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A Comparative Study of Phytoestrogen Action in Mitigating Apoptosis Induced by Oxidative Stress

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Abstract. *The phytoestrogens kaempferol, genistein and genistin were characterized using in vitro assays utilizing iodoacetic acid (IAA)-induced oxidative stress and mitochondrial dysfunction. RGC-5 cells were treated with different concentrations of IAA, and phytoestrogens were administered along with IAA. IAA is cytotoxic to RGC-5 cells and induces the generation of reactive oxygen species (ROS) in vitro. Genistein rescued RGC-5 cells in the presence of IAA, however, it also increased caspase activation and did not inhibit the generation of ROS. Genistein increased phosphorylation of ribosomal s6 kinase (p90RSK), reduced phosphorylation of the ribosomal S6 protein, and had no effect on phosphorylation of protein kinase B (AKT). Kaempferol and genistin rescued RGC-5 cells from IAA-induced cell death, as well as reduced caspase activation and ROS generation. Kaempferol increased phosphorylation of AKT and MAP kinase (p44/42). Genistin reduced phosphorylation of p42 and p90RSK. Although these phytoestrogens are flavonoids and similar in structure, they exhibit different effects on cell signaling.*

Oxidative stress, along with mitochondrial dysfunction, has been implicated in many neurodegenerative diseases such as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, Huntington's (1), and glaucoma (2). Reactive oxygen species (ROS) cause oxidative damage and are found endogenously as by-products of metabolism derived from the mitochondria. When found exogenously or occurring from an imbalanced redox status during disease, they can cause sufficient cellular damage to lead to apoptotic cell death (3).

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There is much evidence for the contribution of oxidative stress to the pathology of optic neuropathies such as glaucoma. Retinal ganglion cells (RGCs) are central nervous system (CNS) neurons, which makes them more susceptible to oxidative stress because of the concentration of polyunsaturated fatty acids making up their membranes, and their oxygen consumption, which is almost 20% of the body's total oxygen (4). RGCs are also more susceptible to oxidative damage than other CNS neurons because of their exposure to direct sunlight (5). Some theorize that the generation of ROS is induced by ischemia (6), others suspect the inhibition of retrograde neurotrophin support due to increased intraocular pressure (IOP) (7), and yet another school of thought points to glutamate excitotoxicity as the culprit for increased ROS generation and RGC cell death (8). Irrespective of the cause of ROS generation, there is mounting evidence implicating oxidative stress as a determining factor for RGC death in neurodegenerative eye disorders, and particularly in patients with primary open-angle glaucoma.

In diseases related to increased oxidative load, antioxidants remain a promising means of intervention. Thus far, a lack of clinical trials that examine the capability of antioxidants to confer neuroprotective benefits has inhibited potential progress of antioxidants as neuroprotectants (9). The current neuroprotective drugs are limited by other non-specific actions (10, 11) and it is difficult to determine whether this effect is afforded by the IOP-lowering capabilities of the drugs or not. 17β-Estradiol has shown neuroprotective capabilities, however it may also have unwanted feminizing side-effects (12).

Our study utilizes an *in vitro* model of RGC cell death that mimics mitochondrial-associated apoptosis, one of the predominant mechanisms of induction of apoptosis. For several reasons, we used the immortalized cell line, RGC-5. RGC-5 cells maintain many characteristics of primary neurons like retinal ganglion cells including the expression of neuronal proteins (13-17). There is also evidence that RGC-5 cells behave as primary neurons would in disease

states (18, 19). However, some have recently suspected the derived neuronal cell line to have a different origin (20).

Our hypothesis is that mitochondrial dysfunctions result in the accumulation of oxidative stress, which in turn causes apoptosis of neurons in neurodegenerative diseases. In order to emulate this condition *in vitro*, we induced a mild, mitochondrial-associated oxidative stress using iodoacetic acid (IAA). IAA is a metabolic inhibitor that irreversibly depletes the amount of ATP present in a cell. The basis of action for IAA is its ability to inhibit the cytosolic glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (21). The degenerative effects of IAA on the vertebrate retina were first described in 1951, and IAA has since been used to simulate many degenerative diseases involving oxidative stress *in vitro* (22). It has been utilized to induce neurodegeneration in many cell types including: cerebellar granule cells (23), primary cortical neurons (24), and chick retinal cells (25). The effects of IAA also seem to mimic the effects seen in animal models of retinal ischemia. This includes loss of ATP (21, 24), increase in ROS (24), breakdown of phospholipids (26) and alterations in membrane potential (27). *In vivo*, the morphology of neurons affected by IAA cytotoxicity resembles the morphology of neurons affected by ischemia (28).

Phytoestrogens are a class of flavonoids found in plants that are consumed in high dietary concentrations. They function as free-radical scavengers utilizing a phenolic structure. There has been an increasing interest in the application of phytoestrogens in human disease due to accumulating evidence that they also have anti-inflammatory, anti-nociceptive (29) and neuroprotective properties (30). Phytoestrogens are a plausible therapeutic intervention to retard the progression of apoptotic cell death in neurodegeneration. For the purpose of this study, we chose a group of phytoestrogens found in many plant foods. This group includes fisetin hydrate, apigenin, kaempferol, genistein, and genistin.

Materials and Methods

Cell culture. Transformed RGC-5 cells (kind gift from Dr. N. Agarwal, UNT Health Science Center, Fort Worth, TX, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Grand Island, NY, USA) in a water-jacketed incubator at 37˚C with an humidified 37˚C atmosphere, consisting of 95% room air and 5% CO_2. The cells were passaged by trypsinization every two days. The doubling time for RGC-5 cells is approximately 20 h (17).

