixed with 70% ethanol for 20 min. The fixed cells were then washed with PBS and stained with 1 μg/ml DAPI. Following 10 min of incubation, the cells were again washed with PBS, and the plates were observed under a fluorescence microscope (Olympus Optical, Tokyo, Japan). The degree of nuclear fragmentation was evaluated based on the percentage of DAPI-stained cells from among 100–120 randomly chosen cells.

2.2.8. DNA fragmentation analysis

Apoptotic cells exhibit unique ladders of nucleotide fragments

during agarose gel electrophoresis. PC12 cells (1.6×10^6 cells/8 ml in a 8.5-cm dish) were incubated at 37℃ with 200 μM H_2O_2 for 24 h with or without pretreatment with decaffeinated coffee or chlorogenic acid, and then washed and collected with ice-cold PBS and centrifuged at 200 × *g* for 10 min. Cellular DNA was isolated using a DNA isolation buffer [10 mM EDTA, 50 mM Tris–HCl (pH 8.0), 0.5% SDS, and 0.5 mg/ml proteinase K] and incubated for 4 h at 50℃. After centrifugation at 10,000 × *g* for 15 min, the supernatants were extracted with an equal volume of phenol, chloroform, and isoamyl alcohol. The DNA was then mixed with 4 M NaCl and 100% ethanol and stored at –70℃ overnight. Each sample was then loaded onto a 1.8% Tris–boric acid–EDTA agarose gel and electrophoresed at 100 V for 30 min.

2.2.9. Measurement of intracellular ROS accumulation

I measured the intracellular production of ROS using a DCFH-DA assay. Dichlorofluorescin diacetate (DCFH-DA) is deacetylated in cells, whereupon its reaction with intracellular radicals (mainly H_2O_2) converts it into a fluorescent product, DCF, which is retained within the cells. PC12 cells (5×10^4 cells/well in 24-well plates) were preincubated in PBS containing 50 μM DCFH-DA for 20 min and then rinsed with PBS. The cells were then incubated at 37℃ with 200 μM H_2O_2 for 15 min with or without

pretreatment with decaffeinated coffee or chlorogenic acid, and then lysed (0.5% Triton X-100, 0.1% CHAPS, and 0.1 mM EDTA) and examined with a fluorescence spectrophotometer (F-4500; Hitachi, Tokyo, Japan) with excitation at 488 nm and emission at 530 nm.

2.2.10. Western blot analysis

PC12 cells (2×10^5 cells in a 6-cm dish) and primary neuronal cultures (4×10^6 cells in a 6-cm dish) were incubated at 37℃ with 200 μM or 50 μM H_2O_2 for 24 h with or without pretreatment with caffeinated coffee, decaffeinated coffee or chlorogenic acid, washed and collected with ice-cold PBS, and then centrifuged at 600 × *g* for 10 min. The cell pellet was then resuspended in 100 μl of ice-cold lysis buffer (Cell Signaling) and incubated on ice for 30 min. After centrifugation at 1000 × *g* for 15 min, the supernatant was separated and stored at –70℃. The protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). The proteins were then separated by SDS-PAGE and transferred onto a polyvinylidene difluoride transfer membrane, which was then blocked with 5% skim milk containing 0.5 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20 for 2 h at room temperature. The membrane was subsequently incubated with the primary antibody. After three washes with TBST (Tris-buffered saline with 0.1% Tween 20), the blots were incubated with

horseradish peroxidase-conjugated secondary antibodies in TBST with 5% skim milk at a 1:5000 dilution for 2 h at room temperature. The blots were then again washed three times in TBST. The blots were developed using an enhanced chemiluminescence (ECL) detection method by immersing them for 5 min in a mixture of ECL reagents A and B (Amersham Bioscience, New York, NY, USA) at a 1:1 ratio and exposing them to photographic film for a few minutes.

2.2.11. Replication and Statistical Analysis

When necessary, the data are expressed as the mean ± SD. Student's *t*-test was used for single comparisons, with a probability-value of $p < 0.05$ as the criterion for statistical significance.

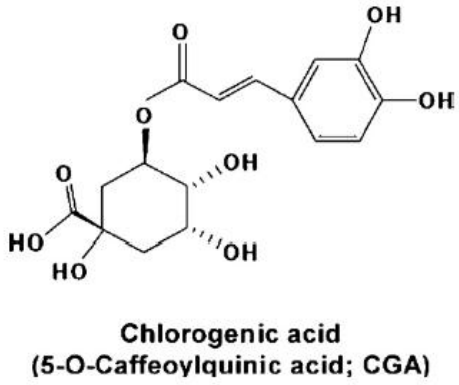

Figure 1. Chemical structure of chlorogenic acid.

2.3. Results

2.3.1. Coffee, decaffeinated coffee and chlorogenic acid inhibit H_2O_2-induced neuronal cell death.

The total phenolic content of the decaffeinated coffee was 330 mg per gram of chlorogenic acid equivalents. I investigated the effect of coffee and decaffeinated coffee against H_2O_2-induced cell death by observing the morphological change and MTT assay (Fig. 2A and B). Treatment with 50 μM H_2O_2 induced neuronal cell death (b) and pretreatment with coffee or decaffeinated coffee at 50 μg/ml for 1 h decreased neuronal cell death induced by H_2O_2 (c and d).

Nuclear condensation is morphological characteristics of apoptosis. Treatment with 50 μM H_2O_2 alone for 24 h resulted in the condensation of primary cortical neuron, as determined by DAPI staining, however, these were significantly decreased by pretreatment with coffee (50 μg/ml) or decaffeinated coffee (50 μg/ml) (Fig. 2 C).

To examine the effect of chlorogenic acid on primary cortical neuronal apoptotic cell death, cells were treated with 50 μM H_2O_2 for 24 h with or without preincubation of 50 μM chlorogenic acid for 1 h (Fig. 2D). Neuronal cells treated to 50 μM H_2O_2 for 24 h exhibited significant condensed nucleus compared to control cells (Fig. 2D, a and b). Pretreatment of 50 μM chlorogenic acid for 1 h significantly suppressed H_2O_2-mediated

nuclear condensation (Fig. 2D, b and c).

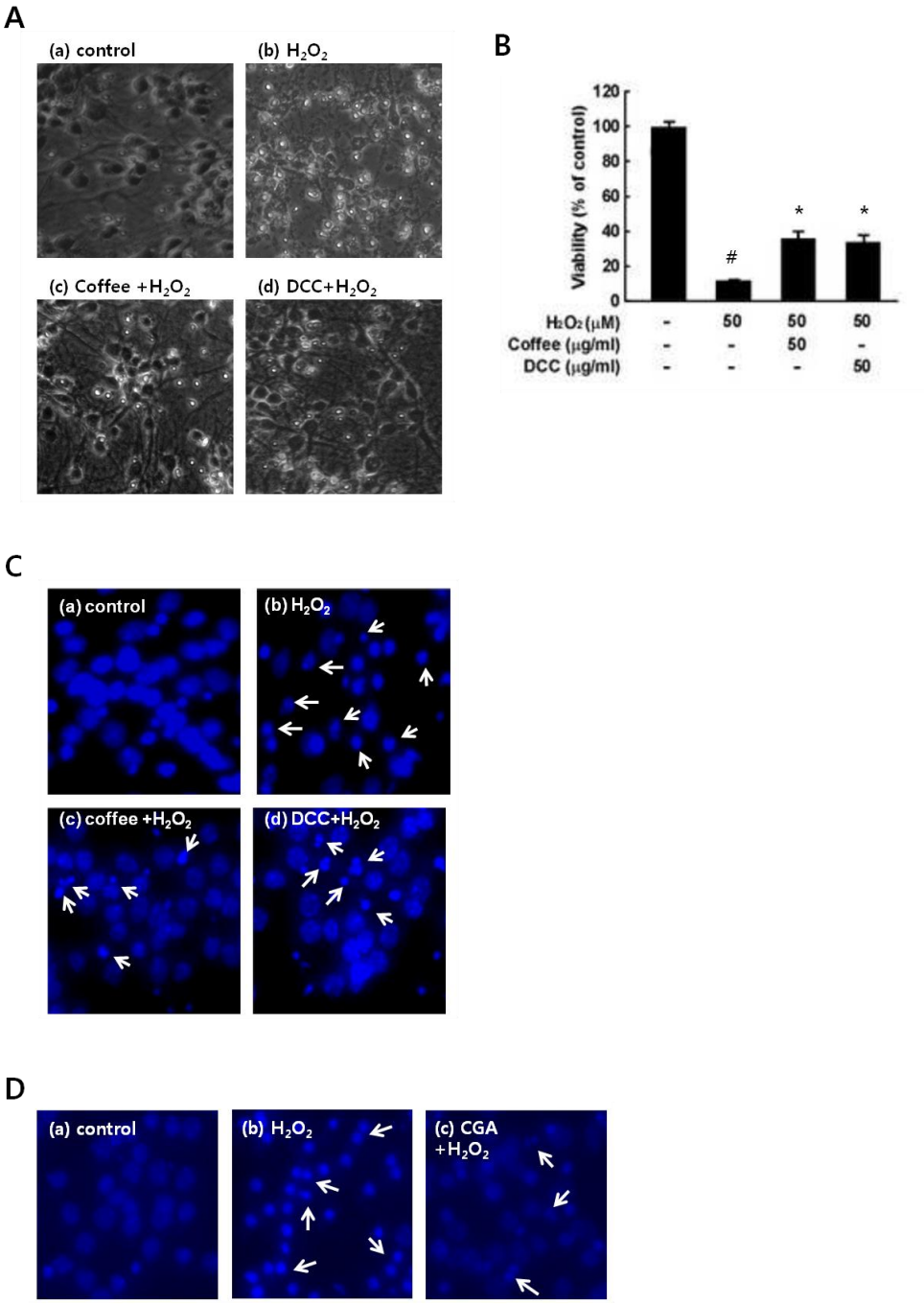

Figure 2. The effects of coffee, decaffeinated coffee and chlorogenic acid on H_2O_2-induced neuronal cell death. (A-C) Primary cortical neurons were

pretreated with coffee and decaffeinated coffee (DCC) at 50 µg/ml for 1 h followed by 50 µM H_2O_2 for 24 h. The viability of neuronal cells was determined by cell morphology visualized under a microscope, and the phase contrast images were presented (A). The viability of the neuronal cells was measured by MTT assay. The relative cell viabilities, expressed as a percentage of control values, are presented as means ± SE (n = 3) (B). Nuclear morphology was examined by DAPI staining under a fluorescence microscope as described in material and method (C). (D) Primary cortical neuron were pretreated with 50 µM chlorogenic acid for 1h followed by 50 µM H_2O_2 for 24 h. The apoptotic neuronal cells were detected by DAPI staining under a fluorescence microscope. $^{\#}p < 0.05$ compared to control cells. * $p < 0.05$ compared to H_2O_2-treated cells.

2.3.2. Decaffeinated coffee and chlorogenic acid inhibit H_2O_2-induced PC12 cell death

According to the results of the MTT reduction assay, the viability of the cells exposed to 200 μM H_2O_2 was 29.7 ± 4.4% of the control value, while preincubation with decaffeinated coffee at 5 μg/ml or chlorogenic acid at 1 and 5 μM significantly increased the viability to 65.0 ± 5.8%, 62.7 ± 11.2%, and 84.4 ± 6.7%, respectively (Fig. 3A). In line with our MTT data, the results of the trypan blue assay revealed that decaffeinated coffee and chlorogenic acid exerted protective effects against H_2O_2-induced neuronal cell death in a dose-dependent manner. Compared to the control group, the viability of cells exposed to 200 μM H_2O_2 for 24 h was 22.6 ± 1.3%, and this value was increased to 48.0 ± 2.8%, 59.7 ± 3.5%, 50.2 ± 3.0%, and 59.9 ± 3.5% by pretreatment with decaffeinated coffee at 1 and 5 μg/ml or chlorogenic acid at 1 and 5 μM, respectively (Fig. 3B). PC12 cells exposed to decaffeinated coffee (1 and 5 μg/ml) or chlorogenic acid (1 and 5 μM) alone for 24 h did not exhibit significant cytotoxicity compared to control cells, as measured by both MTT and trypan blue exclusion assays (data not shown).

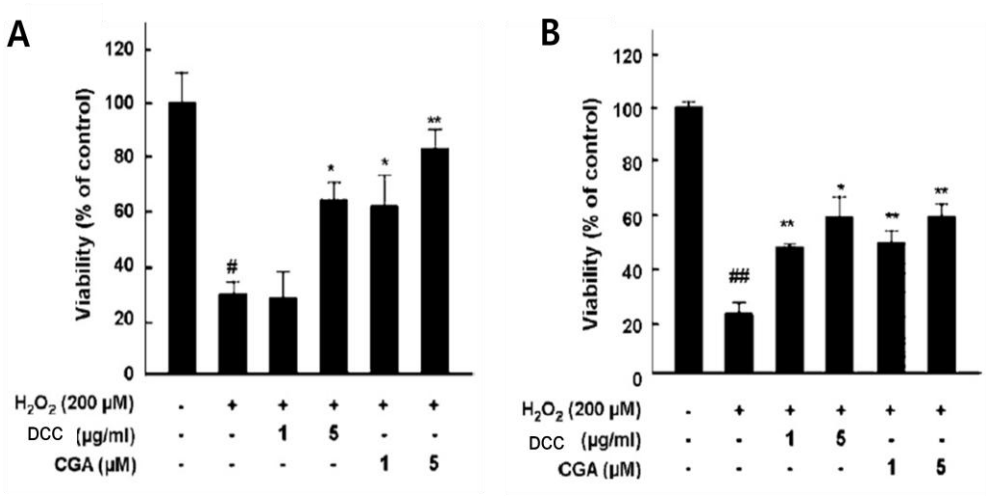

Figure 3. The effects of decaffeinated coffee (DCC) and chlorogenic acid (CGA) on H_2O_2-induced PC12 cell death. Cells were pretreated with DCC or CGA at the indicated concentrations for 30 min, and then further treated with 200 μM H_2O_2 for 24 h at 37°C. The viability of the cells was determined by an (A) MTT reduction assay and (B) a trypan blue exclusion assay as described in the Materials and Methods. The values shown are the percentages of viable cells relative to untreated control cells (taken as 100%). The data are given as the mean ± SD for three independent experiments. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to control cells. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to H_2O_2-treated cells.

2.3.3. Decaffeinated coffee and chlorogenic acid attenuate H_2O_2-induced apoptosis in PC12 cells

Nuclear condensation and DNA fragmentation into 200-bp fragments are morphological characteristics of apoptosis. Treatment with 200 μM H_2O_2 alone for 24 h resulted in the condensation of PC12 nuclei, as determined by DAPI staining; however, this was significantly decreased by pretreatment with decaffeinated coffee (1 and 5 μg/ml) or chlorogenic acid (1 and 5 μM) (Fig. 4, A and B). Pretreatment with decaffeinated coffee or chlorogenic acid also attenuated DNA fragmentation compared to PC12 cells treated with H_2O_2 alone, as determined by agarose gel electrophoresis. Treatment with 5 μg/ml decaffeinated coffee or 5 μM chlorogenic acid dramatically inhibited H_2O_2-induced DNA fragmentation (Fig. 4, C and D). These findings show that decaffeinated coffee and chlorogenic acid protect PC12 cells against H_2O_2-induced apoptosis.

