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Neuroprotective effects of flavones on hydrogen peroxide-induced apoptosis in SH-SY5Y neuroblostoma cells

Sam Sik Kang, Ji Yeon Lee, Yoo Keum Choi, Gap Seok Kim and Byung Hee Han*

College of Pharmacy and Natural Products Research Institute, Seoul National University, Seoul 110-460, South Korea

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Abstract—Neuroprotective effects of flavones were examined. Luteolin and apigenin exhibited neuroprotection against oxidative stress-induced cell death in SH-SY5Y cells. Free radical scavenging activity and neuroprotection assays revealed that flavones exerted their neuroprotective effects via the direct interaction with the apoptotic caspase pathway independently of their antioxidant activity.

The generation of reactive oxygen species (ROS) in normal cells is tightly regulated by biological antioxidants and by antioxidant enzymes.[1,2] In pathophysiologic conditions, the generation of oxidants exceeds the intracellular antioxidant capacity, resulting in oxidative damages to proteins, lipids, and DNA. Numerous studies have demonstrated the implication of ROS in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and ischemic and hemorrhagic stroke.[2–5] ROS plays a critical role in neurodegeneration induced by a variety of insults including excitotoxicity, amyloid β (Aβ), ischemia, and nerve growth factor withdrawal. Recently, the antioxidant strategy has shown promise in the treatment of both acute and chronic neurodegenerative diseases.

Flavonoids are naturally-occurring polyphenolic compounds presented in a variety of fruits, vegetables, and seeds. They can be classified into flavonols, flavanones, flavones, isoflavones, and anthocyanidins.[6] Flavonoids have many biological, pharmacological activities including antioxidative, antiinflammatory, and antitumor effects.[7] Recent studies have demonstrated that both antioxidative and antiinflammatory properties of flavonoids can contribute to their neuroprotective effects in many settings of neurodegeneration.[8] It has been reported that wogonin, derived from the root of *Scutellaria baicalensis* George, protected neurons against ischemia-reperfusion brain injury via the inhibition of inflammatory activation of microglia following stroke.[8] Apigenin and kempferol protected rat cortical neurons against amyloid β (Aβ)-induced neurotoxicity by inhibition of the caspase pathway independently of their antioxidative capacity.[9] Therefore, the molecular mechanisms underlying neuroprotective effects of flavonoids may include not only their antioxidant activities but also their interaction with cell signaling cascades leading to cell death upon cellular oxidative stress. Understanding the molecular basis of neuroprotective actions of flavonoids would provide important clues for drug design as neuroprotectants.

In this study, we wanted to test whether flavones directly influence cell death induced by oxidative stress in SH-SY5Y neuronal cells. We selected four, structurally related flavones: wogonin, chrysin, apigenin, and luteolin, of which neuroprotective effects have not been well explored.

Chrysin was purchased from Sigma–Aldrich Chemical Co. Apigenin and luteolin were isolated from the flowers of *Chrysanthemum boreale* as previously described.[10] Wogonin was isolated from the root of *S. baicalensis* George as previously described.[11]

We first examined the free radical scavenging activity of flavones using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as a free radical donor (Table 1).[12] Luteolin (IC_{50}: 10.4 μM) had more potent free radical scavenging activity than that of vitamin C (IC_{50}: 26.5 μM) used as a standard antioxidant (Table 1). In contrast to luteolin, wogonin, chrysin, and apigenin did not show any ability to

* Corresponding author. Tel.: +82-2-740-8917; fax: +82-2-762-8322; e-mail: hanbh@snu.ac.kr

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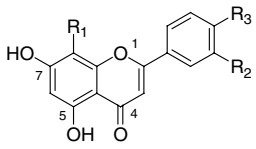
Table 1. Chemical structure and free radical scavenging activity of flavones



Compounds	R_1	R_2	R_3	IC_{50}[a] (μM)
Wogonin	OMe	H	H	>100
Chrysin	H	H	H	>100
Apigenin	H	H	OH	>100
Luteolin	H	OH	OH	10.4
Vitamin C				26.5

[a] IC_{50} values were calculated from the concentration at that 50% DPPH free radical was scavenged by compounds ($n = 5$–7). Vitamin C was used as a reference antioxidant.

scavenge DPPH free radical at a concentration up to 100 μM. Thus, dihydroxyl groups at the R_2 position as well as at the R_3 position seemed to be critical for flavones to elicit the antioxidant activity. In contrast, flavones having hydroxyl groups at position 5 and at position 7 with or without a hydroxyl group at the R_2 position did not provoke the radical scavenging activity.