Cell viability. RGC-5 cells were seeded into 96-well plates at approximately 3,000 cells per well and cultured overnight in DMEM with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin). Before treatment, the cells were washed with DMEM free of serum and antibiotics and it was added to each well. Cells were treated with different concentrations of IAA (Sigma-Aldrich, St. Louis, MO, USA) from 2-10 μM in the presence and absence of one of phytoestrogens at concentrations of 5-50 μM (Sigma-Aldrich) for 24 h. The effects of treatment of IAA and the antioxidants on RGC-5 cell viability were quantified by Calcein AM fluorescent staining (Sigma-Aldrich), as previously described (31). In order to reduce extracellular esterase contribution, cells were washed with and incubated in phosphate buffered saline (PBS) prior to treatment with 2 μM Calcein AM for 30 min at room temperature. Fluorescence was detected at an excitation of 485 nm and emission of 530 nm using a BioTek Synergy2 plate reader and the Gen5 software by BioTek (Winooski, VT). Values were then normalized to background fluorescence and are represented in RFU. Morphological changes in RGC-5 cells after IAA and antioxidant treatment were observed under light microscopy.

Caspase activation. Caspase activity was determined utilizing the fluorimetric dye Ac-DEVD-AMC (7-amino-4-methylcoumarin) (Sigma Aldrich), a substrate that is preferentially cleaved by capase-3. The resultant 7-amino-4methylcoumarin was detected at an excitation of 360 nm and emission at 460 nm using a BioTek Synergy2 plate reader and the Gen5 software by BioTek. Values were normalized to background fluorescence and then to cell number, as determined by Calcein AM values in the same well. Results are expressed in relative fluorescence units (RFU) and represent two separate experiments.

ROS generation. Involvement of (ROS) was determined by H2DCF (dichlorofluorescein)-DA fluorescent assay (Sigma Aldrich) (32). Fluorescence was detected at an excitation of 485 nm and an emission of 530 nm, using a BioTek Synergy2 plate reader and the Gen5 software by BioTek.

Immunoblotting. Phosphorylation status of cell survival proteins was determined using the PathScan Multiplex Western Cocktail I from Cell Signaling Technologies (Danvers, MA). Antibodies included were against: phospho-p90RSK (Ser380), phospho-AKT (Ser473), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), phospho-S6 ribosomal protein (Ser235/236), and eukaryotic translation initation factor, eIF4E.

JC-1 mitochondrial staining. Depolarized mitochondrial membrane potential was determined using JC-1 staining (Calbiochem, Rockland, MA). Cells were seeded into glass bottom dishes at 7,000 cells per well or in 96-well plates as described above. Cells were cultured overnight in DMEM containing 10% FBS and 1% antibiotics. Before treatment, cells were washed twice with and then cultured in serum-free DMEM. Dishes were treated for 24 h with IAA and phytoestrogens as described above. Stable mitochondrial membrane potential (MMP) causes a shift in fluorescence from 530 nm of the monomeric form, to 590 nm from the J-aggregates. 96-Well plate values were quantified using a BioTek Synergy2 plate reader and the Gen5 software by BioTek. Changes in MMP were visualized using the argon laser of a Zeiss laser scanning microscope (Zeiss LSM 510), and quantified using a plate reader.

Mass spectrometry. Mass spectra of phytoestrogens were acquired on a quadrupole ion trap instrument (LCQ, Thermo Fisher, San Jose, CA, USA), operated with the manufacturer's Excalibur 1.4 software.

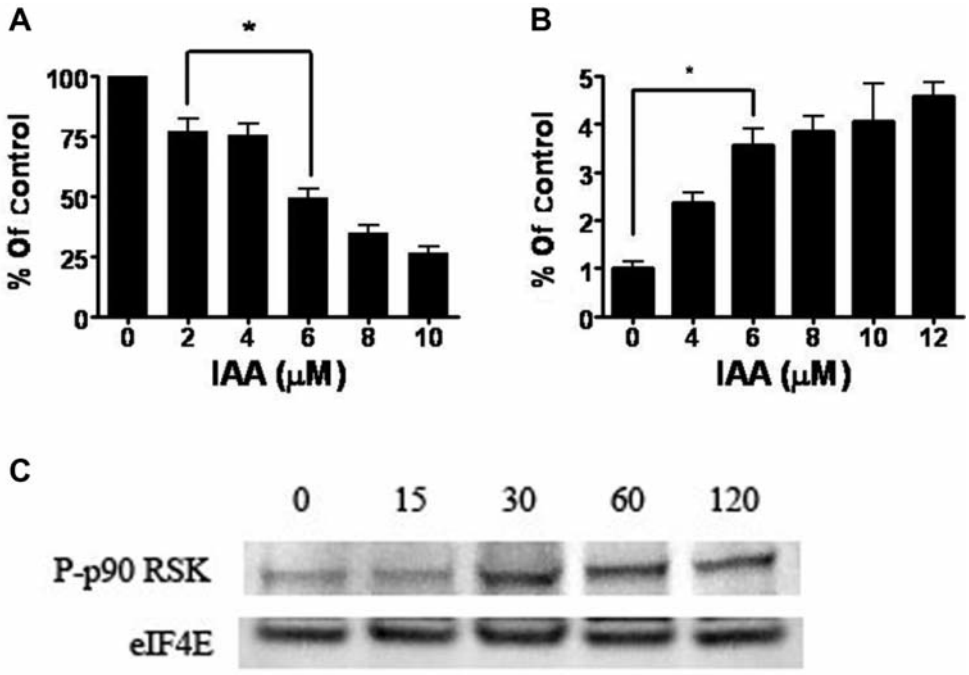

Figure 1. *IAA is cytotoxic to RGC-5 cells via generation of ROS and affects phosphorylation of p90 RSK. A: Survival of RGC-5 cells after 24 h of exposure to IAA as determined by Calcein AM assay. B: Generation of ROS in RGC-5 cells after 24 h of IAA treatment as determined by H2DCF (dichlorofluorescein)-DA assay. C: Immunoblot against phospho-p90 RSK from RGC-5 cell lysates after the first two hours of IAA treatment. *p<0.05.*

Electrospray ionization (ESI) was employed in the negative-ion mode. The capillary voltage and temperature were set to −4.5 kV and 200˚C, respectively. The sheath gas flow was 40 units, and no auxiliary gas flow was used. Aliquots (10 μl) of the phytoestrogens were mixed with methanol (190 μL, HPLC grade) for analysis. A liquid sample flow of 5 μl/min was used for direct sample infusion by the syringe pump built into the instrument. Mass spectra were acquired through automatic gain control to adjust trapping of the ions based on two 200-ms microscans and 1×10^8 as the target ion count. Ten scans were averaged to obtain ESI mass spectra for inspection.