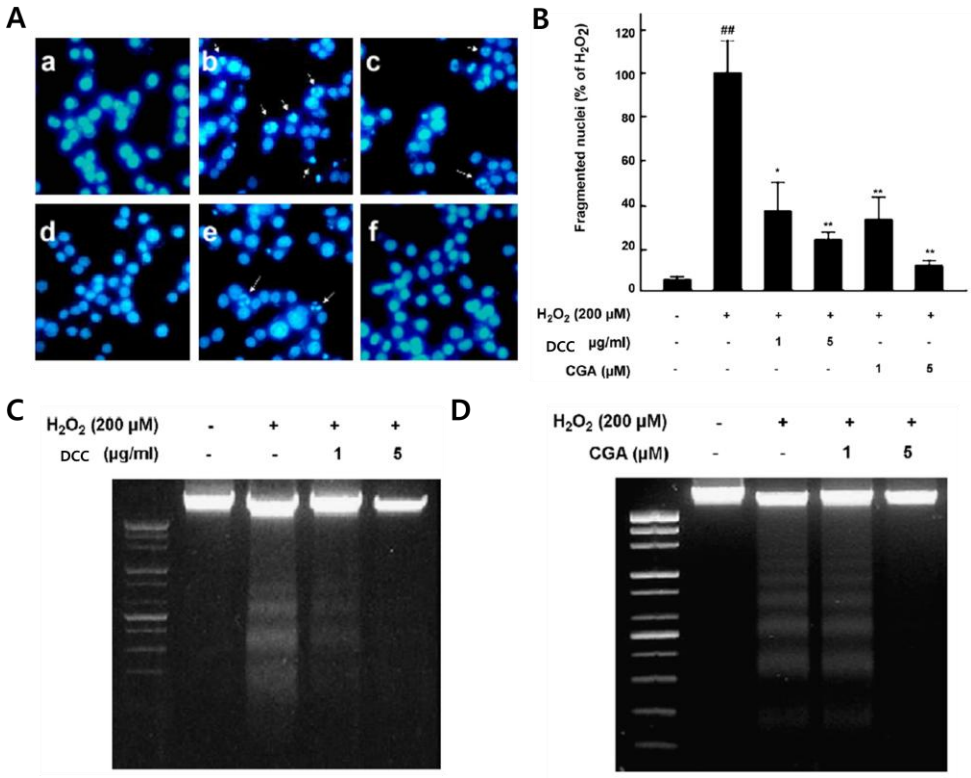

Figure 4. The effects of decaffeinated coffee (DCC) and chlorogenic acid (CGA) on H_2O_2-induced nuclear condensation and DNA fragmentation in PC12 cells. (A-B) DCC and CGA inhibited H_2O_2-induced nuclear condensation. Cells were preincubated for 30 min with DCC or CGA and then exposed to 200 μM H_2O_2 for 24 h: (A) (a) no treatment, (b) 200 μM H_2O_2, (c) 200 μM H_2O_2 + 1 μg/ml DCC, (d) 200 μM H_2O_2 + 5 μg/ml DCC, (e) 200 μM H_2O_2 + 1 μM CGA, and (f) 200 μM H_2O_2 + 5 μM CGA. Nuclear morphology was examined by DAPI staining under a fluorescence microscope as described in the Materials and Methods. (B) Quantitative

analysis of nuclear fragmentation in the PC12 cells. The percentage of cells with fragmented nuclei was determined by DAPI staining as described in the Materials and Methods. The data are given as the mean ± SD for three independent experiments. $^{\#\#}p < 0.01$ compared to control cells. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to H_2O_2-treated cells. (C-D) Cells were preincubated for 30 min with (C) DCC (1 and 5 µg/ml) or (D) CGA (1 and 5 µM) then exposed to 200 µM H_2O_2 for 24 h. Cellular DNA was extracted and visualized on agarose gels as described in the Materials and Methods. The data are representative of three independent experiments.

2.3.4. Coffee, decaffeinated coffee, and chlorogenic acid inhibit H_2O_2-induced down-regulation of anti-apoptotic Bcl-2, Bcl-X_L protein and cleavage of caspase-3 and pro-PARP in primary cortical neuron.

Bcl-2 and Bcl-X_L promote cell survival and down-regulation of Bcl-2 and Bcl-X_L occurs during apoptosis [22-24]. The decreased the anti-apoptotic protein expression of Bcl-2 and Bcl-X_L by 50 μM H_2O_2 were inhibited by pretreatment with coffee or decaffeinated coffee at 50 μg/ml for 1 h (Fig. 5A). The Bcl family of proteins inhibits the formation of mitochondrial transition pores and blocks the release of cytochrome c, thereby attenuating activation of the caspase-3 cascade. Caspase-3 is activated through proteolytic processing from its inactive zymogen into activated fragments, and I assessed the level of cleaved caspase-3 [25]. Western blot analysis revealed that the H_2O_2-induced cleavage of caspase-3 was inhibited by coffee and decaffeinated coffee at 50 μg/ml for 1 h (Fig. 5A). An impaired mitochondrial membrane potential and caspase activation leads to the cleavage of PARP from its full-length form (116 kDa) to its cleaved form (89 kDa) [26, 27]. The exposure of primary cortical neuron to 50 μM H_2O_2 for 24 h induced PARP cleavage; however, the effect was inhibited by pretreatment with 50 μg/ml of coffee or decaffeinated coffee for 1 h (Fig. 5A). These results suggest that coffee and decaffeinated coffee suppress H_2O_2-induced apoptosis by blocking the down-regulation of Bcl-2

and Bcl-$X_{L,}$ and cleavage of caspase-3 and pro-PARP.

The exposure of neuronal cells to 50 μM H_2O_2 for 24 h induced caspase-3 cleavage; however, the effect was inhibited by pretreatment with 50 μM chlorogenic acid for 1 h (Fig. 5B). Western blot analysis showed that cleavage of pro-PARP induced by treatment with 50 μM H_2O_2 for 24 h was attenuated by pretreatment with 50 μM chlorogenic acid for 1 h (Fig. 5B). Decreased protein level of Bcl-2 and Bcl-X_L by H_2O_2 were significantly attenuated by pretreatment with 50 μM chlorogenic acid for 1 h (Fig. 5C). These results indicate that chlorogenic acid attenuates the H_2O_2-induced apoptosis of neuronal cells by blocking the down-regulation of Bcl-2 and Bcl-X_L and subsequent cleavage of caspase-3 and pro-PARP.

Next, I compared chlorogenic acid with caffeic acid, another coffee phytochemical and caffeine in terms of the inhibitory effect on downregulation of Bcl-2 and Bcl-X_L. Caffeic acid at 50 μM and caffeine at 50 μM failed to inhibit downregulation of Bcl-2 and Bcl-X_L induced by H_2O_2 (Fig. 5C).

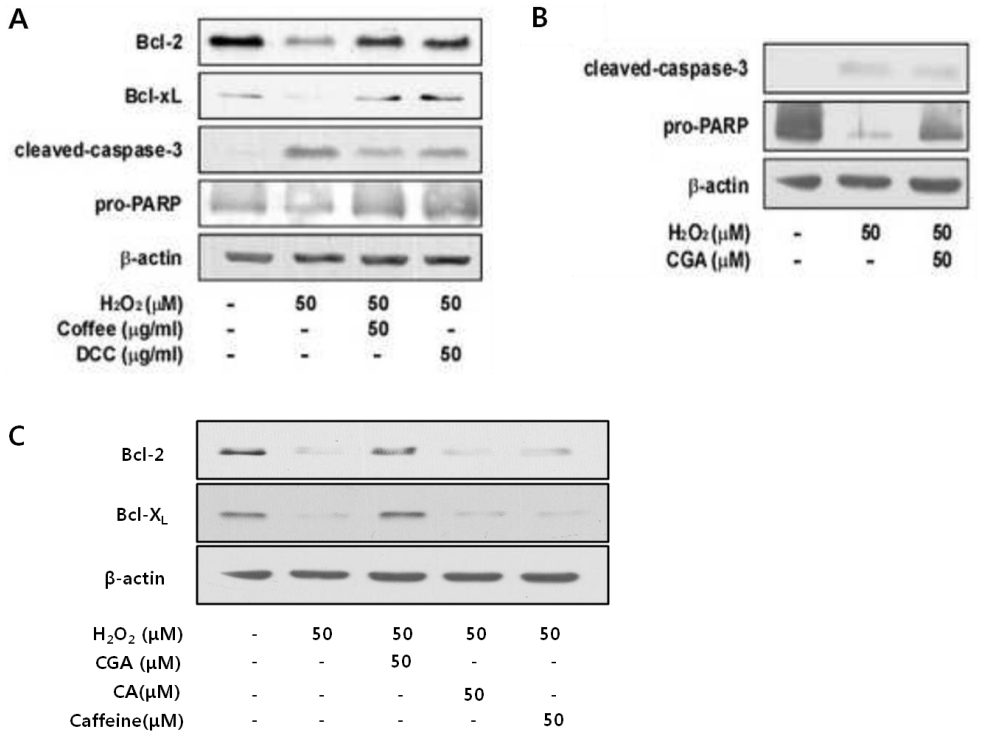

Figure 5. The effects of coffee, decaffeinated coffee (DCC), and chlorogenic acid on H_2O_2-induced down-regulation of Bcl-2 and Bcl-X_L as well as cleavage of caspase-3 and pro-PARP in primary cortical neuron. (A-B) Neuronal cells were pretreated with coffee, decaffeinated coffee (DCC) (50 μg/ml) or chlorogenic acid (CGA) (50 μg/ml) for 1 h followed by 50 μM H_2O_2 for 24 h. The levels of cleaved caspase-3 and pro-PARP were determined by Western blot analysis. (C) Neuronal cells were pretreated with chlorogenic acid (CGA) (50 μM), caffeic acid (CA) (50 μM) and caffeine (50 μM) for 1 h followed by 50 μM H_2O_2 for 24 h. The levels of Bcl-2 and Bcl-X_L were determined by Western blot analysis. β-Actin was measured to

confirm uniform protein loading.

2.3.5. Decaffeinated coffee and chlorogenic acid prevent H_2O_2-induced PARP cleavage, Bcl-X_L downregulation, and caspase-3 downregulation

The exposure of PC12 cells to 200 μM H_2O_2 induced PARP cleavage; however, the effect was inhibited in a dose-dependent manner by pretreatment for 30 min with 5 μg/ml decaffeinated coffee or 5 μM chlorogenic acid as shown by Western blotting (Fig. 6A and B). H_2O_2 decreased Bcl-X_L expression, but the effect was markedly attenuated by pretreatment with decaffeinated coffee or chlorogenic acid (Fig. 6A and B). Next, we assessed whether the increased expression of Bcl-X_L in response to decaffeinated coffee and chlorogenic acid leads to a lack of caspase-3 activation. Western blot analysis revealed that the H_2O_2-induced downregulation of caspase-3 was inhibited by decaffeinated coffee and chlorogenic acid (Fig. 6A and B). These results suggest that decaffeinated coffee and chlorogenic acid attenuate the H_2O_2-induced apoptosis of PC12 cells by blocking the downregulation of Bcl-X_L and caspase-3 expression.

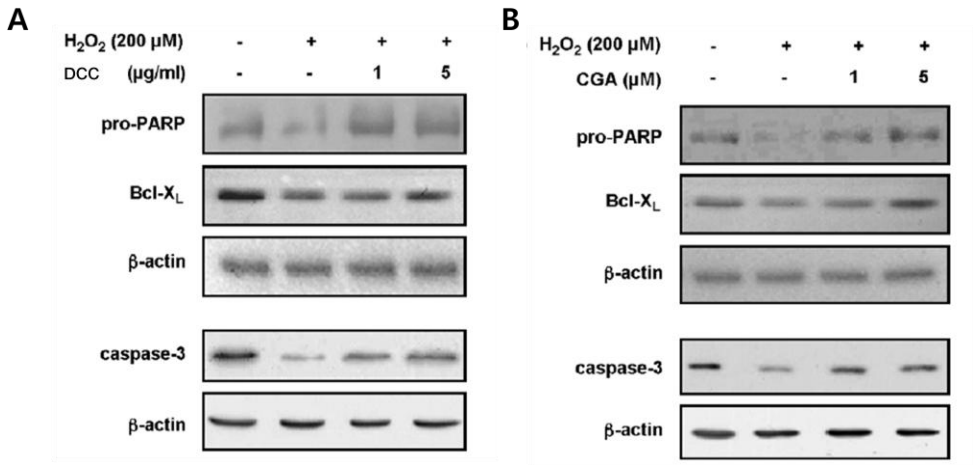

Figure 6. The effects of decaffeinated coffee (DCC) and chlorogenic acid (CGA) on H_2O_2-induced cleavage of PARP and the downregulation of Bcl-X_L and caspase-3 expression in PC12 cells. Cells were preincubated for 30 min with (A) DCC (1 and 5 μg/ml) or (B) CGA (1 and 5 μM) then exposed to 200 μM H_2O_2 for 24 h. The levels of PARP, caspase-3, Bcl-X_L, and β-actin were examined by Western blotting as described in the Materials and Methods. β-actin was measured to confirm uniform protein loading. The data are representative of three independent experiments.

1.3.6. Decaffeinated coffee and chlorogenic acid prevent H_2O_2-induced accumulation of intracellular ROS

To determine whether decaffeinated coffee and chlorogenic acid is able to block the accumulation of intracellular ROS induced by H_2O_2, we examined the ROS level in PC12 cells exposed to H_2O_2 using DCFH-DA. PC12 cells exposed to 200 μM H_2O_2 for 15 min showed increased fluorescence, but the effect was significantly suppressed by pretreatment with decaffeinated coffee (1 and 5 μg/ml) or chlorogenic acid (1 and 5 μM) in a dose-dependent manner (Fig. 7A and B)

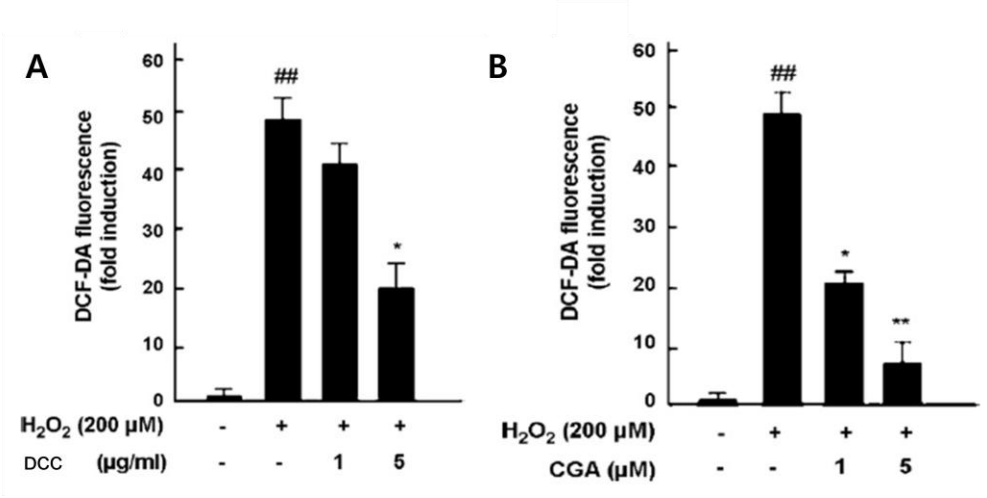

Figure 7. The effects of decaffeinated coffee (DCC) and chlorogenic acid (CGA) on the H_2O_2-induced accumulation of intracellular ROS in PC12 cells. ROS accumulation was assessed by DCF-derived fluorescence in PC12 cells treated with 200 μM H_2O_2 for 15min in the presence or absence of (A) DCC or (B) CGA using the DCFH-DA assay described in Section 2. The data are given as the mean±S.D. for three independent experiments. ##$p < 0.01$ compared to control cells. *$p < 0.05$ and **$p < 0.01$ compared to H_2O_2-treated cells.

2.3.7. Decaffeinated coffee and chlorogenic acid prevent the H_2O_2-induced activation of JNK and p38 MAPK

Increasing evidence indicates that MAPK activation is involved in the pathogenesis of AD [28]. Exposure to 200 μM H_2O_2 strongly activated JNK and p38 MAPK in PC12 cells, but the effect was greatly attenuated by pretreatment with decaffeinated coffee (1 and 5 μg/ml) or chlorogenic acid (1 and 5 μM) (Fig. 8). These results indicate that the inhibitory effects of decaffeinated coffee and chlorogenic acid on the H_2O_2-induced apoptosis of PC12 cells involve reduced JNK and p38 MAPK activation.

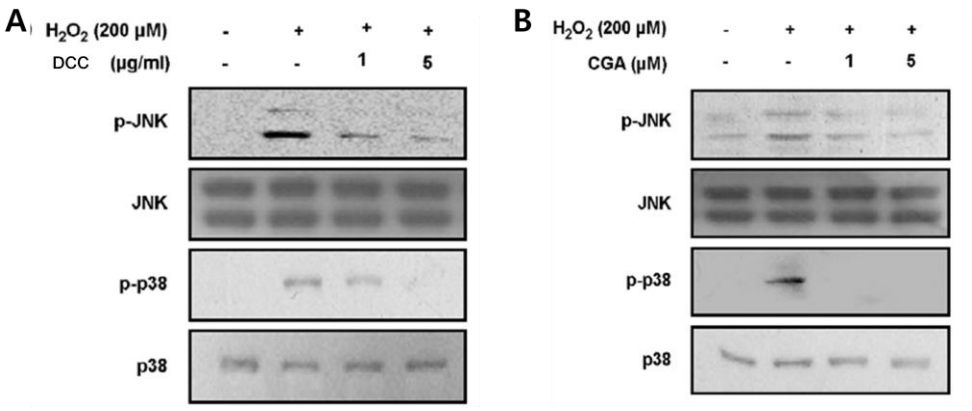

Figure 8. The effects of decaffeinated coffee (DCC) and chlorogenic acid (CGA) on the H_2O_2-induced activation of JNK and p38 MAPK in PC12 cells. Cells were preincubated for 30min with (A) DCC (1 and 5μg/ml) or (B) CGA (1 and 5 μM) and then exposed to 200 μM H_2O_2 for 15 min. The amounts of phosphorylated or total JNK and p38 MAPK were determined by Western blotting as described in Section 2. Total JNK and p38 MAPK were measured to confirm uniform protein loading. The data are representative of three independent experiments.