We then tested whether these flavones protected cells against oxidative stress-induced cell death in SH-SY5Y human neuroblastoma cells. As determined by MTT assay,[13] oxidative stress by hydrogen peroxide resulted in a decrease in the cell viability by 21.5 ± 1% as compared with control group (Fig. 1). Luteolin significantly inhibited cell death induced by hydrogen peroxide, resulting in an increase in the cell viability by 76.4 ± 1.5%, 84.0 ± 0.2%, 77.5 ± 2.3% at concentrations of 2, 10, 50 μM, respectively. Apigenin also remarkably increased the cell viability by 24.6 ± 2.9%, 52.4 ± 1.2%, 63.7 ± 3.1% at concentrations of 2, 10, 50 μM, respectively. Chrysin at a concentration of 50 μM was able to inhibit cell death but by far lesser extent than that of luteolin and apigenin. Wogonin, however, had no effect on hydrogen peroxide-induced cell death. Therefore, the order of potency for neuroprotection was luteolin > apigenin ≫ chrysin≈wogonin. These data suggest that a hydroxyl group at the R_3 position was essential to exert inhibitory effects against oxidative stress-induced cell death.

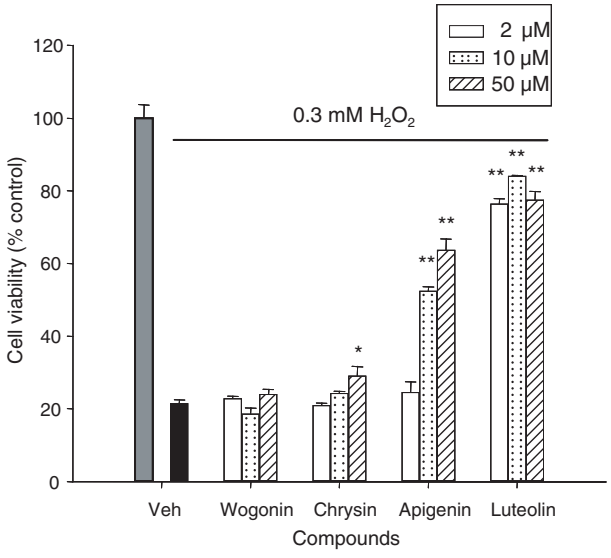


Figure 1. Neuroprotective activity of flavones. SH-SY5Y cells were treated with hydrogen peroxide in the presence or absence of various concentrations of flavones. Twenty-four hours later, cell viability was determined by MTT assay. Data are presented as mean ± SEM from triplicate samples. *: $P < 0.05$; **: $P < 0.001$ as compared to vehicle treated samples (*t*-test).

Among tested compounds, luteolin showed both antioxidant activity and neuroprotection against oxidative stress-induced cell death. In contrast, neuroprotective effect of apigenin was not through its antioxidant activity. Although further study is required to clarify how apigenin protects cells against insult, it is likely that it directly interacts with cellular events leading to cell death after oxidative stress. A previous study has demonstrated that apigenin attenuated the Aβ-induced cytotoxicity without intervening the oxidative stress in rat cortical neurons.[9] It was of interest that wogonin had no direct effect on oxidative stress-induced cell death in SH-SY5Y cells. In contrast, a recent study showed that wogonin protected neurons against NMDA-mediated excitotoxicity in vitro as well as against ischemic injury in vivo by inhibition of inflammatory activation of microglia.[8] Furthermore, wogonin inhibited lipopolysaccharide-induced *i*-NOS expression and NO production in microglia.[14] Unlike wogonin, our findings suggest that both luteolin and apigenin directly influence the cell death pathway in neurons.