Statistical analysis. Statistical significance was determined by one-way analysis of variance. $p<0.05$ was considered significant for all experiments.

Results

Chemical stability of phytoestrogens. A pilot stability study emulating assay conditions and employing negative-ion ESI mass spectrometric detection revealed no apparent loss of IAA and phytoestrogens, no formation of specific degradation or conjugation products thereof, after 8 and 24 h of incubation at 37˚C. The de-protonated test agents ($[M-H]^-$: *m/z* 285 for kaempferol, m/z 269 for genistein, and *m/z* 431 for genistin) were the predominant species detected in the mass spectra.

Effects of IAA on RGC-5 cells. RGC-5 cells were susceptible to IAA-induced cytotoxicity, with a half maximal inhibitory concentration (IC_{50}) value of approximately 6 μM (Figure 1A). Morphological differences were apparent compared with no treatment after 24 h of 6 μM IAA exposure, including rounding up and lifting off, characteristic of apoptosis. IAA induced the generation of ROS in RGC-5 cells (Figure 1B). The basal level of phosphorylation of p90 RSK was increased after 2 h of 6 μM IAA treatment (Figure 1C). Mitochondrial membrane potential collapses during mitochondria-related apoptosis. The mitochondrial membrane potential detecting dye, JC-1, allows detection of the changes in MMP by forming a red fluorescent monomer when the membrane potential is intact, which is capable of crossing the mitochondrial membrane. In the cytoplasm it forms a green polymer. Twenty-four hours after treatment with 6 μM IAA, the mitochondrial membrane potential had collapsed in RGC-5 cells (Figure 2). 17β-Estradiol was neuroprotective to RGC-5 cells in the face of IAA treatment and reduced ROS generation (Figure 3).

Fisetin hydrate and apigenin were not neuroprotective against IAA in our *in vitro* model and are therefore not discussed further (Figure 3).

Kaempferol mitigates the cytotoxic effects of IAA. The chemical structure of kaempferol allows it to act as a free-

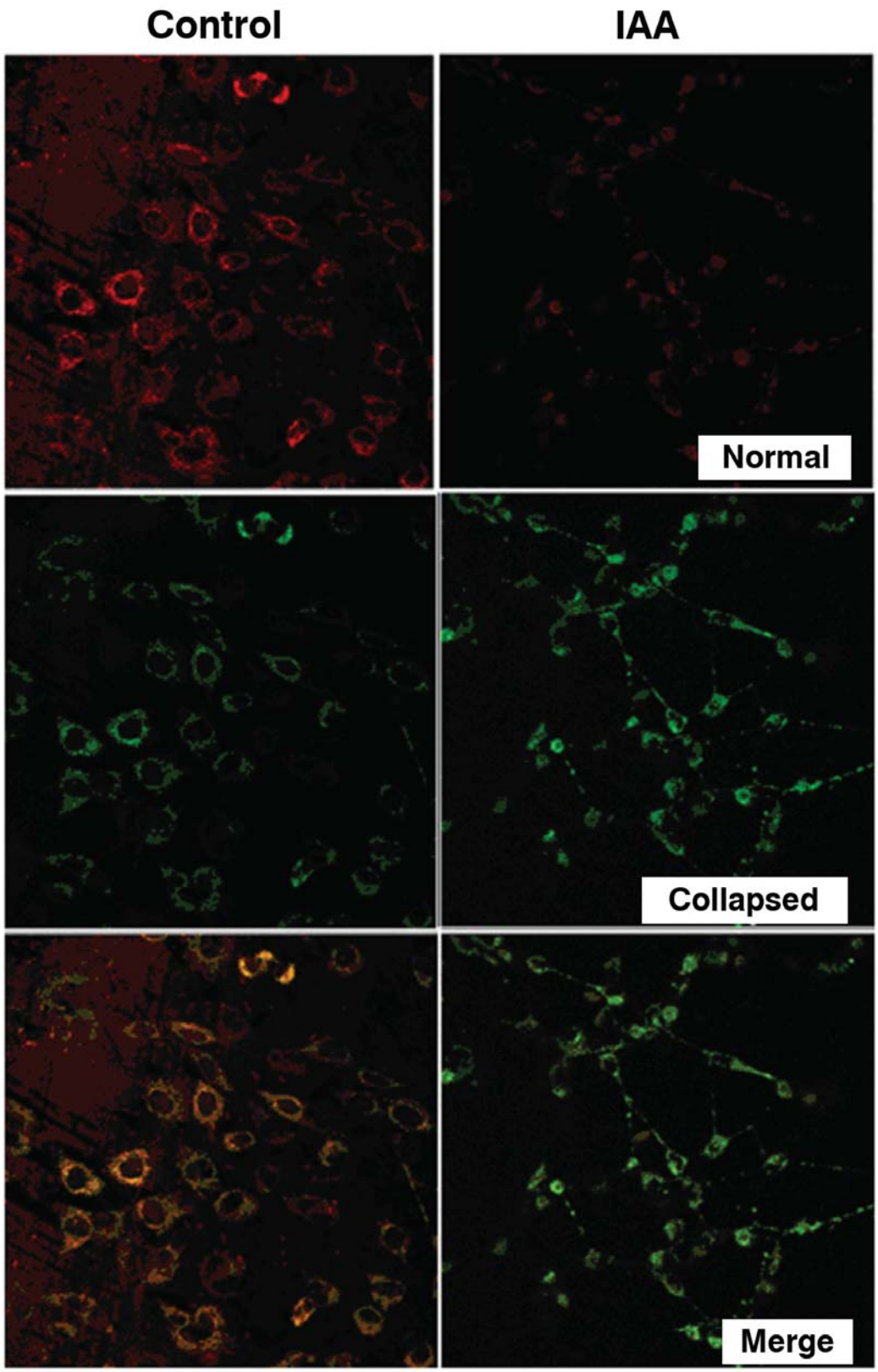

Figure 2. *IAA causes collapse of mitochondrial membrane potential. Collapse of MMP as determined by JC-1 fluorescent dye for control and IAA-treated RGC-5 cells after 24 h.*