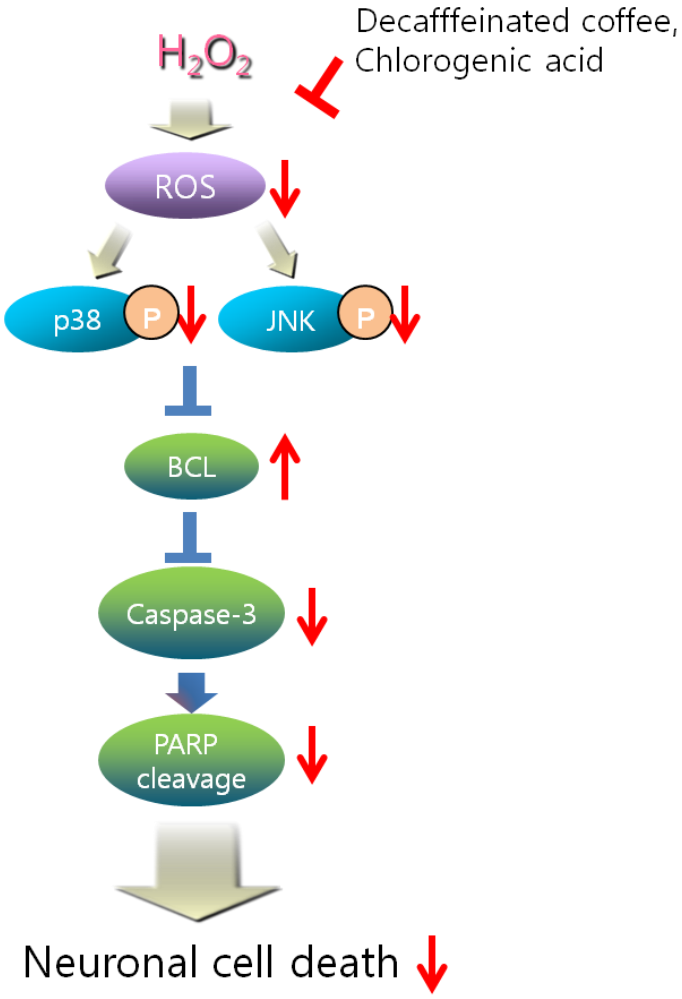

Figure 9. Hypothetical scheme for the mechanism of the neuroprotective action of decaffeinated coffee and chlorogenic acid.

2.4. Discussion

Oxidative stress increases neuronal apoptosis during early stage of AD through the production of free radicals such as H_2O_2, which can damage cellular lipids, proteins, and nucleic acid [29, 30]. In this study, I investigated that the potential neuroprotective effects of coffee, decaffeinated coffee and chlorogenic acid. Results have shown that both coffee and decaffeinated coffee protect neuron against H_2O_2-induced apoptosis similarly. Chlorogenic acid, a major phytochemical found in both coffee and decaffeinated coffee, also attenuated H_2O_2-induced apoptotic neuronal death. A simplified depiction of proposed mechanism is shown in Fig. 9.

Oxidative stress-induced apoptosis is closely related to mitochondrial dysfunction. Oxidative stress induced by ROS such as H_2O_2 increases mitochondrial permeability, leading to the release of cytochrome *c* and the subsequent activation of caspases [29]. Caspases are cysteine proteases that mediate cell death, and caspase-3 has been shown to be an important regulator of neuronal apoptosis. Caspase-3 is cleaved and activated during the final step of apoptosis; it also mediates H_2O_2-induced apoptosis by cleaving full-length PARP (116 kDa) into an 89-kDa fragment [31]. In the present study, exposure to H_2O_2 resulted in the caspase-3 downregulation and PARP cleavage, but this effect was attenuated by pretreatment with coffee, decaffeinated coffee and chlorogenic acid. These results indicate that

H_2O_2-induced apoptosis may be prevented by coffee, decaffeinated coffee and chlorogenic acid through the inhibition of caspase-3 cleavage and subsequent PARP cleavage.

The Bcl-2 family of apoptosis regulatory genes is also involved in mitochondrial membrane dysfunction during apoptosis. During the initiation phase of apoptosis, proapoptotic Bcl-2 family members such as Bax are translocated to the mitochondrial membrane where they increase mitochondrial permeability [29]. The intracellular concentration of the antiapoptotic protein Bcl-2 acts as a molecular rheostat that influences whether a cell lives or dies [21]. Bcl-X_L is structurally and functionally analogous to Bcl-2, and it blocks the induction of apoptosis by a wide array of death signals. The protective effect of Bcl-X_L overexpression against apoptosis induced by various death stimuli has also been investigated [7]. I analyzed the level of Bcl-2 and Bcl-X_L expression to determine the molecular mechanisms underlying the antiapoptotic effects of coffee, decaffeinated coffee and chlorogenic acid. PC12 cells or primary neuronal culture exposed to H_2O_2 showed decreased Bcl-2 or Bcl-X_L expression, but the effect was greatly reduced by treatment with coffee, decaffeinated coffee and chlorogenic acid. However, caffeic acid, another coffee phytochemical, and caffeine did not suppress H_2O_2-induced Bcl-2 and Bcl-X_L downregulation. These results indicated that the inhibitory effect of coffee

and decaffeinated coffee on Bcl-2 and Bcl-X_L downregulation might be attributable to chlorogenic acid but not caffeic acid and caffeine .

Highly reactive hydroxyl radicals are generated from H_2O_2 via the metal-catalyzed Fenton reaction. The oxidation of DCFH-DA in the presence of endogenous ROS including hydroxyl radicals results in fluorescence. In this study, PC12 cells treated with H_2O_2 produced fluorescence, indicating that H_2O_2 significantly increased the accumulation of intracellular ROS; however, the effect was reduced by pretreatment with decaffeinated coffee or chlorogenic acid. These data demonstrate that decaffeinated coffee and chlorogenic acid attenuate ROS accumulation in PC12 cells. Accumulating literatures still have focused on the protective effect of chlorogenic acid against human diseases because of its high antioxidant properties and positive relationship between antioxidant effects of coffee and chlorogenic acid contents [32]. In accordance with previous literatures, my results revealed that chlorogenic acid has strong effect on preventing ROS accumulation, which is partly attributed to its antioxidant activity. However, ROS such as H_2O_2 act not only as cellular messengers capable of causing oxidative damage to macromolecules, but also as signaling molecules that activate protein kinase cascades [33]. Accumulating evidence suggests that ROS activate stress-activated protein kinases such as JNK and p38 MAPK by inhibiting MAP kinase phosphatases [34, 35]. SP600125, a specific

inhibitor of JNK, and SB203580, a specific inhibitor of p38 MAPK, have been shown to prevent the induction of PC12 cell death by H_2O_2, indicating that JNK and p38 MAPK activation is required for H_2O_2-induced neuronal apoptosis [36].

Recent studies have highlighted several important mechanisms involving phenolic phytochemicals that complement their direct antioxidant activities, including their ability to influence gene expression and cell signaling by binding to cellular kinases, which has been shown to have chemoprotective effects against oxidative stress-induced human diseases [37]. Therefore, phenolic phytochemicals may inhibit ROS-induced apoptosis by downregulating protein kinase signaling in addition to eliminating ROS production. To examine these possibilities, I investigated the effects of decaffeinated coffee and chlorogenic acid on the activation of JNK and p38 MAPK. JNK and p38 MAPK were rapidly activated by H_2O_2 in PC12 cells, but the effect was inhibited by pretreatment with decaffeinated coffee or chlorogenic acid. Inactivation of JNK and p38 MAPK is likely to result in the inhibition of apoptosis because the activation of these molecules reportedly involves the downregulation of Bcl-X_L, an antiapoptotic protein. Previous study revealed that JNK acts as an upstream mediator downregulating Bcl-X_L expression in neuronal cells [38]. This study showed that amyloid-beta-induced Bcl-X_L downregulation was inhibited by

SP600125, indicating that JNK can be involved in regulating Bcl-X_L expression and subsequently inducing apoptosis. p38 MAPK was also identified as a positive regulator of TNF-induced endothelial cell apoptosis via the downregulation of Bcl-X_L expression [39]. In this study, they suggested that p38 MAPK phosphorylates Bcl-X_L, leading to the proteasomal degradation of Bcl-X_L. These previous studies showed the strong evidence that JNK and p38 MAPK are involved in the Bcl-X_L regulation and the subsequent apoptosis. Other studies also suggested that JNK and p38 MAPK can also upregulate proapoptotic Bcl-2 family proteins and induce apoptosis. A recent study showed that JNK and p38 MAPK phosphorylated proapoptotic Bax, leading to its mitochondrial translocation prior to apoptosis [40]. Since the increased ratio of Bax to Bcl-X_L leads to cells more susceptible to apoptosis, this JNK and p38 MAPK induced-Bax translocation should be regarded as another mechanism causing the JNK and p38 MAPK-involved apoptosis. My results indicated that decaffeinated coffee and chlorogenic acid prevent apoptosis by blocking the activation of JNK and p38 MAPK, which are linked to the regulation of Bcl-X_L.

There is no direct evidence whether chlorogenic acids can pass through the blood–brain barrier. However, one study showed that chlorogenic acid is neuroprotective against scopolamine-induced amnesia via anti-acetylcholinesterase and anti-oxidative activities in mice [41]. The

blood-brain barrier has been found to be permeable to epigallocatechin gallate (EGCG, MW 458), the bioactive phenolics in green tea [42]. The molecular weight of chlorogenic acid is 354 which is smaller than that of EGCG. These results suggest that chlorogenic acid are likely permeable to blood-brain barrier [43].

One study showed that 1 h after oral ingestion of 250 ml of a drink containing green coffee bean extract which includes 300 mg chlorogenic acid, 7.39±2.64 ng/ml of chlorogenic acid is detected in human plasma [44]. In contrast, another recent study reported 3.14 ± 1.64 μM, much higher concentration of chlorogenic acid, was detected in human plasma after the consumption of 190ml of coffee drink containing 1068 ± 49 μmol chlorogenic acid [45], implying that the concentration of chlorogenic acid used in our study can be achievable after coffee consumption. Even so, further studies will be necessary to manifest whether the neuroprotective antioxidant effects of coffee and chlorogenic acid that I have shown here are of relevance to the reported protective effects of coffee consumption against AD.

In summary, the results of this study show that coffee and decaffeinated coffee inhibited H_2O_2-induced apoptotic neuronal death and that chlorogenic acid might be largely responsible for these effects. This protection occurs through the attenuation of intracellular ROS accumulation

and the inhibition of JNK and p38 MAPK activation.

2.5. References

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Chapter 3.

Decaffeinated coffee prevents scopolamine-induced memory impairment in rats

Abstract

Several human studies have reported that coffee consumption improves cognitive performance. In the present study, I investigated whether instant decaffeinated coffee also ameliorates cognitive performance and attenuates the detrimental effects of scopolamine on memory. Memory performance was evaluated in Morris water maze test and passive avoidance test. Instant decaffeinated coffee (p.o.) at 120 or 240 mg/kg in Sprague-Dawley rats, which is equivalent to approximately three or six cups of coffee, respectively, in a 60-kg human, was administered for two weeks. Oral gavage administration of instant decaffeinated coffee inhibited scopolamine-induced memory impairment, which was measured by Morris water maze test and passive avoidance test. Instant decaffeinated coffee suppressed scopolamine-mediated elevation of tumor necrosis factor-α (TNF-α) and stimulation of nuclear factor-κB (NF-κB) pathway (i.e., phosphorylation of IκBα and p65) in the rat hippocampus. These findings suggest that caffeine-free decaffeinated coffee may prevent memory impairment in human through the inhibition of NF-κB activation and subsequent TNF-α production.

Key words: *Decaffeinated coffee; Memory; NF-κB; Scopolamine; TNF-α*

3.1. Introduction

Several seminal studies have reported that coffee consumption is associated with better cognitive performance and is inversely associated with neurodegenerative diseases such as Alzheimer's disease (AD) [1]. Along the same lines, there are seminal epidemiological, pharmacological and genetic evidences showing an inverse relation between coffee intake and Parkinson's disease (PD) [2-4]. Caffeine, an adenosine A2A receptor antagonist, is considered to be primarily responsible for the neuropharmacological effects of coffee, because of its well-known psycho-stimulating effects and the ability to cross the blood-brain barrier [5-7]. Large cohorts and meta-analysis have confirmed the impact of caffeine on AD [8-10]. Especially, many studies showed that caffeine prevented short- or long term memory impairment in animal models [11-15]. Caffeine was also reported to attenuate scopolamine-induced memory impairment in humans [16]. On the other hand, the effect of decaffeinated coffee on memory performance has not been elucidated.

Coffee is a complex chemical mixture consisting of a number of bioactive compounds called phytochemicals. Although climatic conditions, agricultural practices, processing, and storage vary its composition, coffee contains approximately 7-9% phenolic phytochemicals, and only 1% caffeine in general [17, 18]. Chlorogenic acid, 5-feruloylquinic acid, 4-

caffeoylquinic acid, and caffeic acid have been noted as major phytochemicals found in coffee [19], and these phytochemicals themselves have been reported to have neuroprotective activities. For example, chlorogenic acid significantly improved the scopolamine-induced impairment of short-term or working memory [20]. Caffeic acid was also found to be neuron-protective *in vivo* under pathological conditions of focal cerebral ischemia [21]. Kahweol and cafestol were suggested as antioxidative and neuroprotective components in coffee as well [22, 23]. Taken together, combination of these phytochemicals in coffee might contribute to prevent memory impairment.

Scopolamine is a non-selective muscarinic receptor antagonist that is well known to pharmacologically interfere with memory performance in a transient manner [24]. Animals with scopolamine-induced memory impairment have been widely used to probe drugs attenuating cognitive deficits. I investigated whether instant decaffeinated coffee (IDC) attenuates learning and memory impairment induced by scopolamine in Sprague-Dawley rats. Oral gavage administration of IDC at 120 or 240 mg/kg in rats, which is equivalent to approximately three or six cups of coffee, respectively, in a 60-kg human, was used to test the preventive effects of IDC on learning and memory impairment.

3.2. Materials and methods

3.2.1. Reagents

IDC (Maxim Decaffeinated, carbohydrate 625 mg, chlorogenic acid 55 mg, caffeine 0.939 mg per gram of IDC) was purchased from Dongsuh Food (Seoul, South Korea). Scopolamine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibody against TNF-α was obtained from R&D Systems (Minneapolis, MN, USA). Antibodies against phosphorylated p65 and phosphorylated IκBα were purchased from Cell Signaling Technology (Danvers, MA, USA).

3.2.2. Animals

Male Sprague-Dawley rats weighing 200–250 g (age, 7 weeks) were purchased from the Hyochang Science (Taegu, South Korea)4. All experiments were performed according to the institutional guidelines for the care and use of laboratory animals. The rats were housed 3 or 4 per cage, allowed access to water and food *ad libitum*, and maintained at an ambient temperature of 21 ± 2 ℃ with 50 ± 10% humidity and a 12-h diurnal light cycle (lights on 06:00–18:00 h) prior to testing. The rats were habituated for 5 days before the drug administration. All behavioral experiments were carried out in a room adjacent to the housing room under the same ambient conditions.

3.2.3. Drug administration

IDC was dissolved in distilled water and scopolamine in saline for use. In the scopolamine-induced memory impairment study (Fig. 1A), IDC (1 ml, 120 or 240 mg/kg, p.o.) or distilled water was administered once a day for 6 days and then given 1 h before the first trial session every consecutive 5 days in the water maze task and 1 h before the acquisition trial and the retention trial in the passive avoidance task for the next 2 days. Memory impairment was induced by scopolamine treatment (0.75 mg/kg, i.p.) 30 min before each task. In control group, vehicle solution (distilled water, p.o. and saline, i.p.) was administered using the same time schedule. Each group contained 6 rats.

In the study to investigate the effect of IDC as a memory enhancer (Fig. 1B), IDC (1 ml, 120 or 240 mg/kg, p.o.) or distilled water (p.o.) was administered once a day for 6 days and then given 1 h before the first trial session every consecutive 5 days in the water maze task and 1 h before the acquisition trial and the retention trial in the passive avoidance task for the next 2 days. In the control group, vehicle solution (distilled water, p.o.) was administered using the same time schedule. Each group contained 7 rats.

3.2.4. Morris water maze test

The Morris water maze is a circular pool (180 cm in diameter and 60

cm in height) with a featureless inner surface. The pool was filled with water maintained at 21 ± 2 ℃. The tank was placed in a dimly lit, sound proof test room with various visual cues. The pool was conceptually divided into quadrants, and a hidden escape platform (12 cm in diameter and 38 cm in height) was placed in one of the pool quadrants and submerged 2 cm below the water surface so that it was not visible at water level. During the 5 subsequent days, the rats underwent three trials per day with the platform in place. For each training trial, rats were placed in the water facing the pool wall in different pool quadrants, with a variable order each day. When a rat located the platform, it was permitted to remain on the platform for 30 s. If the rat did not locate the platform within 90 s, it was placed on the platform for 30 s. The animal was taken to its home cage and was allowed to dry under an infrared lamp after each trial. During each trial, the time taken to find the hidden platform (latency) was recorded using a video camera-based Ethovision System (Nodulus, Wageningen, Netherlands). Immediately after the last training trial session, rats were subjected to a probe trial session in which the platform was removed from the pool and rats were allowed to swim for 90 s to search for it. A record was kept of the swimming time in the pool quadrant where the platform had previously been placed.