Next, we explored whether flavones blocked cell death through interaction with the caspase pathway. We have previously demonstrated that activation of caspase-3 is a hallmark feature of neuronal apoptosis following neonatal hypoxic–ischemic brain injury.[15,16] Caspases belong to a family of cysteine aspartyl-specific proteases. Caspase-3 among effector caspases has been implicated in neuronal apoptosis during normal brain development and in delayed neuronal cell death after brain injury in the developing and adult brains.[16–20] Once activated, caspase-3 is directly responsible for proteolytic cleavages of a variety of fundamental proteins including cytoskeletal proteins, kinases, and DNA-repairing enzymes.[21–24] At least two different initiator caspases can be responsible for activation of caspase-3 through distinct cellular signaling pathways. Procaspase-8 is an initiator caspase that can process itself upon stimulation of FAS-tumor necrosis factor family of death receptors. Another mechanism for caspase-3 activation is the mitochondrial pathway that can be initiated by cytochrome c release from the mitochondria followed by activation of caspase-9 and caspase-3 up on loss of trophic support, mitochondrial dysfunction, and certain cytotoxic agents.[25–27]

To test whether apigenin and luteolin influenced the activation of caspase-3 induced by hydrogen peroxide,

we performed DEVD–AMC cleavage assay as we described previously.[16,28,29] The activation of caspase-3 was detectable at 4 h, peaking at 8 h after treatment with hydrogen peroxide in SH-SY5Y cells (data not shown). Caspase-3 activity was 11.5-fold increased at 8 h after the hydrogen peroxide treatment as compared with control (Fig. 2). Unexpectedly, all flavones tested attenuated the activation of caspase-3 induced by hydrogen peroxide at a concentration of 10 μM, but both luteolin and apigenin had greater effects than wogonin and chrysin. In fact, both apigenin and luteolin almost completely blocked the caspase-3 activity. These data suggest that flavones, without having the antioxidant activity, inhibited apoptosis, presumably, via the direct interaction with the caspase pathway. It is of interest to note that apigenin, which did not have the antioxidant activity, had a similar effect to luteolin in terms of inhibition of the caspase-3 pathway. Further study is necessary to understand the exact molecular basis of neuroprotective action of these compounds.

In conclusion, our data suggest that flavones protect neurons against oxidative stress-induced cell death via different molecular mechanisms. Hydroxyl groups at the R_2 position and at the R_3 position of flavones may be critical for biological activities including the antioxidant activity, and the direct influence on the cellular events leading to apoptosis. The present study provides insights into the design of flavonoid derivatives with optimal neuroprotective activities.

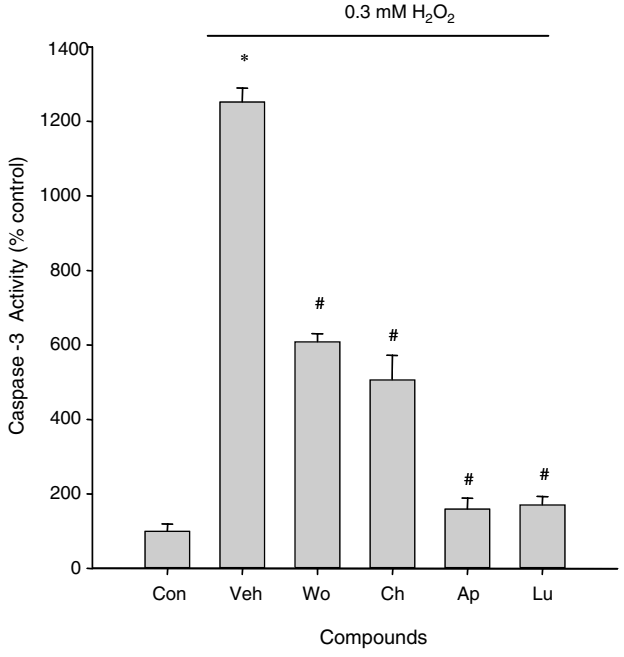


Figure 2. Effects of flavones on the activation of caspase-3 induced by hydrogen peroxide. SH-SY5Y cells were treated with hydrogen peroxide in the presence of vehicle (Veh) or 10 μM of wogonin (Wo), chrysin (Ch), apigenin (Ap), or luteolin (Lu). Eight hours later, cells were harvested and subjected to DEVD–AMC cleavage assay. Data are presented as mean ± SEM from triplicate samples. *: $P < 0.001$ versus Con group; #: $P < 0.001$ versus Veh group as analyzed by *t*-test.

Acknowledgements

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13. Neuroprotective activity of flavones was assessed in human neuroblastoma cells SH-SY5Y cells. Cells were plated at 1×10^5 cells per well in a 48-well plate in DMEM medium containing 10% fetal bovine serum and antibiotics at a humidified CO_2 incubator. A day after plating, cells were pre-treated with test compounds for 1 h and then treated with 0.3 mM hydrogen peroxide for 24 h. To assess the cell viability, cells were incubated with MTT for 3 h at 37 °C, and OD was read at 595 nm using a spectrophotometry.
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