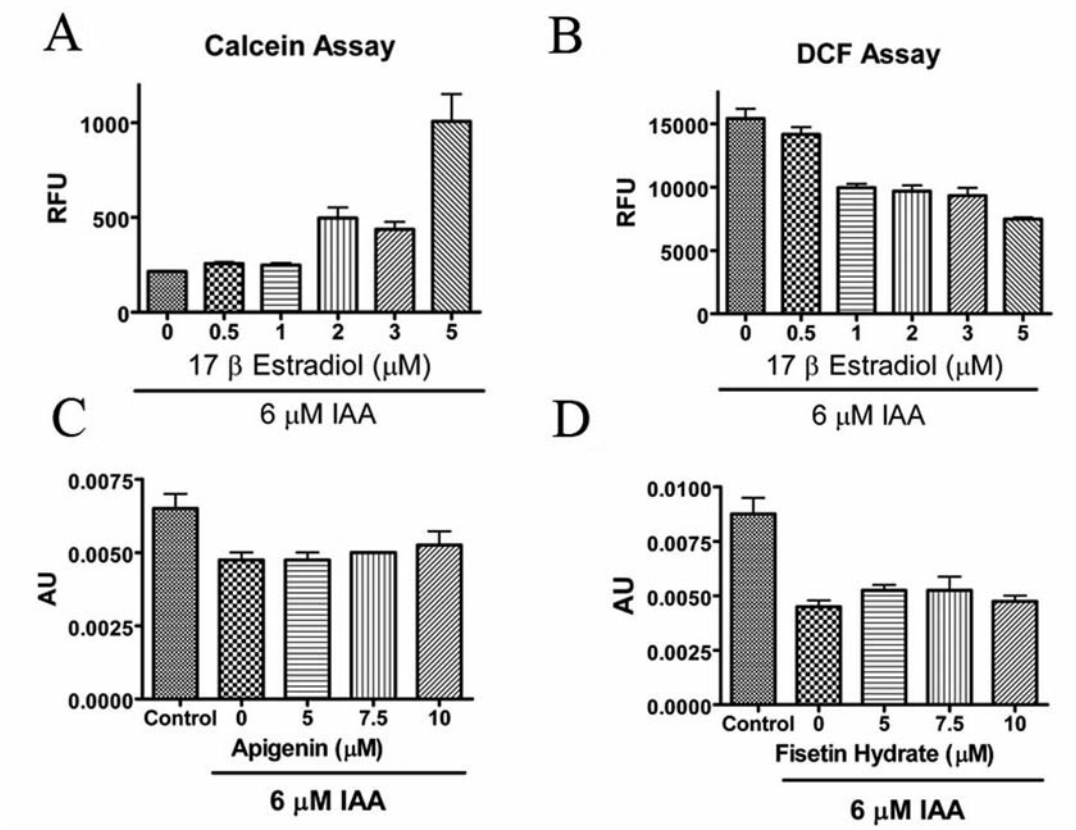

Figure 3. *A: Survival of RGC-5 cells upon exposure to IAA and 17β-Estradiol simultaneously after 24 h, as determined by Calcein AM. B: Generation of ROS after 24 h of simultaneous treatment with IAA and 17β-Estradiol, as determined by H2DCF (dichlorofluorescein)-DA assay. C: Survival of RGC-5 cells after simultaneous exposure to IAA and Apigenin for 24 h as determined by Calcein AM. D) Survival of RGC-5 cells after simultaneous exposure to IAA and fisetin hydrate for 24 h as determined by Calcein AM.*

radical scavenger, utilizing hydroxyl groups and a conjugated ring structure (Figure 4A). When simultaneously treated with 6 μM IAA and increasing doses of kaempferol, we observed increased cell viability using the Calcein AM assay, indicating that kaempferol mitigated IAA-induced cytotoxicity (Figure 4B). Kaempferol also mitigated caspase activation (Figure 4C) and the generation of ROS (Figure 4D). Basal levels of phosphorylation of cell survival-related proteins remained stable during the first two hours in control, serum deprived RGC-5 cells. Kaempferol is an RSK inhibitor and thereby reduced phosphorylation of p90RSK and S6. Kaempferol also reduced activation of AKT and increased activation of p44/p42 at 60 min (Figure 4E).

Genistein mitigates cytotoxity but not caspase activation or ROS generation. Like kaempferol, the structure of genistein allows it to function as a free-radical scavenger (Figure 5A). When simultaneously treated with 6 μM IAA and escalating doses of genistein, RGC-5 cells exhibited increased viability, suggesting genistein mitigates IAA-induced cell death (Figure 5B). Genistein did not inhibit caspase activation due to IAA, and alone actually induced caspase activation at concentrations higher than 30 μM (Figure 5C). Genistein did not inhibit the generation of ROS at concentrations of 20 μM or less, but did at higher concentration, which also activated caspases (Figure 5D). Basal levels of phosphorylation of cell survival-related proteins remained stable during the first two hours in control, serum-deprived RGC-5 cells. Genistein is a tyrosine kinase inhibitor and thereby reduces activation of p44/p42 and activated AKT levels. Genistein increased phosphorylation of p90RSK and reduced phosphorylation of S6 (Figure 5E).