3.2.5. Passive avoidance test

The passive avoidance test is a well-established experimental procedure used to assess short-term reference memory, which depend on cortical and hippocampal circuitries [25]. The step-through passive avoidance test was performed in identical illuminated and dark chambers (Gemini Avoidance System, San Diego, CA, USA). The illuminated compartment contained a bulb, and the floor of the non-illuminated compartment was composed of stainless steel rods. These compartments were separated by a guillotine door. For the acquisition trial, rats were initially placed in the illuminated compartment and the door between the two compartments was opened 20 s later. When the rats entered the dark compartment, the door closed automatically and an electrical foot shock (0.5 mA) of 5-s duration was delivered through the stainless steel rods. Twenty-four hours after the acquisition trial, the rats were again placed in the illuminated compartment for retention trials. The time taken for a rat to enter the dark compartment after the door opened was measured as the latency time in both acquisition and retention trials, with a maximum of 300 s.

3.2.6. Western blot analysis

The hippocampus was homogenized using Ultra Turrax homogenizer (Next Advance, Averill Park, NY, USA) in ice-cold tissue protein extraction solution (Thermo Fisher Scientific, Rockford, IL, USA)

containing phosphatase inhibitor cocktail and 0.1 mM phenylmethanesulfonylfluoride (PMSF). The lysate was centrifuged at 16,000×g for 15 min. The protein concentration of the supernatant was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). The protein (90 μg) was subjected to 10% SDS-PAGE and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked in 5% fat-free dry milk for 1 h and then incubated with primary antibodies for 2 h at room temperature. After incubation with the horseradish-peroxidase-conjugated secondary antibodies, protein bands were detected using an enhanced chemiluminescence [26] detection kit (GE Healthcare, St. Giles, United Kingdom).

3.2.7. Statistical analysis

All analyses were performed using the PASW 18 Statistical Package (SPSS 12.0 KO for Windows, SPSS Inc., Chicago, IL, USA). One-way analysis of variation [27] and one-way repeated ANOVA were conducted to assess the effects of IDC. Post-hoc analyses (t-test or least significant difference (LSD)) were subsequently conducted to determine the effects of the scopolamine or the IDC treatment. Data were expressed as mean ± standard error of the mean (SEM). $P < 0.05$ was considered significant.

3.3. Results

3.3.1. IDC inhibited scopolamine-induced memory impairment

To determine the effects of IDC on memory impairment, the Morris water maze test was performed in rats treated with IDC (120 or 240 mg/kg, p.o.) and scopolamine (0.75 mg/kg, i.p.) (Fig. 2A). One-way repeated ANOVA showed that the interaction effects between the group and the training session were not significant ($F_{(12,80)}$=0.877, n.s). On the other hand, the between group effects were significant ($F_{(3,20)}$=10.286, $P < 0.001$) as were the training session effects ($F_{(4,80)}$=36.563, $P < 0.001$). As shown in Fig. 2A, the vehicle-treated rats (VEH) quickly became proficient at locating the submerged platform during the training sessions; however, the scopolamine rats (SCO) did not show much improvement over the course of training when compared with the vehicle-treated rats (VEH). The scopolamine-treated rats that were administered IDC (120 or 240 mg/kg, p.o.) showed significantly better performances than the scopolamine rats ($P = 0.038$ or $P = 0.009$, respectively). Fig. 2C depicts the representative swim paths of these rats on the 5th day of the Morris water maze test. Vehicle-treated rats (a, VEH) swam a shorter distance to find the platform compared to scopolamine-treated rats (b, SCO). IDC treatment at 120 mg/kg (c, SCO+IDC 120 mg/kg) or 240 mg/kg (d, SCO+IDC 240 mg/kg) shortened the distance needed to find platform.

Spatial learning was also assessed by the probe test, in which the platform was removed from the pool and rats were given 90 s to look for it. I measured how long rats spent in the quadrant that had previously held the platform (Fig. 2B). The one-way ANOVAs on the probe test showed that the between group effects were significant ($F_{(3,20)}$=5.389, $P < 0.01$). The vehicle-treated rats were found to have the spatial bias when compared with the scopolamine rats ($P = 0.004$). Vehicle-treated rats spent about 37.4 s in the platform quadrant, whereas scopolamine rats spent about 22.6 s in that quadrant. Scopolamine rats treated with IDC (120 or 240 mg/kg, p. o.) did show statistically significant ameliorative effects on spatial learning when compared with scopolamine rats ($P = 0.040$ or $P = 0.004$, respectively). The speed of rats was measured on day 5 and the between group effects were not significant (Supplementary Fig. 1).

Retention of the passive avoidance response was measured to confirm the effects of IDC on memory impairment (Fig. 3). Scopolamine (0.75 mg/kg, i.p.) was administered 30 min before the acquisition trial and the retention trial. The one-way ANOVAs on the passive avoidance test showed that the between group effects were significant ($F_{(3,20)}$=31.157, $P < 0.001$). Scopolamine-treated rats had a significantly shorter step-through latency compared to the vehicle-treated rats ($P = 0.006$). Administration of IDC (240 mg/kg, p.o.) 30 min before the scopolamine treatment significantly

lengthened the step-through latency (P = 0.034). Latency times during the acquisition trial were not affected by any of these drugs (Fig. 3). These observations suggest that IDC acted as a memory stabilizer against scopolamine-mediated deficits.

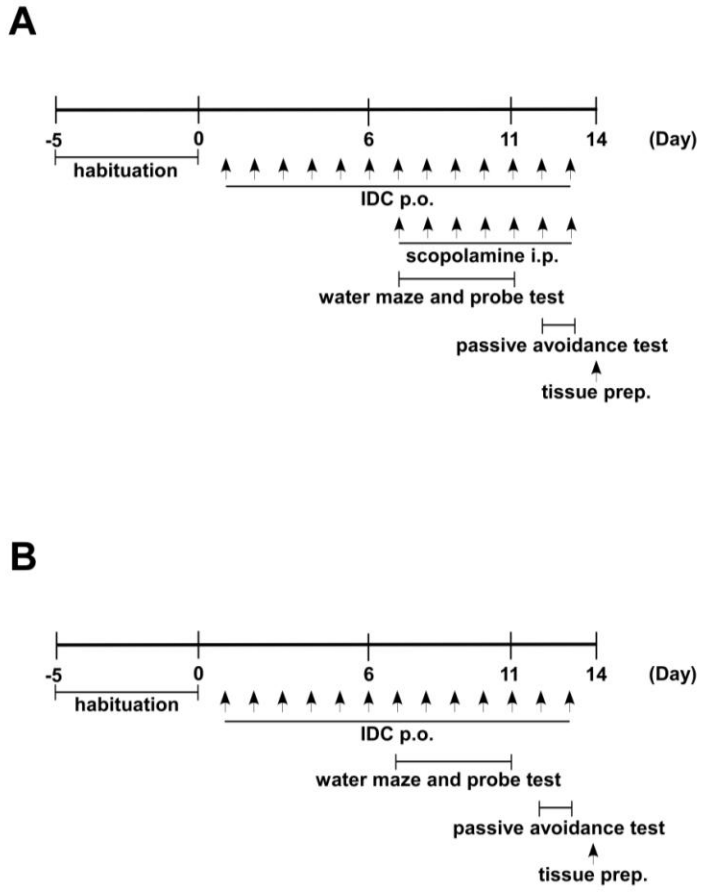


Figure 1. Experimental schedule to determine the effect of instant decaffeinated coffee (IDC) on memory impairment (A) and memory enhancement (B). (A) After a 5-day habituation period, rats were given IDC (120 or 240 mg/kg, p.o.) for a total of 13 days. IDC alone was treated for 6 days, and then scopolamine (0.75 mg/kg, i.p.) was administered together with IDC for another 7 days. Rats underwent the Morris water maze test for 5 days, and the probe test was conducted after the last training trial of the

Morris water maze test. The day after completion of the probe test, the passive avoidance test was conducted for 2 days. The day after passive avoidance test, the rats were sacrificed and hippocampus was removed for Western blot analysis. (B) After a 5-day habituation period, rats were given IDC (120 or 240 mg/kg, p.o.) for a total of 13 days. Rats underwent the Morris water maze test for 5 days, and the probe test was conducted after the last training trial of the Morris water maze test. The day after completion of the probe test, the passive avoidance test was conducted for 2 days. The day after the passive avoidance test, the rats were sacrificed and hippocampus was removed for Western blot analysis.

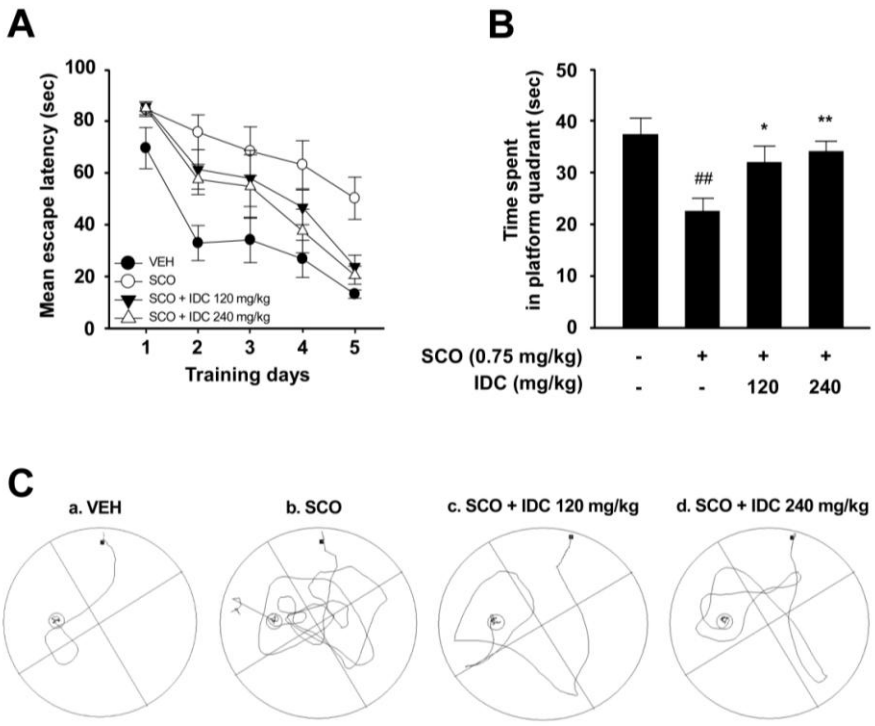


Figure 2. Effect of instant decaffeinated coffee (IDC) on scopolamine-induced memory impairment in the Morris water maze test. (A) IDC reduced the scopolamine (SCO)-induced escape latency. IDC (120 or 240 mg/kg, p.o.) was administered to rats 1 h before the training trials, and memory impairment was induced by scopolamine treatment (0.75 mg/kg, i.p.) 30 min before the training trials. Data are expressed as mean ± SEM (n=6). (B) IDC increased time spent in the platform quadrant during the probe test, which had been reduced by scopolamine treatment. Data are expressed as mean ± SEM (n=6). $^{\#\#}P < 0.05$ versus vehicle–treated group; *p < 0.05 versus the

scopolamine-treated group; **p < 0.01 versus the scopolamine-treated group. (C) Representative swimming paths of rats from each group in the Morris water maze test on the training day 5.

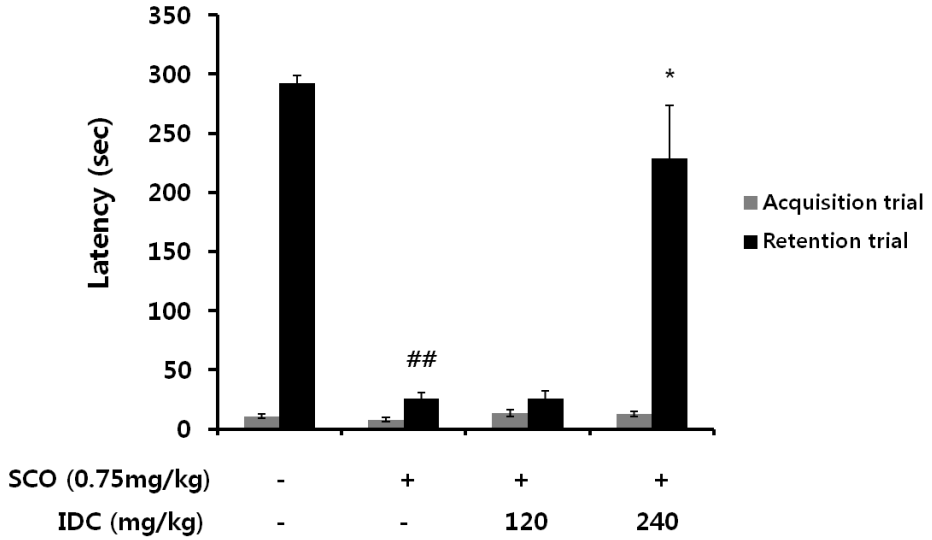
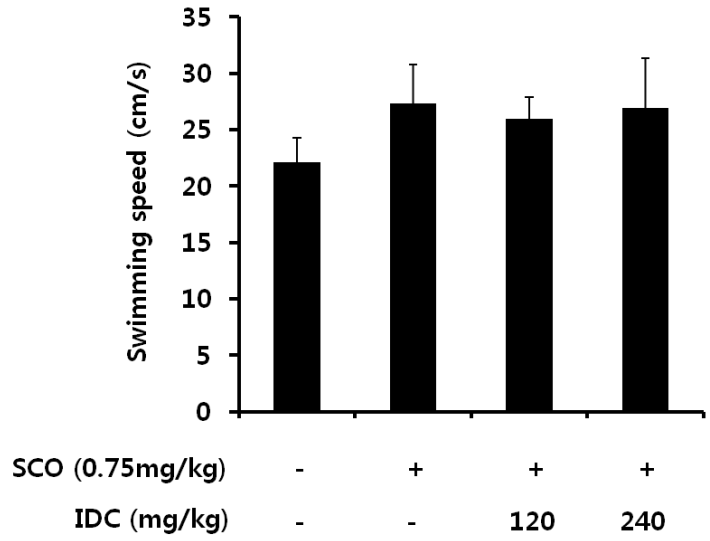


Figure 3. Effect of instant decaffeinated coffee (IDC) on scopolamine-induced memory deficits in the passive avoidance test. IDC increased the step-through latency of scopolamine (SCO)-treated rats. IDC (120 or 240 mg/kg, p.o.) was administered to rats 1 h before the acquisition trial and the retention trial, and the memory impairment was induced by scopolamine treatment (0.75 mg/kg, i.p.) 30 min after IDC treatment. Data are expressed as mean ± SEM (n=6). $^{\#\#}P < 0.01$ versus vehicle-treated rats; $*p < 0.05$ versus scopolamine-treated rats.



Supplementary Figure 1. Average swimming speed on day 5 in the Morris water maze test.

3.3.2. IDC did not enhance memory per se

To know whether IDC *per se* enhances memory, rats were treated with IDC (120 or 240 mg/kg, p.o.) without scopolamine and underwent the Morris water maze test (Fig. 4A and B). One-way repeated ANOVA showed that the interaction effects between the group and the training session were not significant ($F_{(8,72)}$=0.463, n.s.). The between group effects were not significant ($F_{(2,18)}$=1.574, n.s.), either, even though the training session effects were ($F_{(4,72)}$=41.505, $P < 0.001$). As shown in Fig. 4A, the vehicle-treated rats (VEH) became proficient at locating the submerged platform during the training sessions, and the IDC (120 or 240 mg/kg, p.o.)-treated rats did not show much difference over the course of training when compared with the vehicle treated rats (VEH). There were no significant differences between the vehicle-treated rats (VEH) and IDC (120 or 240 mg/kg, p.o.)-treated rats ($P = 0.097$ or $P = 0.275$), indicating that IDC does not enhance spatial memory *per se*.

I also measured how long rats spent in the quadrant that had previously held the platform after IDC treatment by the probe test (Fig. 4B). Vehicle-treated rats and IDC-treated rats spent similar times in that quadrant. The one-way ANOVAs on the probe test showed that the between group effects were not significant ($F_{(2,18)}$=0.511, n.s.). The speed of rats was measured for 5 days during the training trials of the Morris water maze test

and the between group effects were not significant (data not shown).

The effects of IDC as a memory enhancer were tested by the passive avoidance test. The one-way ANOVAs on the passive avoidance test showed that the between group effects in step-through latency were not significant ($F_{(2,18)}$=0.143, n.s.) (Fig. 4C). During the acquisition trial, no differences in latent time were observed either. Overall, these observations suggest that IDC has no effect as a memory enhancer in itself.

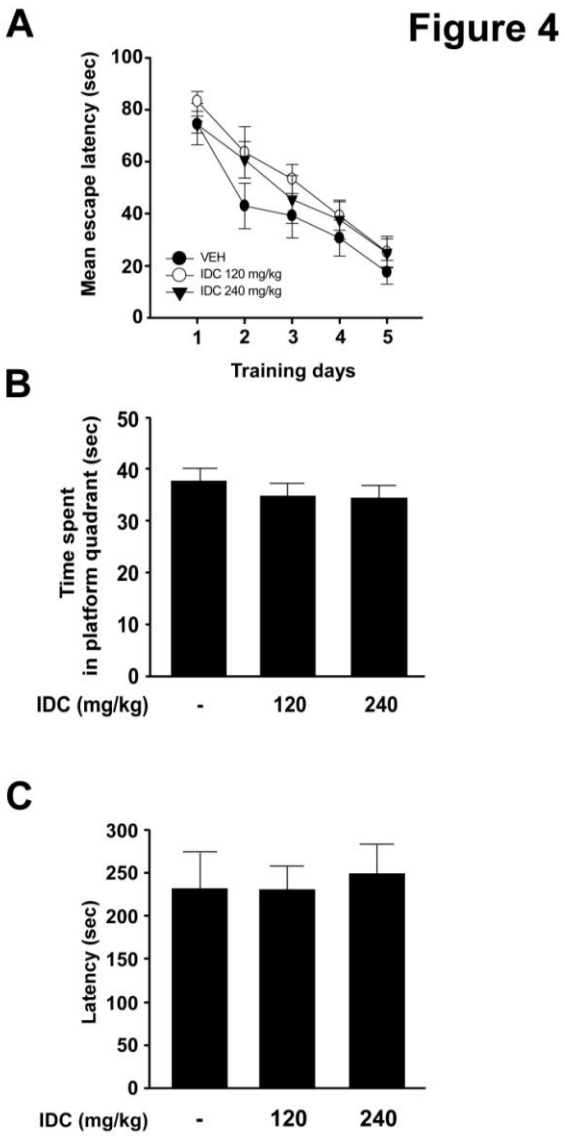


Figure 4. Effects of instant decaffeinated coffee (IDC) on memory enhancement in the Morris water maze test and the passive avoidance test. IDC (120 or 240 mg/kg, p.o.) was administered to rats 1 h before the trials.

(A) IDC did not alter escape latency *per se* in the water maze test. (B) IDC did not increase time spent in the platform quadrant during the probe test. (C) IDC did not increase the step-through latency in the passive avoidance test. Data are expressed as mean ± SEM (n=7).

3.3.3. IDC suppressed scopolamine-induced TNF-α production

Accumulating evidence suggests that inflammation is involved in impaired learning and memory [28-31]. Pro-inflammatory cytokine such as TNF-α is up-regulated in brains affected by dementia [32]. To determine the effects of IDC on TNF-α production, I performed Western blot analysis of proteins from the hippocampus of rats treated with IDC and scopolamine (Fig. 5A and B). One-way ANOVA analysis of the TNF-α showed that the between group effects were significant ($F_{(3,8)}$=12.326, $P < 0.01$). The hippocampal TNF-α levels in the scopolamine (0.75 mg/kg, i.p.)-treated rats were strongly up-regulated when compared with the vehicle rats (Fig. 5B; $P = 0.016$). These increases induced by scopolamine were attenuated by treatment with IDC (120 or 240 mg/kg, p.o.; $P = 0.029$ or $P = 0.000$, respectively). These results suggest that IDC significantly inhibited scopolamine-induced TNF-α production.

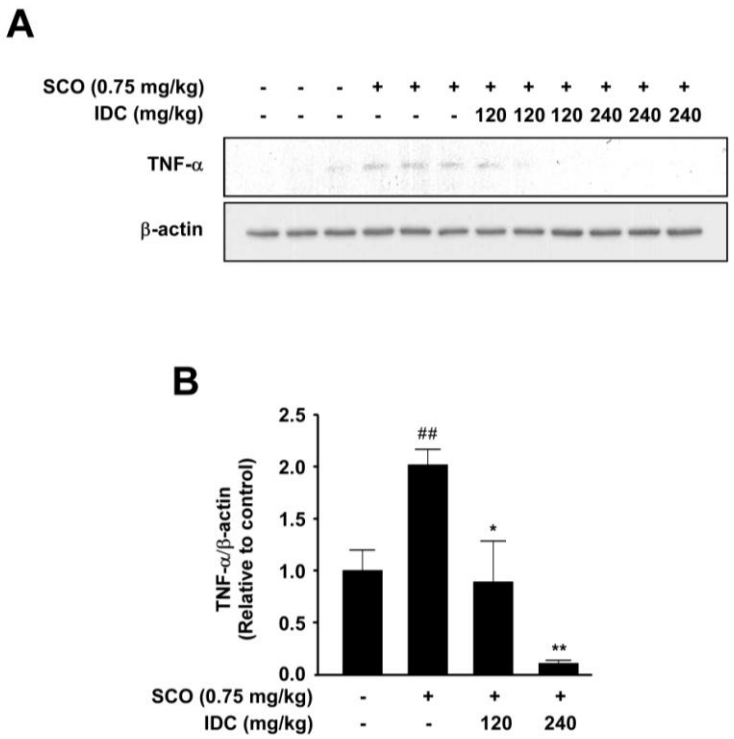


Figure 5. Effects of instant decaffeinated coffee (IDC) on scopolamine (SCO)-induced TNF-α production. (A) IDC suppressed the scopolamine-induced increase in TNF-α production in the hippocampus of rats. Levels of TNF-α were determined by Western blot analysis. β-actin was used as a loading control. (B) TNF-α protein levels of (A) were quantified. Data are expressed as fold increase relative to vehicle-treated group (mean ± SEM; n=3). $^{\#}P < 0.05$ versus vehicle-treated rats; $*p < 0.05$ versus scopolamine-treated rats; $**p < 0.01$ versus scopolamine-treated rats.

3.3.4. IDC suppressed scopolamine-induced NF-κB activation

To determine the inhibitory mechanism of IDC on TNF-α production, I evaluated the nuclear factor-κB (NF-κB) signaling molecules in the hippocampus by Western blot analysis (Fig. 6A–C). One-way ANOVA analysis of protein levels of p-p65 and p-IκBα showed that the between group effects were significant ($F_{(3,8)}$=14.973, $P < 0.01$, and $F_{(3,8)}$=25.509, $P < 0.001$, respectively). Hippocampal p-p65 and p-IκBα levels in the scopolamine-treated rats were strongly up-regulated when compared with the vehicle rats (Fig. 6B and C; $P = 0.018$ and $P = 0.049$, respectively). Increase in the level of p-p65 induced by scopolamine was attenuated by treatment with IDC (240 mg/kg, p.o.; $P = 0.02$, Fig. 6B) and the scopolamine-induced phosphorylation of IκBα was also inhibited by treatment of IDC (120 or 240 mg/kg, p.o.; $P = 0.004$ or $P = 0.002$, respectively, Fig. 6C). Hypothetical scheme for the inhibitory effect and mechanism of decaffeinated coffee in scopolamine-induced memory impairment in rats is shown in Figure 7.

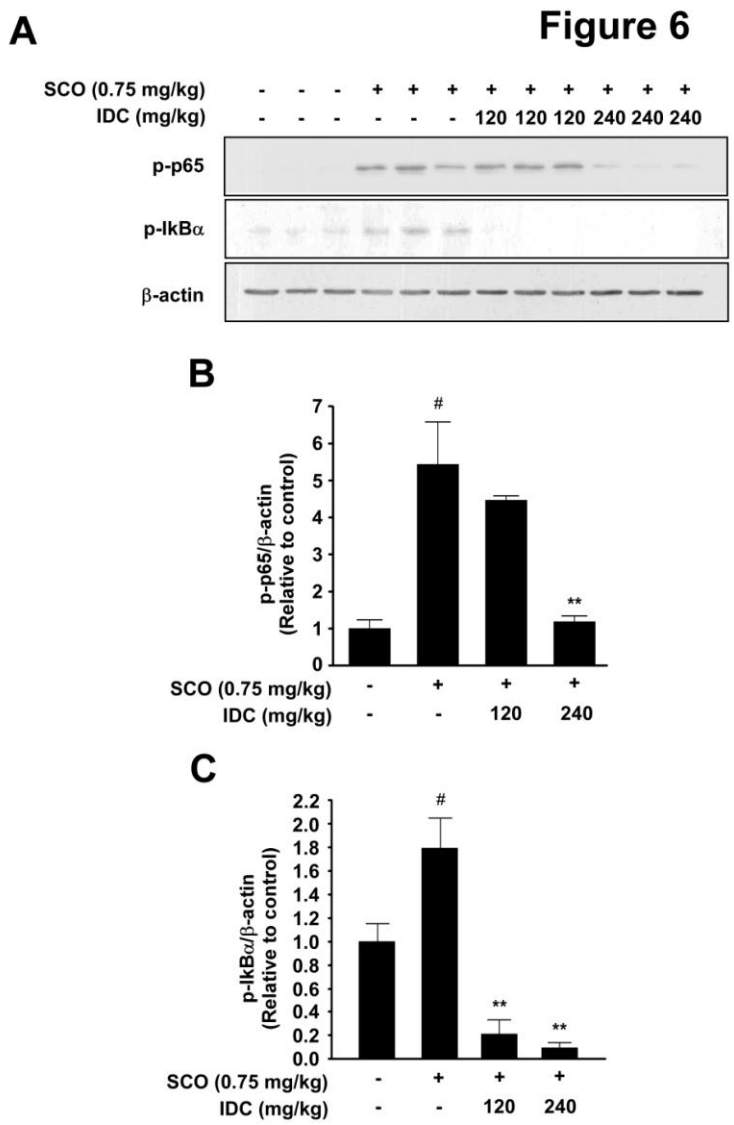


Figure 6. Effects of instant decaffeinated coffee (IDC) on scopolamine (SCO)-induced activation of the NF-κB pathway. (A) IDC inhibited scopolamine-induced phosphorylation of p65 and IκBα in the hippocampus of rats. Levels of phosphorylated p65 and IκBα were determined by Western

blot analysis. β-actin was used as a loading control. (B and C) Protein levels of phosphorylated p65 and IκBα (A) were quantified. Data are expressed as fold increase relative to vehicle-treated group (mean ± SEM; n=3). $^{\#}P < 0.05$ versus vehicle-treated rats; $*p < 0.05$ versus scopolamine-treated rats; $**p < 0.01$ versus scopolamine-treated rats.

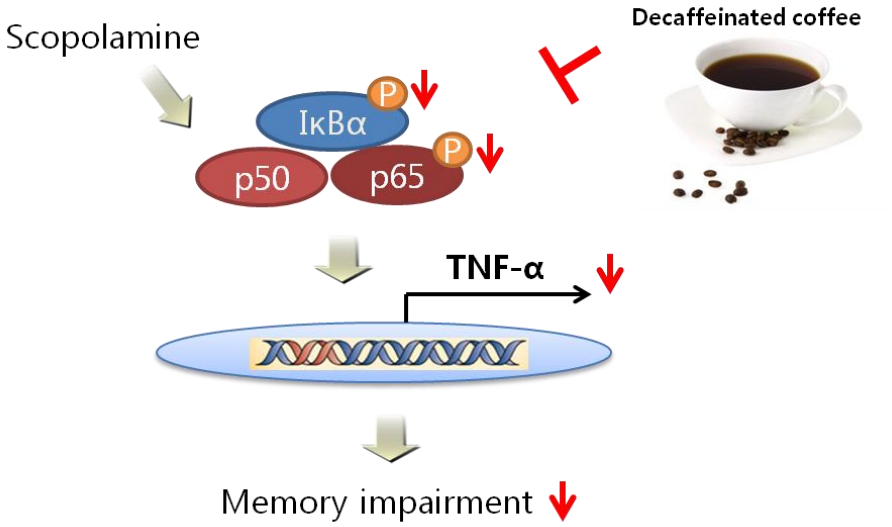


Figure 7. Hypothetical scheme for the inhibitory effect and mechanism of decaffeinated coffee in scopolamine-induced memory impairment in rats.

3.4. Discussion

I found that IDC-treated rats without scopolamine did not improve the learning and memory compared to vehicle-treated rats, suggesting that decaffeinated coffee is not a general memory bolsterer (Fig. 4). Instead, IDC attenuated scopolamine-induced purported memory deficit (Fig 2 and 3). Oral administration of IDC at 120 or 240 mg/kg in rats, which is equivalent to approximately three or six cups of coffee, respectively, in a 60-kg human, prevented the effects of scopolamine on memory impairment, indicating that IDC acted as a memory stabilizer against scopolamine.

Results of recent studies have indicated that decaffeinated coffee is neuroprotective. For example, decaffeinated coffee attenuated H_2O_2-induced oxidative neuronal cell death by inhibiting the accumulation of intracellular reactive oxygen species (ROS) [33]. Decaffeinated coffee up-regulated NADPH:quinone oxidoreductase 1 (NQO1) expression and prevented H_2O_2-induced apoptosis in primary cortical neuron [34]. In the transgenic fly models of Alzheimer's disease, Parkinson's disease, and Huntington's disease, decaffeinated coffee activated cytoprotective transcription factor NF-E2-Related Factor 2 (Nrf2) and showed neuroprotective effects [22]. Dietary supplementation with decaffeinated green coffee improved diet-induced brain energy metabolism dysfunction in a high-fat diet mouse [35]. Taken together with our observations, these reports suggest that

decaffeinated coffee drinks may exert beneficial effects on brain.

Accumulating evidence suggests that increased inflammation and microglial activation is associated with cognitive deficits [28]. High level of pro-inflammatory cytokines including TNF-α, interleukin (IL)-6, and IL-1 are shown in the brains of dementia [32, 36, 37]. TNF-α and inducible nitric oxide synthase (iNOS) are required for amyloid β (Aβ)-induced learning and memory impairment [38]. In particular, TNF-α participates in the Aβ-induced inhibition of long-term potentiation, a form of synaptic plasticity closely associated with learning and memory [39]. Therefore, inhibiting TNF-α production and neuroinflammation may be a promising strategy to prevent memory impairment [40, 41].

I found that IDC attenuated the scopolamine-mediated up-regulation of TNF-α in the hippocampus. Muscarinic receptors in the central nervous system inhibit systemic inflammation in endotoxemic rats and activation of muscarinic cholinergic transmission in the central nervous system lowers serum TNF levels [42]. Since scopolamine is a non-selective muscarinic receptor antagonist, blockage of muscarinic receptor by scopolamine might increase the expression of TNF-α in the hippocampus. Acetylcholinesterase is activated by scopolamine, and acetylcholinesterase can enhance inflammation [20, 43]. Scopolamine is also reported to increase Ca^{2+} level in rat hippocampus by increasing the expression of IP_3 receptor and L-type

calcium channel [44]. Inhibition of Ca^{2+} channel reduces inflammation [26, 45]. Collectively, the inhibition of muscarinic receptor by scopolamine treatment is closely related with pro-inflammatory process. It has been reported that coffee contains cholinomimetic compounds distinct from caffeine [46], and the compounds might act as a muscarinic receptor ligand and inhibit the scopolamine-mediated induction of TNF-α. IDC pretreatment also significantly decreased the phosphorylation of IκBα and p65 in scopolamine-treated rats, suggesting that the ability of IDC to lower the level of TNF-α might be mediated by suppressing NF-κB activation.

Coffee is a rich source of chlorogenic acids and contains many bioactive phenolic phytochemicals [17, 18]. It has been reported that chlorogenic acid inhibits staphylococcal exotoxin-induced proinflammatory cytokines TNF-α, IL-1β, and IL-6 [47]. Chlorogenic acid was reported to protect ischemia/reperfusion injury in rat liver by antioxidant and anti-inflammatory properties [48]. Chlorogenic acid also inhibited scopolamine-induced amnesia via anti-acetylcholinesterase and anti-oxidative activities in mice [20]. Caffeic acid lowered renal and cardiac levels of TNF-α, IL-1β, and IL-6 in diabetic mice and inhibited lipopolysaccharide-induced TNF-α release from human monocytes [49, 50]. These results suggest that coffee phenolic phytochemicals including chlorogenic acid and caffeic acid might also be the bioactive neuroprotective candidates in IDC that attenuate TNF-α

levels in the rat hippocampus.

It is possible that IDC control memory in a manner dependent of adenosine A2A receptors which is the target of caffeine. First, it should be considered that most decaffeinated coffees actually have caffeine, albeit in lower amounts than regular coffee; second, apart from caffeine, there are several other xanthines that can interfere with the function of adenosine A2A receptors; third, it cannot be excluded that other components present in coffee might be antagonists of adenosine A2A receptors. On the other hand, because caffeine have anti-inflammatory effects decreasing cytokine such as TNF-a, it is also possible that a tiny amount of caffeine in decaffeinated coffee contribute to neuroprotective effect of decaffeinated coffee [51]. Moreover, it has been reported that caffeine synergized with another coffee component to enhance cognitive performance [52].