Genistin mitigates cytotoxicity due to IAA. Genistin, a glycosylated form of genistein, acts as a free-radical scavenger similarly utilizing the hydroxyl groups and a conjugated ring structure (Figure 6A). Simultaneous treatment with 6 μM IAA and increasing doses of genistin led to increased cell viability during Calcein AM assay, indicating that genistin mitigated IAA-induced cytotoxicity (Figure 6B). Genistin also mitigated caspase activation (Figure 6C) and the

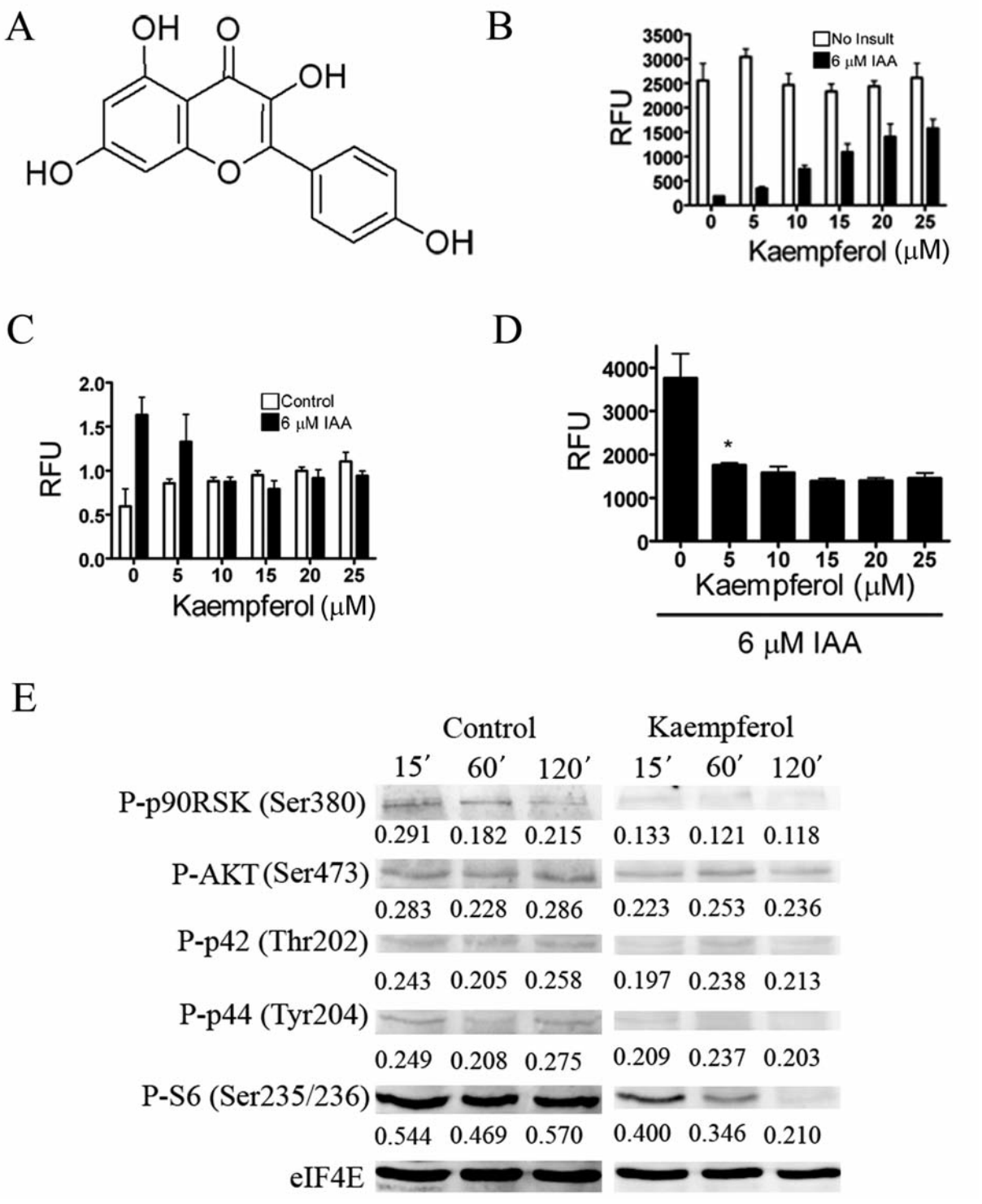

Figure 4. *Kaempferol rescues RGC-5 cells from cell death, mitigates caspase-3 activation and the generation of ROS after 24 h of IAA treatment. A: Chemical structure of kaempferol. B: Survival of RGC-5 cells after 24 h exposure to IAA and kaempferol simultaneously as determined by Calcein AM assay. C: Caspase-3 activation after 24 h of simultaneous treatment with IAA and kaempferol, as determined by fluorogenic Caspase-3/7 assay normalized to cell number by Calcein AM. D: Generation of ROS after 24 h of simultaneous treatment with IAA and kaempferol as determined by H2DCF (dichlorofluorescein)-DA assay. E: Time course of expression of control RGC-5 cells during two hours of serum deprivation and those treated with 30 μM kaempferol. Kaempferol is a RSK inhibitor and thereby reduces phosphorylation of p90RSK and S6. Kaempferol affects activation of AKT and p44/p42 at 60 min of treatment. *p<0.05.*

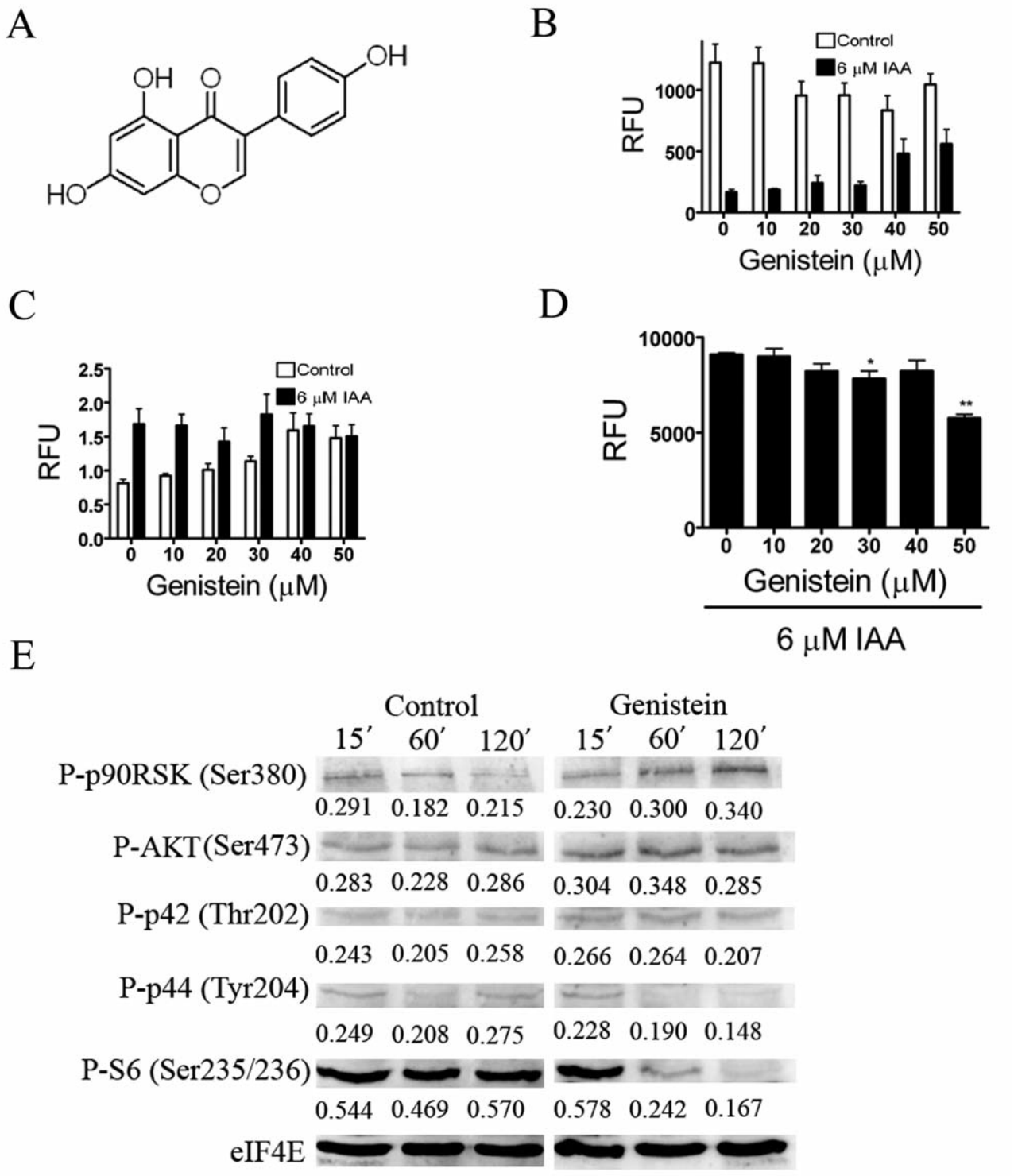

Figure 5. *Genistein rescues RGC-5 cells from cell death due to IAA insult in vitro, does not mitigate caspase activation, but does inhibit the generation of ROS after 24 h of IAA treatment. A: Chemical structure of genistein. B: Survival of RGC-5 cells after exposure to 24 h of IAA and genistein simultaneously, as determined by Calcein AM assay. C: Caspase-3 activation after 24 h of simultaneous treatment with IAA and genistein determined by fluorogenic Caspase 3/7 assay, normalized to cell number by Calcein AM. D: Generation of ROS after 24 h of simultaneous treatment with IAA and genistein determined by H2DCF (dichlorofluorescein)-DA assay. E: Time course of expression of control RGC-5 cells during two hours of serum deprivation and those treated with 30 μM Genistein. Genistein is a tyrosine kinase inhibitor and thereby affects levels of activated p42 and is expected to affect levels of activated AKT. Genistein increases phosphorylation of p90RSK and reduces phosphorylation of S6. *p<0.05.*