In conclusion, our findings demonstrate that IDC did not enhance memory *per se*, however, stabilized the memory impairment induced by scopolamine. The memory stabilizing effects of IDC in scopolamine-treated rats appeared to be mediated by suppressing NF-κB activation, thereby reducing TNF-α levels in the hippocampus. IDC might protect brain against memory impairment by attenuating NF-κB-TNF-α-mediated tissue injury in the hippocampus. These results suggest that regular consumption of decaffeinated coffee might be beneficial on brain health.

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Chapter 4.

Kaempferol Attenuates 4-Hydroxynonenal-Induced Apoptosis in PC12 Cells by Directly Inhibiting NADPH Oxidase

Abstract

Kaempferol, a natural flavonoid isolated from various plant sources, has been identified as a potential neuroprotectant. In this study, I investigated the protective effect of kaempferol against 4-hydroxynonenal-induced apoptosis in PC12 rat pheochromocytoma cells. Kaempferol inhibited 4-HNE-mediated apoptosis, characterized by nuclear condensation, down-regulation of antiapoptotic protein Bcl-2, and activation of proapoptotic caspase-3. Kaempferol inhibited 4-HNE-induced phosphorylation of c-Jun N-terminal protein kinase (JNK). More importantly, kaempferol directly bound p47*phox*, a cytosolic subunit of NADPH oxidase (NOX), and significantly inhibited 4-HNE-induced activation of NOX. The antiapoptotic effects of kaempferol were replicated by the NOX inhibitor apocynin, suggesting that NOX is an important enzyme in its effects. These results suggest that kaempferol attenuates 4-HNE induced activation of JNK and apoptosis by binding p47*phox* of NOX and potently inhibiting activation of the NOX-JNK signaling pathway in neuron-like cells. Altogether, these results suggest that kaempferol may be a potent prophylactic against NOX-mediated neurodegeneration.

Key words: *apoptosis; kaempferol; NADPH oxidase;4-hydroxynonenal*

4.1. Introduction

4-Hydroxynonenal is a major aldehyde product generated by lipid peroxidation of cellular membranes [1]. Numerous studies have reported that 4-HNE has prooxidant effects, increasing intracellular reactive oxygen species and eliciting apoptosis in cells [2, 3]. 4-HNE is considered a possible cause of numerous diseases, including obesity, diabetes, and associated vascular and neurodegenerative disorders [4]. 4-HNE is highly elevated in the brains of patients with Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis [5, 6].

NADPH oxidase (NOX) is a transmembrane/cytosolic multisubunit enzyme that transfers electrons from NADPH to molecular oxygen to produce superoxide. Superoxide produced by NOX can spontaneously form hydrogen peroxide, which undergoes further reactions to generate ROS [7]. Originally described in neutrophils, where it is the enzyme responsible for killing bacteria and fungi [8], the essential function of NOX has been identified in many other cell types [9]. NOX is involved in a number of cellular signaling pathways, including kinase activation, regulation of ion channels, and Ca^{2+} signaling [9]; in the brain, it may modulate neuronal activity and play a role in cognitive function [10]. Overactivation of NOX generates excessive amounts of ROS and contributes to neurotoxicity and neurodegeneration [10]. Overactivated NOX has been observed in the brains

of patients with Alzheimer's disease and Parkinson's disease [11, 12]. NOX is composed of multiple cytosolic regulatory components (p47*phox*, p67*phox*, p40*phox*, and rac) and a transmembrane catalytic heterodimer (p22*phox* and gp91*phox*) [9]. When NOX is activated, regulatory cytosolic components are translocated to cellular membranes, where they form a functional complex that generates superoxide radicals [9]. It has been reported that 4-HNE increases the membrane translocation of p47*phox* and promotes NOX activity [13].

Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one) (Fig. 1) is a natural flavonoid isolated from tea, mushrooms, kale, broccoli, and other plant sources [14]. Kaempferol has been reported to have several health-promoting effects. For example, kaempferol possesses antioxidative and anti-inflammatory properties [15, 16] and exhibits antitumor activity [17]. Kaempferol is neuroprotective against excitotoxic insults [18] and reduces the incidence of cerebrovascular diseases [19]. Kaempferol at blood micromolar concentrations protects against ischemia/reperfusion-induced damage in rat brain [20]. On these grounds, the possibility that kaempferol could afford neuroprotection against 4-HNE-induced NOX activation and apoptosis deserves to be assessed experimentally.

In the present study, I sought to determine whether kaempferol

prevents 4-HNE-induced NOX activation and apoptosis in PC12 rat pheochromocytoma cells. I confirmed that 4-HNE induces apoptosis through the activation of NOX in PC12 cells and showed that kaempferol protects the neuron-like cells against 4-HNE-induced c-Jun N-terminal protein kinase (JNK) activation and apoptosis by direct binding to p47*phox* of NOX and blocking the activation of NOX.

4.2. Materials and Methods

4.2.1. Chemicals and reagents

Kaempferol was purchased from Indofine Chemicals (Hillsborough, NJ. 4-HNE was obtained from Cayman Chemical (Ann Arbor, MI). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue solution (0.4%), and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium, fetal bovine serum, horse serum, and penicillin/streptomycin mixture were obtained from Invitrogen (Carlsbad, CA). Antibodies against poly(ADP-ribose) polymerase (PARP), Bcl-2, caspase-3, JNK, and p47*phox* were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phosphorylated-JNK and anti-β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA) and Sigma-Aldrich, respectively. Lucigenin was obtained from Tokyo Chemical Industry (Tokyo, Japan). Hanks' balanced salt solution (HBSS) was purchased from Mediatech (Herndon, VA). NADPH was obtained from AppliChem (Darmstadt, Germany). Cyanogen bromide-Sepharose 4B was purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Apocynin (4-hydroxy-3-methoxyacetophenone) was obtained from Calbiochem (San Diego, CA). EDTA, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), and all other chemicals used were purchased from Sigma-Aldrich

and were of analytical grade.

4.2.2. Cell culture

Rat PC12 pheochromocytoma cells have been used widely to investigate oxidative stress-mediated neuronal damage [21, 22]. PC12 cells were provided by Dr. Y.-J. Surh (Seoul National University, Seoul, Republic of Korea) and grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, and 0.1% penicillin/streptomycin at 37℃ in a humidified 10% CO_2 atmosphere. Treatment with 4-HNE, kaempferol, and apocynin was performed on cells in serum-free media.

4.2.3. MTT assay.

The MTT assay measures the reduction of the tetrazolium salt MTT to insoluble formazan as a result of mitochondrial activity and thus serves as a metabolic indicator of live cells. After PC12 cells (2×10^4 cells/well in 96-well plates) were incubated with 20 μM 4-HNE for 24 h with or without kaempferol, MTT solution (final concentration, 1 mg/ml) was added, and cells were incubated for an additional 2 h. The dark-blue formazan crystals formed in viable cells were dissolved in dimethyl sulfoxide, and absorbance at 570 nm was measured with a microplate reader. Results are expressed as

the percentage of MTT reduction, measured as absorbance values relative to those of control cells.

4.2.4. Trypan blue exclusion assay

The trypan blue exclusion assay is based on the interaction of trypan blue dye with damaged membranes of dead cells. Trypan blue dye is excluded from viable cells. PC12 cells (1 x 10^5 cells/well in six-well plates) were resuspended after incubating with 20 µM 4-HNE for 24 h with or without kaempferol. After centrifugation at 600*g* for 6 min, cells were resuspended in 200 µl of phosphate-buffered saline (PBS). Suspended cells then were mixed with 200 µl of trypan blue staining solution (0.4%) and incubated for 5 min at room temperature. The cells then were loaded onto a hemocytometer and stained. Dye-excluding cells were counted under a microscope, and the percentage of stained cells was determined by scoring 150 cells.

4.2.5. DAPI staining assay

The DNA-specific fluorescent dye DAPI was used to detect nuclear fragmentation characteristic of apoptotic cells. PC12 cells (5 x 10^4 cells/well in 24-well plates) were incubated with 20 µM 4-HNE for 24 h with or without kaempferol, washed with PBS, and fixed with 70% ethanol for 20

min. The fixed cells were washed with PBS again and incubated with DAPI (1 μg/ml) for 10 min. The cells then were washed with PBS and observed under a fluorescence microscope (Olympus, Tokyo, Japan). The degree of nuclear fragmentation was evaluated by counting the percentage of DAPI-stained cells in 100 to 120 randomly selected cells.

4.2.6. Western blot analysis

PC12 cells (4 x 10^5 cells/ml in a 6-cm dish) were incubated with 20 μM 4-HNE with or without kaempferol, washed, collected with ice-cold PBS, and centrifuged at 600*g* for 10min. The cell pellet was resuspended in 100 μl of ice-cold lysis buffer (Cell Signaling Technology) and incubated on ice for 30 min. After centrifugation at 1000*g* for 15 min, the supernatant was separated and stored at -70℃. The protein concentration in lysates was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Protein lysates were separated on an SDS-polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane. Membranes were blocked with a 5% skim milk solution containing 0.5mM Tris-HCl (pH 7.5), 150mM NaCl, and 0.05% Tween 20 for 2 h at room temperature and then incubated with primary antibody. After three washes with Tris-buffered saline containing 0.1% Tween 20 (TBST), the blots were incubated for 2 h at room temperature with horseradish peroxidase-conjugated secondary

antibodies diluted 1:5000 in TBST/5% skim milk. The blots then were washed three times in TBST and developed using an enhanced chemiluminescence detection kit (GE Healthcare).

4.2.7. NOX activity assay

NOX activity was measured using superoxide-sensitive lucigenin chemiluminescence (Kim et al., 2007). Cells were grown on a 96-well plate (2 x 10^4 cells/well) for 24 h, and then the medium was removed and replaced with HBSS buffer. Cells were preincubated with the indicated drugs for 30 min before exposure to 20 μM 4-HNE for an additional 5 min. Cells then were loaded with HBSS buffer containing 25 μM lucigenin and 200 μM NADPH. Chemiluminescence was measured using Veritas microplate luminometer software (Tuner Biosystems, Sunnyvale, CA).

4.2.8. Kaempferol-sepharose 4B pulldown of p47*phox*

A kaempferol-Sepharose 4B complex was generated by activating Sepharose 4B freeze-dried powder (0.3 g) in 30 ml of HCl (1 mM). Kaempferol (2mg) was mixed with activated Sepharose 4B in coupling buffer [0.1MNaHCO3 (pH 8.3) and 0.5 M NaCl] and rotated at 4℃ overnight. Coupling buffer was replaced with 0.1 M Tris-HCl buffer (pH 8.0), and the slurry was rotated again at 4℃ overnight. The kaempferol-Sepharose

4B complex then was washed once with 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl, followed by a second wash containing 0.5 M NaCl. Proteins from PC12 cells were incubated with kaempferol-Sepharose 4B beads or uncomplexed Sepharose 4B beads (100 µl, 50% slurry) in reaction buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 2 mg/ml bovine serum albumin, 0.02 mM PMSF, and 1 µg of protease inhibitor mixture] and rotated overnight at 4℃. The beads then were washed five times with washing buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, and 0.02 mM PMSF]. p47*phox* bound to kaempferol-Sepharose 4B was analyzed by Western blotting.

4.2.9. Replication and Statistical Analysis

Experiments were repeated at least three to four times with consistent results. Means ±S.E. have been presented in figures. All of the statistical analyses were performed using SPSS software (SPSS 19.0 for Windows; SPSS Inc., Chicago, IL). Treatment effects were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. A probability cutoff of $p < 0.05$ was used as the criterion for statistical significance.

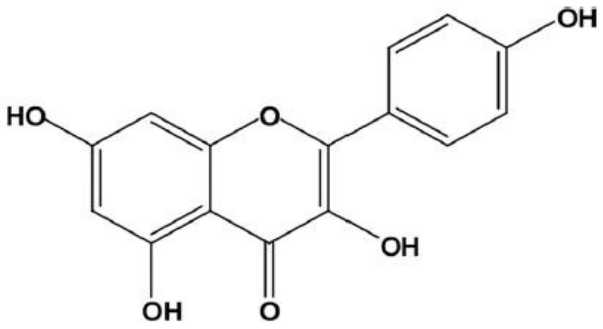

Figure 1. Chemical structure of kaempferol.

4.3. Results

4.3.1. Kaempferol inhibited 4-HNE-induced cell death.

I first examined the cytoprotective effect of kaempferol against 4-HNE-induced cell death using MTT and trypan blue exclusion assays in PC12 cells. 4-HNE at a concentration of 10 to 20 μM has been widely used to investigate oxidative stress mediated neuronal damage [23, 24]. Treatment of PC12 cells with 20 μM 4-HNE for 24 h reduced cell viability to 45.37 ± 6.65% that of untreated controls. Pretreatment with 5 or 10 μM kaempferol for 30 min increased the viability of 4-HNE-treated PC12 cells to 96.44 ± 18.71% and 117.07 ± 12.51%, respectively (Fig. 2A). Similar results were obtained using the trypan blue exclusion assay, which showed that 20 μM 4-HNE reduced cell viability to 61.02 ± 3.24% of untreated control values, and pretreatment with 5 or 10 μM kaempferol for 30 min increased these values to 80.34 ± 4.58% and 86.18 ± 7.10%, respectively (Fig. 2B). These observations indicate that kaempferol inhibits 4-HNE-induced cell death in PC12 cells.

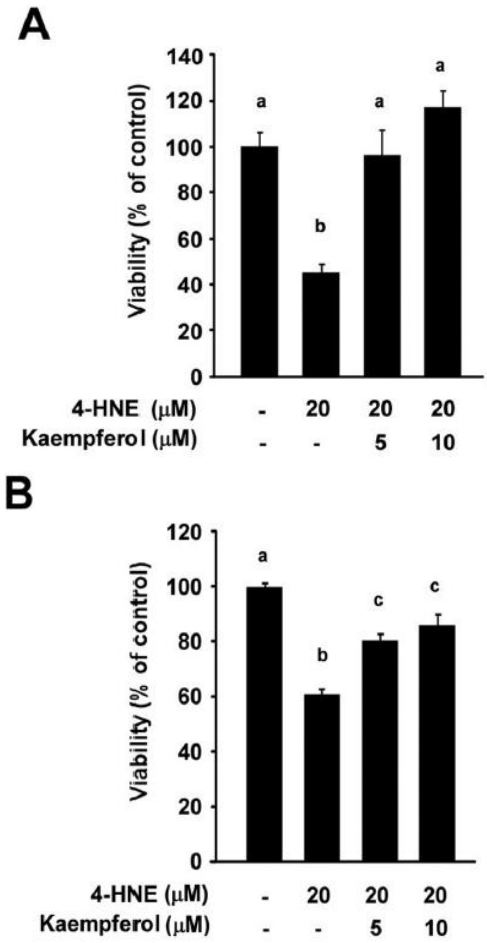

Figure 2. Effects of kaempferol on 4-HNE-induced cell death. PC12 cells were pretreated with kaempferol (5 or 10 μM) for 30 min and then exposed to 20 μM 4-HNE for 24 h. Cell viability was determined using MTT assays (A) and trypan blue exclusion assays (B). Cell viabilities are presented as means ± S.E. (n = 3) expressed as a percentage of control values. Different letters on top of bars indicate a significant difference at $p < 0.05$ analyzed using ANOVA followed by Duncan's multiple range test.

4.3.2. Kaempferol suppressed 4-HNE-induced apoptosis.

Apoptotic cells undergo nuclear condensation, as determined by DAPI staining. Treatment with 20 μM 4-HNE for 24 h resulted in an increase in the number of cells undergoing nuclear condensation (Fig. 3, Ab and B). Pretreatment with 5 or 10μM kaempferol for 30 min significantly decreased the number of cells with condensed nuclei (Fig. 3, A, c and d, and B).

Bcl-2 is an antiapoptotic protein that stabilizes mitochondrial function and suppresses oxidative stress-mediated cellular damage [25]. In addition, it has been reported that Bcl-2 inhibits the activation of caspase-3, which is an important mediator of apoptosis [26]. I thus investigated whether the protective effect of kaempferol against 4-HNE-induced apoptosis in PC12 cells involved changes in Bcl-2 levels and/or caspase-3 activation. Treatment of PC12 cells with 20 μM 4-HNE for 24 h decreased the amount of Bcl-2 and induced the cleavage of procaspase-3 (Fig. 3, C–E). Pretreatment with 10 μM kaempferol for 30 min completely reversed the effects of 4-HNE on Bcl-2 (Fig. 3, C and D), and pretreatment with 5 or 10 μM kaempferol for 30 min blocked 4-HNE-induced procaspase-3 cleavage (Fig. 3, C and E). Collectively, these results indicate that kaempferol protects PC12 cells against 4-HNE-induced apoptosis.