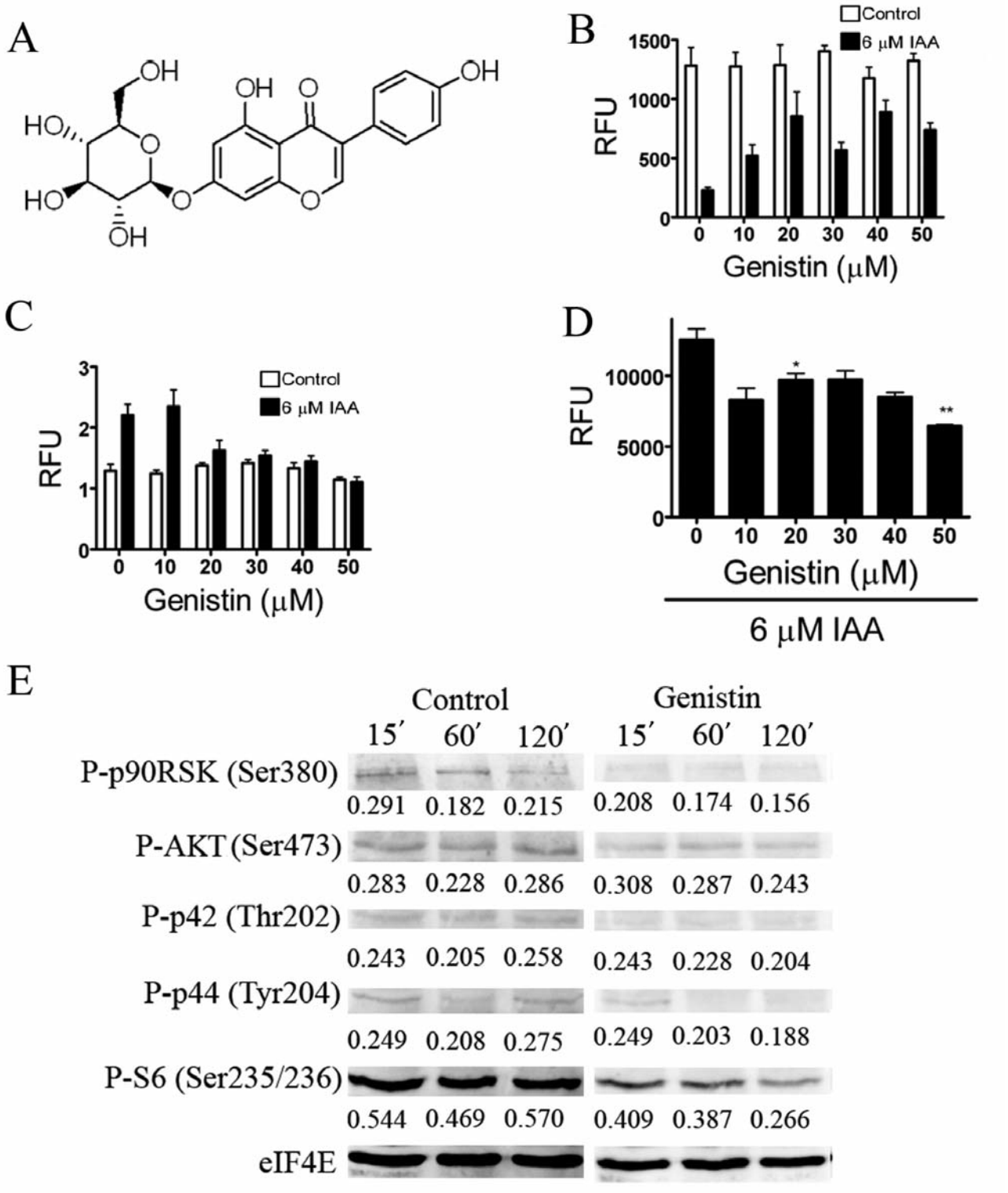

Figure 6. *Genistin rescues RGC-5 cells from cell death, mitigates Caspase-3 activation and the generation of ROS after 24 h of IAA treatment. A: Chemical structure of genistin. B: Survival of RGC-5 cells after 24 h exposure to IAA and genistin simultaneously as determined by Calcein AM assay. C: Caspase-3 activation after 24 h of simultaneous treatment with IAA and genistin, as determined by fluorogenic Caspase-3/7 assay normalized to cell number by Calcein AM. D: Generation of ROS after 24 h of simultaneous treatment with IAA and genistin as determined by H2DCF (dichlorofluorescein)-DA assay. E: Time course of expression of control RGC-5 cells during two hours of serum deprivation and those treated with 30 μM Genistin. Genistin decreases p42 and p90 RSK activation when compared to control. *$p<0.05$.*

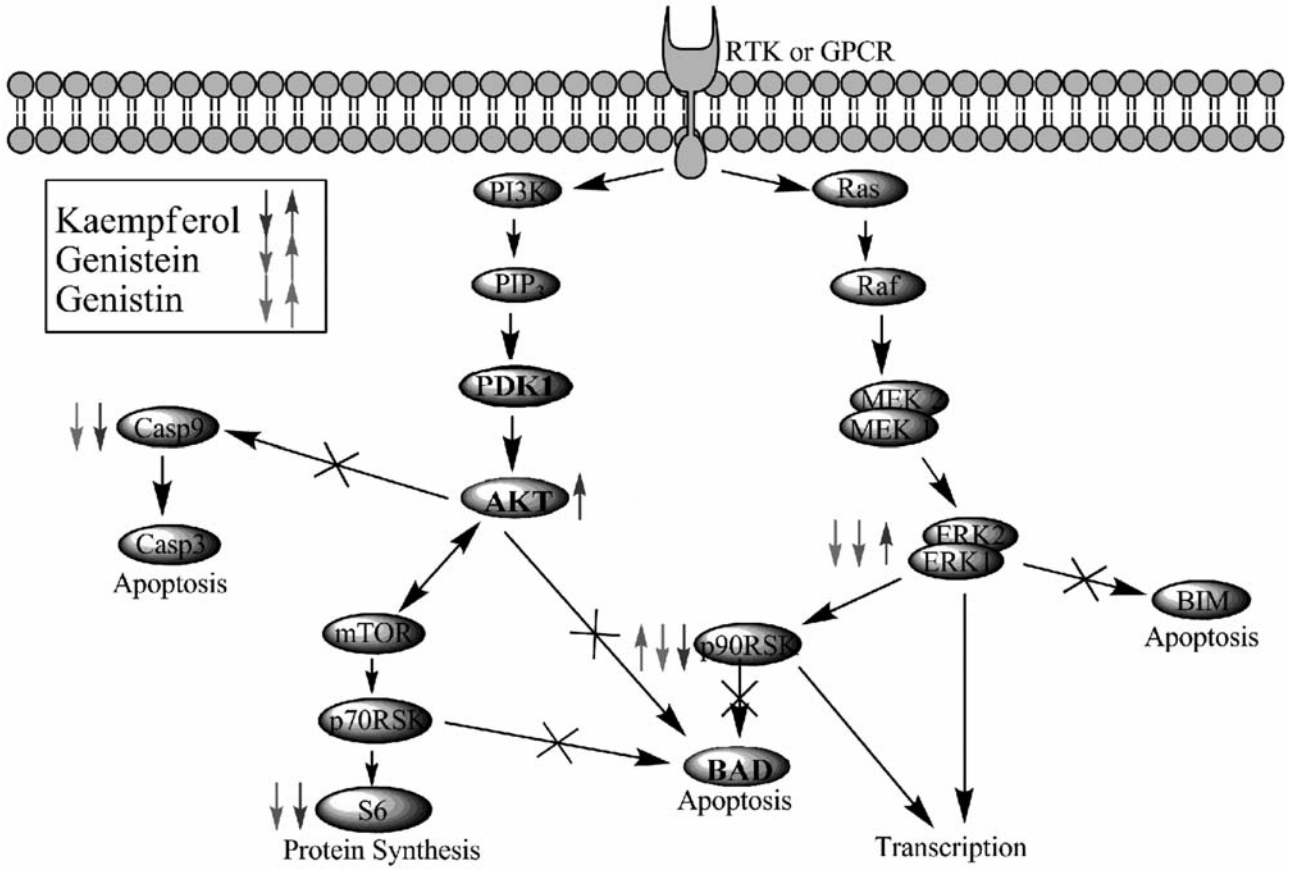

Figure 7. *Pathway illustrating the effects of phytoestrogens on classical antiapoptotic cell signaling. (Abbreviations: RTK: receptor tyrosine kinase, GPCR: G-protein coupled receptor, PI3K: Phosphatidylinositol 3-kinase, PIP2: Phosphatidylinositol 4,5-bisphosphate, PDK1: 3-phosphoinositide dependent protein kinase-1, AKT: protein kinase B, mTOR: mammalian target of rapamycin , RSK: ribosomal s6 kinase, S6: ribosomal s6, Ras: Ras GTPase, Raf: proto-oncogene serine/threonine-protein kinase, MEK: Mitogen-activated protein kinase kinase, ERK: extracellular-signal-regulated kinases, BIM: pro-apoptotic protein, BAD: Bcl-2-associated death promoter, Casp9: Caspase-9, Casp3: Caspase-3).*

generation of reactive oxygen species (Figure 6D). Basal levels of phosphorylation of cell survival-related proteins remained stable during the first two hours in control, serum-deprived RGC-5 cells. Genistin reduced p42 and p90 RSK activation when compared to control (Figure 6E).

Discussion

Phytoestrogens, dietary flavonoids and other dietary supplements derived from plants, and food component are being widely studied due to their multiple actions. One of the advantages of these agents is their neuroprotective activity. Interest in phytoestrogens as neuroprotectants was spurred by a group of studies involving beneficial foods containing dietary flavonoids. One study showed a reversal of age-related cognitive and motor decline in mice after a lifetime of diets supplemented with blueberries, strawberries, and spinach (33). Dietary flavonoids have since been shown to induce Phase II detoxification enzymes, in particular heme-oxygenase-1 (34). Phytoestrogens are also seen as plausible alternatives for estradiol and its analogs because these phenolic compounds of similar structure only weakly interact with estrogen receptors, resulting in weak estrogenic or antiestrogenic outcomes (35). Therefore, use of such agents for neuroprotection avoids the feminizing effects of estradiol therapy.

Our observations give insight into the neuroprotective mechanisms of phytoestrogens in response to oxidative challenge induced by IAA. Moreover, we further delineated the cell signaling pathway which is responsible for the antioxidant action of these phytoestrogens. We found that the IAA-mediated cytotoxicity was reversed in the presence of exogenous antioxidants, kaempferol, genistein and genistin. The protective effect of phytoestrogen was found to be due to loss of MMP of RGC-5 cells, however, there might be other pathways

involved as the mechanism of action. The DCF assay demonstrated generation of ROS by IAA treatment, which was reversed by the phytoestrogens kaempferol and genistin. In addition we found that the caspase was activated in the presence of IAA, which was rescued by kaempferol and genistin. Kaempferol and genistin not only mitigated cell death, but also prevented the activation of mitochondria-associated apoptosis and reduced ROS generation. Genistein is not as plausible as an antioxidant therapy due to its effects on caspase activation at concentrations that are minimal for sequestering ROS, however, it activates key anti-apoptotic signaling events that make it a candidate for neuroprotection. Taken together, these results suggest that although these compounds are structurally very similar, functional differences do exist.

Interestingly, we found that the way these phytoestrogens affect cell signaling differs between them, despite their similar chemical structure. For example, kaempferol is a potent RSK inhibitor (36) and thereby negatively affects phosphorylation of p90RSK, whereas genistein increases phosphorylation of p90RSK. On the other hand, kaempferol positively affects phosphorylation of AKT and p44/42, whereas genistein is a tyrosine kinase inhibitor and reduces phosphorylation of p42 and activation of AKT. Our data lead us to the conclusion that these compounds act differently towards the same goal.

Our novel results include the effects of kaempferol and genistein on AKT phosphorylation, and the preliminary characterization of genistin compared its more popular counterpart, genistein. We also found IAA to induce phosphorylation of p90RSK, a result previously not shown. Although our results indicate that genistein exacerbates caspase activation at minimum concentrations to scavenge ROS, and previously collected evidence shows that it binds estrogen receptor-β with considerable affinity and functions as a potent tyrosine kinase inhibitor, which would invariably affect neuron function, genistein activates key anti-apoptotic/pro-survival pathways that inhibit mitochondria-induced and cytoplasmic apoptosis. Kaempferol, while it has the unwanted side-effect of being an RSK inhibitor, is also a potent neuroprotective compound which probably protects against apoptosis through activation of AKT. Genistin does not exhibit any deleterious inhibitory or receptor-binding properties while functioning as a neuroprotectant, which makes it a good drug candidate. When taking into account the previous evidence presented along with the evidence we collected, we suggest that kaempferol and genistin are good candidates for cytoprotective pharmaceuticals in neurodegenerative diseases (Figure 7).

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

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