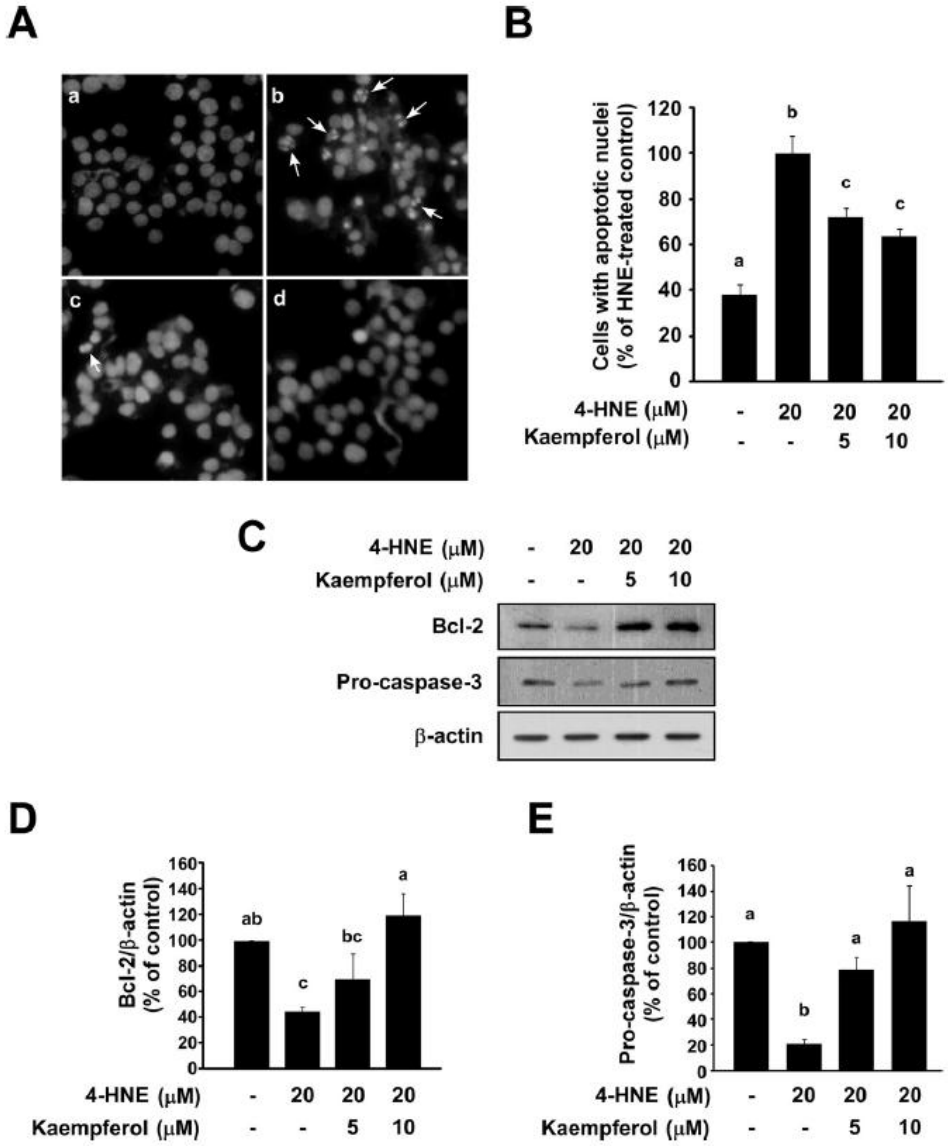

Figure 3. Effects of kaempferol on 4-HNE-induced apoptosis. PC12 cells were pretreated with kaempferol (5 or 10 μM) for 30 min and then exposed to 20 μM 4-HNE for 24 h. A and B, apoptotic cells (condensed nuclei) were measured by DAPI staining assay. Nuclear morphologies were examined by

fluorescence microscopy (A), and the numbers of apoptotic cells were calculated and presented as means ± S.E. (n = 3) expressed as a percentage of apoptotic cells in the 4-HNE-only group (B). C, the protein levels of Bcl-2, cleaved-caspase-3, and β-actin were determined by Western blot analysis. β-Actin was used as a loading control. D and E, the ratios of Bcl-2/β-actin (D) and procaspase-3/ β-actin (E) were determined by densitometry. Data are presented as means ± S.E. of three independent experiments. Different letters on top of bars indicate a significant difference at $p < 0.05$ analyzed using ANOVA followed by Duncan's multiple range test.

4.3.3. Kaempferol blocked 4-HNE-induced JNK phosphorylation.

Because JNK is an important signaling molecule that leads to Bcl-2 down-regulation and caspase-3 activation [3, 27, 28], I investigated the effect of kaempferol on JNK activation (phosphorylation). Treatment with 20 μM 4-HNE for 5 min substantially increased JNK phosphorylation (Fig.4), an effect that was suppressed by pretreatment with 10 μM kaempferol for 30 min (Fig. 4). Collectively, these results indicate that kaempferol might inhibit 4-HNE-induced apoptosis, Bcl-2 down-regulation, and caspase-3 activation by inhibiting JNK phosphorylation.

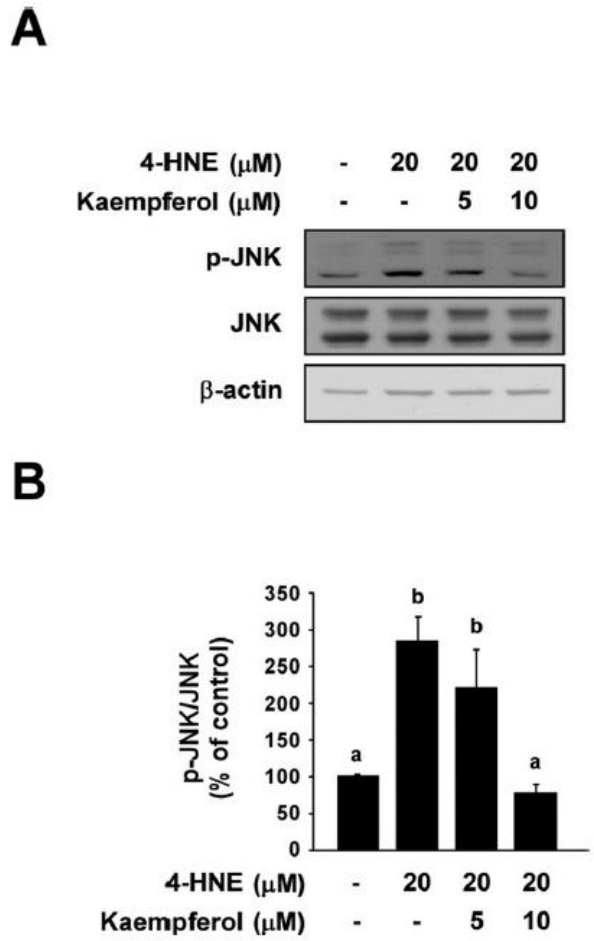

Figure 4. Effects of kaempferol on 4-HNE-induced phosphorylation of JNK. PC12 cells were pretreated with kaempferol (5 or 10 μM) for 30 min and then exposed to 20 μM 4-HNE for 5 min. A, the protein levels of phosphorylated JNK, total JNK, and β-actin were determined by Western blot analysis. β-Actin was used as a loading control. B, the ratio of phospho-JNK/JNK was determined by densitometry. Data are presented as means ± S.E. of three independent experiments. Different letters on top of bars

indicate a significant difference at $p < 0.05$ analyzed using ANOVA followed by Duncan's multiple range test.

4.3.4. Kaempferol attenuated 4-HNE-induced activation of NOX.

To investigate the effect of kaempferol on NOX activity, I performed NOX activity assays. Treatment with 20 μM 4-HNE for 5 min induced a 1.6-fold increase in NOX activity. This 4-HNE-induced increase in NOX activity was suppressed by pretreatment for 30 min with 5 or 10 μM kaempferol, which exerted an inhibitory effect comparable to that of apocynin, a well known pharmacological inhibitor of NOX (Fig. 5, A and B).

Because kaempferol inhibits NOX activity, I investigated whether kaempferol directly binds p47*phox*, a cytosolic subunit of NOX, using kaempferol-Sepharose 4B pulldown assays. Cellular p47*phox* did not bind unconjugated Sepharose 4B (Fig. 5C, lane 2) but did bind kaempferol-Sepharose 4B, suggesting that kaempferol interacts directly with p47*phox* (Fig. 5C, lane 3). These observations indicate that kaempferol might inhibit 4-HNE-mediated activation of NOX by binding directly to p47*phox* in PC12 cells.

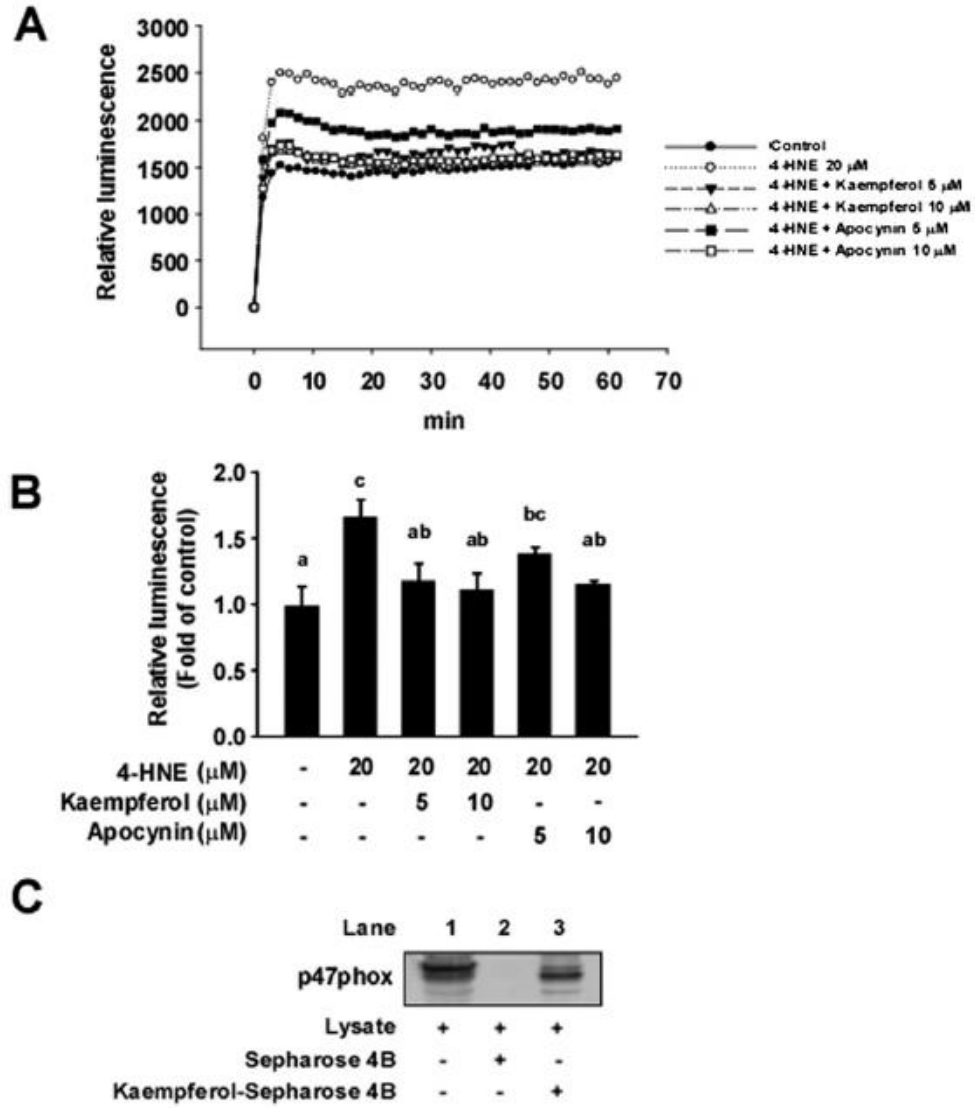

Figure 5. Effects of kaempferol on 4-HNEinduced activation of NOX. A and B, PC12 cells were pretreated with kaempferol or apocynin (5 or 10 μM) for 30 min and then exposed to 20 μM 4-HNE for 5 min. NOX activity was measured by lucigenin luminescence as described under *Materials and Methods*. Fold increases in luminescence after 5 min of 20 μM 4-HNE

treatment, presented as means ± S.E. (n = 3) (B). Different letters on top of bars indicate a significant difference at $p < 0.05$ analyzed using ANOVA followed by Duncan's multiple range test. C, binding of kaempferol to p47*phox* ex vivo was confirmed by kaempferol-Sepharose 4B pulldown assays and Western blotting for bound p47*phox*: lane 1(input control), whole-cell lysates from PC12 cells; lane 2 (binding control), whole-cell lysates of PC12 cells after pulldown with Sepharose 4B beads; lane 3, whole-cell lysates from PC12 cells after pulldown by kaempferol-Sepharose 4B affinity beads.

4.3.5. Apocynin, an inhibitor of NOX, inhibited 4-HNE-induced apoptosis.

To demonstrate that NOX plays a role in 4-HNE-induced apoptosis, I examined the effect of apocynin, a well known inhibitor of NOX, on 4-HNE-induced apoptosis. Pretreatment of PC12 cells with apocynin (5 or 10 μM) for 30 min inhibited 4-HNE-induced cell death (Fig. 6A). I examined the levels of cleaved PARP, an important molecular indicator of the chromatin condensation process associated with apoptosis. Treatment with 20 μM 4-HNE for 24 h increased the level of cleaved PARP, and this increase was suppressed by pretreatment with 10 μM apocynin for 30 min (Fig. 6, B and C). Apocynin at a concentration of 5 or 10 μM attenuated 4-HNE-induced down-regulation of Bcl-2 and procaspase-3 cleavage (Fig. 6, B, D, and E). In addition, pretreatment with apocynin suppressed the 4-HNE-induced phosphorylation of JNK (Fig. 6, F and G). Thus, blocking the activity of NOX prevented the 4-HNE-induced increase in cell death and inhibited 4-HNE-mediated JNK phosphorylation, PARP cleavage, Bcl-2 down-regulation, and caspase-3 activation, indicating that NOX plays an important role in 4-HNE-induced apoptosis in PC12 cells.

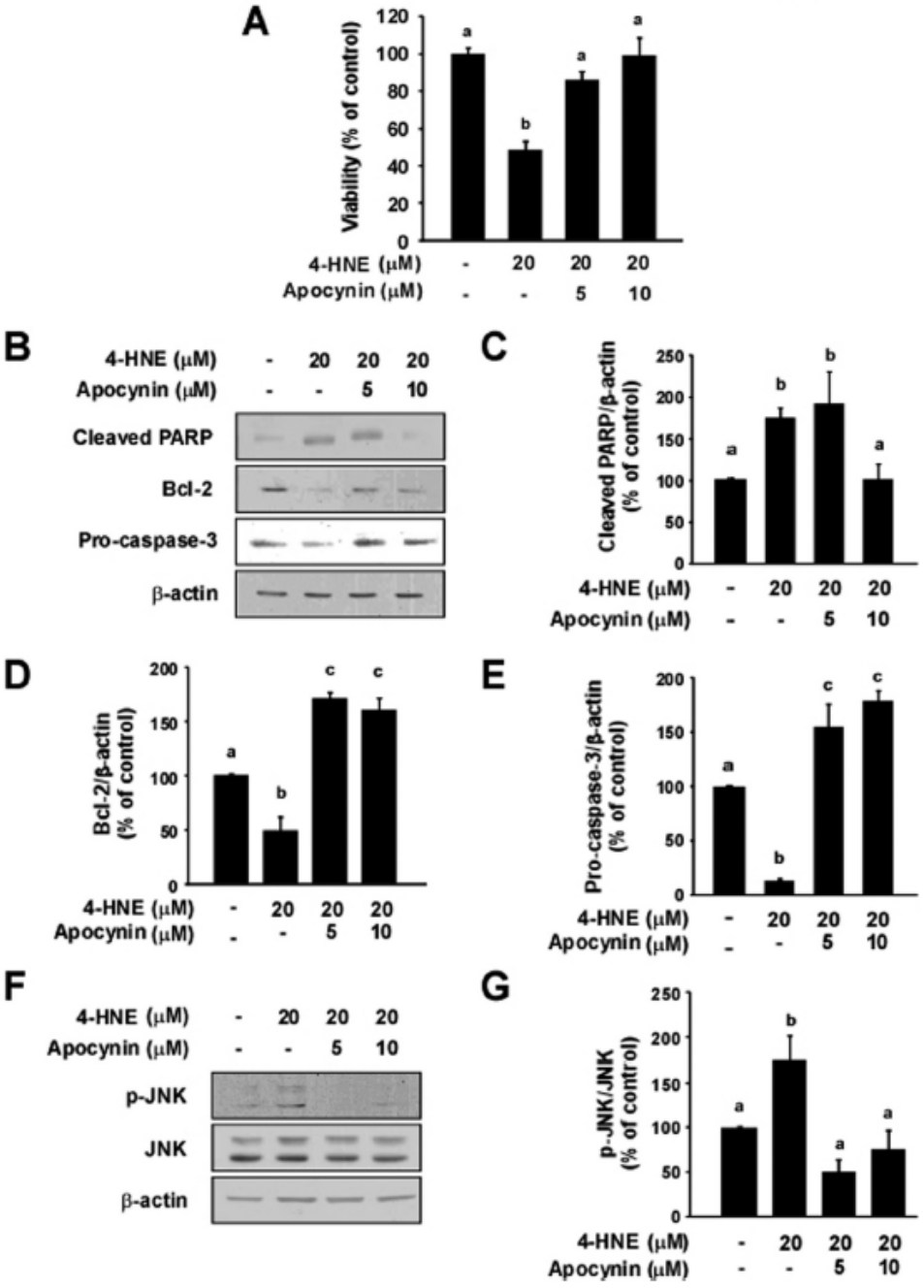

Figure 6. Involvement of NOX in 4-HNE induced apoptosis. A to E, PC12 cells were pretreated with apocynin (5 or 10 μM) for 30 min and then

exposed to 20 μM 4-HNE for 24 h. A, cell viability was determined using MTT assays. Values are presented as means ± S.E. (n = 3) expressed as a percentage of cell viability in control (untreated) cells. B, the protein levels of cleaved-PARP, Bcl-2, pro-caspase-3, and β-actin were determined by Western blot analysis. β-Actin was used as a loading control. C to E, the ratios of cleaved PARP/ β-actin (C), Bcl-2/ β-actin (D), and pro-caspase-3/ β -actin (E) were determined by densitometry. F and G, PC12 cells were pretreated with apocynin (5 or 10 μM) for 30 min and then exposed to 20 μM 4-HNE for 5 min. The protein levels of phosphorylated JNK, total JNK, and β-actin were determined by Western blot analysis. β-Actin was used as a loading control. G, the ratio of phospho-JNK/JNK was determined by densitometry. Data are presented as means ± S.E. of three independent experiments. Different letters on top of bars indicate a significant difference at $p < 0.05$ analyzed using ANOVA followed by Duncan's multiple range test.

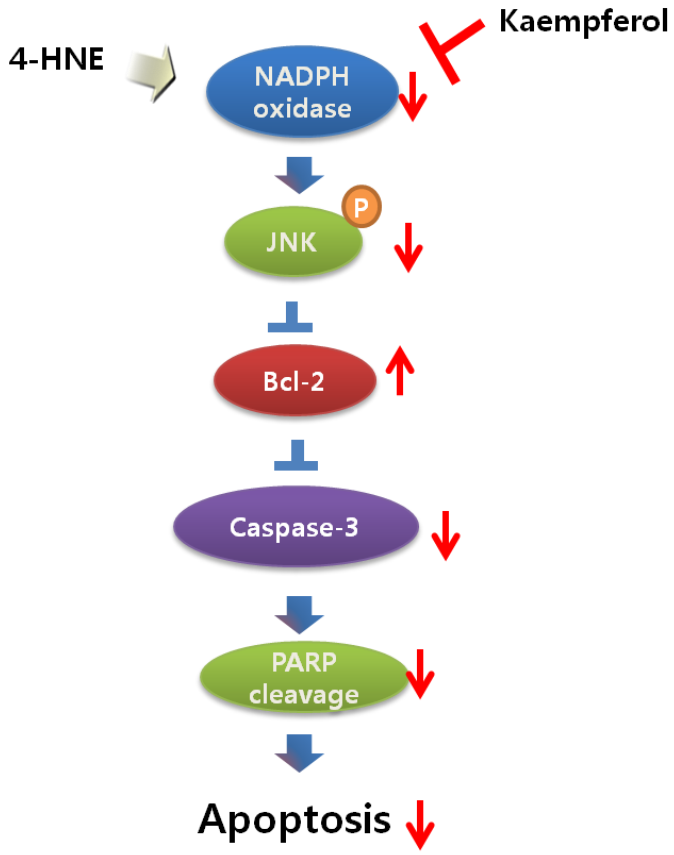

Figure 7. The neuroprotective effect of kaempferol on 4-HNE-induced PC12 cell death.

4.4. Discussion

The consumption of flavonoid-rich foods and beverages has been suggested to limit the neurodegeneration associated with a variety of neurological disorders and to prevent or reverse deteriorations in brain [29]. Originally, it was thought that such actions were mediated by the antioxidant capacity of flavonoids [29]. However, the multiplicity of their effects appears to be underpinned by a number of routes, including interactions with important neuronal and glial signaling cascades in the brain [29]. In this study, I introduced the molecular interaction of kaempferol with p47*phox* of NOX to explain its antiapoptotic and neuroprotective effects.

NOX requires p47*phox*, a key cytosolic subunit, to be activated [30]. Antisense oligonucleotides against p47*phox* have been shown to suppress NOX-mediated apoptosis [13], suggesting that p47*phox* plays an important role in NOX-induced apoptosis. *N*-Methyl-D-aspartate induced superoxide production and cell death are attenuated in p47*phox*(-/-) neurons compared with those in wild-type neurons [7], indicating that p47*phox* is required for *N*-methyl-D-aspartate-mediated NOX activation and neuronal death. Therefore, attenuation of excessive activation of NOX through blocking p47*phox* might be a useful therapeutic strategy for preventing neuronal injury.

In this study, I report that kaempferol could be a potent prophylactic against NOX-mediated neurodegeneration through direct binding to p47*phox*.

The interaction may be responsible for its inhibition of 4-HNE-mediated NOX activation and apoptosis. Kaempferol might bind functional regions in p47*phox*: the PX domain, which interferes with membrane binding, or the SH3 domain, which interacts with the catalytic subunit p22*phox* [31]. However, the results of kaempferol-Sepharose 4B pulldown assays showed that kaempferol did not interact with p67*phox*, the other cytosolic regulatory component of NOX (data not shown). More precise structure-activity relationship studies such as virtual computer modeling or X-ray cocrystallography might define the exact molecular interaction of kaempferol with p47*phox*.

JNK is an important signaling molecule in pathways that lead to 4-HNE-induced apoptosis [32] and has been shown to be involved in caspase-3 activation [3]. My observations demonstrate that apocynin, a specific inhibitor of NOX, blocks 4-HNE-induced JNK phosphorylation, caspase-3 activation, and apoptosis, suggesting that JNK is involved in the NOX-mediated signaling pathway leading to apoptosis. I also found that apocynin did not decrease the phosphorylation of mitogen-activated protein kinase kinase 4, an upstream kinase of JNK, indicating that mitogen-activated protein kinase kinase 4 is not regulated by NOX (data not shown). Therefore, these results indicate that kaempferol might suppress NOX activation through direct binding to p47*phox* and subsequently attenuate JNK-caspase-3

activation. Previous reports have similarly demonstrated that kaempferol inhibits neuronal apoptosis by regulating the c-Jun and caspase cascade [33].

The pharmacokinetic parameters after intravenous administration of 25 mg/kg kaempferol in male rats showed that the concentration of kaempferol reaches up to approximately 30 µM in plasma [34]. Kaempferol can be transported into the neurons in a concentration-dependent manner when the neurons were incubated with the culture medium containing kaempferol [35]. Because of its permeability in neurons, kaempferol at concentrations of 5 and 10 µM might directly bind cytosolic p47*phox* and interfere with 4-HNE-induced NOX activity in PC12 cells. Consistent with my result, NOX has been suggested as a molecular target that might interact with kaempferol [36, 37]. Kaempferol inhibits NOX activity at an IC_{50} value of 92.0 ± 18.3 µM in human neutrophils [36]. Kaempferol has an IC_{50} value of 10.0 ± 3.6 µM against NOX activity in human endothelial cells, making it more potent than the known NOX inhibitor apocynin (IC_{50} = 50.0 ± 9.1 µM) in these cells [37]. Even though there is a lack of direct pharmacokinetic data in brain, kaempferol has been shown to be neuroprotective under pathological conditions in animal models [20, 38]. These observations suggest that kaempferol might be able to cross the blood-brain barrier and reach the brain tissue.

Several studies show that certain food consumption can increase the

level of 4-HNE in brain. High fructose consumption with an omega-3 fatty acid deficient diet increases the level of 4-HNE in rat brain and impairs cognitive functions [39]. The high fat diet promotes 4-HNE-lysine and 4-HNE-histidine in the rat hippocampus [40]. Ethanol consumption changes antioxidant ability and enhances oxidative modification of lipids such as 4-HNE in rat brain [41]. These results suggest that excessive consumption of particular nutrient can induce oxidative stress and increase 4-HNE in brain.

In conclusion, 4-HNE-induced apoptosis, characterized by the induction of nuclear condensation, down-regulation of antiapoptotic protein Bcl-2, and activation of proapoptotic caspase-3, was attenuated by kaempferol treatment. This inhibition was associated with the binding of kaempferol to p47*phox* and suppression of the NOX-JNK pathway. Taken together, these results indicate that kaempferol may act through direct binding to p47*phox* and inhibit 4-HNE-induced NOX activation and JNK-mediated apoptosis in neuron-like PC12 cells.

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Chapter 5.

Conclusion

5.1. Conclusion

In the present study, I sought to investigate various molecular mechanisms that could provide further insights into the potential neuroprotective effects of coffee, decaffeinated coffee and chlorogenic acid. Results show that coffee, both normal and decaffeinated, protect neurons against H_2O_2-induced apoptosis with similar effect. I found that chlorogenic acid, a major phytochemical found in both types of coffee, attenuates H_2O_2-induced apoptotic neuronal cell death. This protection was a result of the suppression of H_2O_2-induced down-regulation of the anti-apoptotic factors Bcl-2 and Bcl-X_L, as well as the attenuation of caspase-3 activation and PARP cleavage. Decaffeinated coffee and chlorogenic acid were also found to inhibit intracellular ROS accumulation and the activation of MAPKs, which further contributed to the attenuation of neuronal apoptosis.

Although I demonstrated that decaffeinated coffee did not enhance memory *per se*, I found that it stabilized scopolamine-induced memory impairment. The protective effect of decaffeinated coffee on memory in scopolamine-treated rats appears to be mediated by the suppression of NF-κB activation, thereby reducing TNF-α levels in the hippocampus. Decaffeinated coffee could therefore be protecting the brain against memory impairment by attenuating NF-κB-TNF-α-mediated tissue injury in the hippocampus.

These findings shed light on the molecular mechanisms underpinning reported neuroprotective effects of coffee in human epidemiologic studies. These results suggest that regular consumption of decaffeinated coffee might be beneficial for reducing memory impairment. Further studies could be conducted to elucidate the potential effects of coffee and decaffeinated coffee in AD and PD transgenic mice.

Both oxidative stress and inflammatory damage increase during the aging process [1, 2]. It therefore stands to reason that the inhibitory effects of decaffeinated coffee on oxidative stress and inflammation could influence age-related cognitive decline. Interestingly, one study has reported that coffee consumption was inversely associated with total and cause-specific mortality [3]. It will be interesting and highly beneficial to identify the molecular determinants responsible for such relationships.

I also found that 4-HNE-induced apoptosis (characterized by the induction of nuclear condensation, down-regulation of Bcl-2 and activation of pro-apoptotic caspase-3), was attenuated by kaempferol treatment. This inhibition was associated with the binding of kaempferol to p47*phox* and subsequent suppression of the NOX-JNK pathway. These findings provide new insights into the molecular mechanisms of the neurotoxic effects of oxidative stress and suggest targets for the development of novel drugs to fight oxidative stress-induced neuronal apoptosis.

The neurodegenerative pathways culminating in disease could well be activated years before the disease itself becomes overt. For example, some forms of AD pathology have been postulated to require 20–30 years before significant symptoms arise. Therefore, longterm consumption of dietary phytochemicals that inhibit neurodegenerative pathways could be an effective way to prevent such diseases progressing. Critical research should be undertaken to identify and investigate further dietary phytochemicals for the development of effective dietary supplements.

A greater understanding of the molecular mechanisms influenced by chlorogenic acid and kaempferol may be helpful in designing more effective functional foods and novel therapeutics. I suggest that to further elucidate such molecular mechanisms, NMR and X-ray crystallography studies will provide important information on the nature of interactions occurring between each bioactive compound and its protein target.

5.2. References

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국문초록

산화적 스트레스와 염증은 알츠하이머 병, 파킨슨 병 및 근위축성 측색 경화와 같은 신경 퇴생성 질환과 밀접하게 연관되어 있다. 산화적 손상은 과산화 수소와 같은 활성 산소에 의해서 유도되며 베타 아밀로이드는 활성 산소를 생산함을 통해서 알츠하이머 질환의 발달과 진행에 있어서 중요한 역할을 담당한다. 또한 TNF-α와 같은 사이토카인들은 알츠하이머 질환에서의 염증 과정에 있어서 중요한 역할을 담당한다. 실제로 염증 유발 사이토카인들은 치매환자의 뇌에서 높은 수준으로 발현된다. 산화적 스트레스와 염증은 신경 세포의 자가사멸반응을 조절하고 이는 뇌의 기능을 손상시키는 결과를 가져온다. 그러므로 신경퇴행성 질환을 예방하기 위해서 산화적 스트레스와 염증을 표적하는 천연물을 찾는 것이 중요하다.

최근 적당한 커피 섭취는 알츠하이머나 파킨슨 병과 같은 신경퇴행성 질환의 위험을 감소시킨다는 연구가 보고되었다. 카페인은 이러한 커피의 신경약리학적인 효능을 설명할 수 있는 물질로 생각 되어 왔으며, 카페인의 신경보호 효능은 몇몇 연구들에 의해 증명되었다. 그러나 커피는 페놀성 화합물의 주요 식이 근원이기도 하다. 클로로젠산(5-O-caffeoylquinic acid)은 커피에 있는 주요 페놀성 화합물이다. 그러나 탈카페인 커피나 클로로젠산과 같은 커피 파이토케미컬의 신경 보호 효능 및 기작에 대해서는 아직 명확하게 규명되지 않았다.

이 연구에서 카페인 커피, 탈카페인 커피 그리고 클로로젠산이 과산화 수소가 유도하는 PC12 및 피질 신경세포의 사멸 및 자가사멸을 완화하는 것을 확인하였다. 이것은 Bcl-2 혹은 Bcl-X_L과 같은 단백질의 감소와 caspase-3의 활성, poly(ADP-ribose)polymerase의 분해를 저해함을 통해 세포 내 핵 응축 및 DNA 분열이 억제 되는 것으로 확인할 수 있었다. 과산화 수소에 의한 세포 내 활성 산소의 축적과 c-Jun N-terminal protein kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) 의 활성이 탈카페인 커피와 클로로젠산에 의해 저해되었다. 커피와 탈카페인 커피 모두 비슷한 효과로 과산화수소에 의한 세포 자가 사멸으로부터 신경을 보호하였으며 클로로젠산과 같은 화합물이 커피의 신경 보호 효능에 기여할 것으로 생각된다.

이 연구에서 기억력에 대한 탈카페인 커피의 효능을 증명하기 위해 스코폴라민에 의해 기억력 손상을 유도한 동물 모델을 사용하였다. 탈카페인 커피를 경구 투여 하였을 때 스코폴라민에 의한 기억력 손상이 억제 되었고, 이것은 모리스 물 미로 실험과 수동 회피 반응 실험을 통해 측정되었다. 탈카페인 커피는 스코폴라민에 의해 증가된 tumor necrosis factor-α (TNF-α) 의 발현과 nuclear factor-κB (NF-κB) 경로를 저해하였다. 이러한 발견은 커피가 기억력에 효능을 보인다는 인간에서 이루어진 연구를 뒷받침하며 탈카페인 커피가 NF-κB 활성과 TNF-α 생성을 저해함을 통한 것이라는 것을 나타낸다.

캠페롤 (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one)은 차, 버섯, 케일, 브로폴리와 같은 식물에 존재하는 천연 플라보노이드이다. 캠페롤은 Bcl-2 단백질의 감소와 caspase-3의 활성을 억제하고 세포 내 핵 응축을 억제함을 통해 세포 자가사멸을 저해하였다. 또한 캠페롤은 JNK의 인산화를 저해하였다. 더욱이 캠페롤은 NADPH oxidase (NOX)의 구성 성분인 p47phox에 직접 결합하여 4-HNE에 의한 NOX의 활성을 저해하였다. 이러한 캠페롤의 세포 자가사멸 억제 효능은 NOX 저해제인 apocynin에 의해서도 동일하게 나타남을 확인하였다. 이러한 결과는 캠페롤이 NOX의 p47phox에 결합하여 NOX-JNK 활성 경로를 저해함을 통해 세포 자가 사멸을 억제함을 의미한다. 따라서 캠페롤은 NOX가 조절하는 신경퇴행에 대하여 가능성 있는 예방물질이 될 것이다.

종합적으로 커피와 탈카페인 커피 모두가 산화적 스트레스에 의한 신경세포 사멸을 억제하고 클로로젠산이 커피의 이런 효능에 기여할 것이다. 탈카페인 커피는 해마에서 NF-κB-TNF-α 에 의한 조직 손상을 저해함을 통해 기억력 손상으로부터 뇌를 보호한다. 캠페롤은 p47phox에 결합하여 NOX의 활성과 JNK에 의해 조절되는 세포 자가사멸을 억제한다. 이런 결과들은 커피, 탈카페인 커피, 클로로젠산 및 캠페롤의 지속적인 섭취가 뇌 건강에 이로운 역할을 할 수 있음을 시사한다.