17β-エストラジオールはHT22細胞においてグルタミン酸に誘起されるAkt及びその下流標的分子の減少を抑制する

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17β-Estradiol Prevents the Glutamate-Induced Decrease of Akt and Its Downstream Targets in HT22 Cells

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ABSTRACT. Estradiol is known to exert neuroprotective effect against glutamate toxicity in hippocampal-derived cell line (HT22). This study investigated whether estradiol modulates the anti-apoptotic signal through the phosphorylation of Akt and its downstream targets, including Bad, forkhead transcription factors FKHR and FKHRL1. Pretreatment with 17β-estradiol decreased glutamate toxicity-induced cell death in HT22 cells. Also, pretreatment with 17β-estradiol significantly decreased the positive cells of TUNEL stain, compared to that of only glutamate-treated cells. Potential activation was measured by phosphorylation of Akt at Ser^{473}, Bad at Ser^{136}, FKHR at Ser^{256}, and FKHRL1 at Thr^{32} using Western blot analysis. 17β-estradiol pretreatment prevented the glutamate-induced decrease of pAkt, pBad, pFKHR, and pFKHRL1. These findings clearly confirm that 17β-estradiol plays a potent neuroprotective role against glutamate-induced toxicity and suggest that phosphorylation of Akt and its downstream targets by 17β-estradiol mediated these protective effects.
KEY WORDS: Akt, Bad, estradiol, FKHR, FKHRL1.

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It is known that estradiol plays potent neurotrophic and neuroprotective roles in immature and adult brains [6, 12]. In particular, clinical studies have shown that estradiol decreases the risk or severity of neurodegenerative conditions such as Alzheimer's disease, stroke, and Parkinson's disease [8, 10]. In *in vitro* studies, estradiol exerts neuroprotective effects and reduces neuronal damage caused by serum deprivation, β-amyloid treatment, and exposure to glutamate [4, 14]. Also, numerous studies have demonstrated that estradiol inhibits cell death by decreasing the extent of apoptotic cell death and enhancing cell survival signals [12, 13].

The phosphatidylinositol 3-kinase (PI3-K)/Akt signal pathway is an important mediator of survival in response to growth factors [3]. In the presence of survival factors, activated Akt phosphorylates the specific residences of pro-apoptotic proteins, such as Bad, forkhead transcription factors FKHR and FKHRL1, leading to suppression of their apoptotic activity, thereby promoting cell survival [3, 9]. However, in the survival factor withdrawal, Bad dephosphorylates and interacts with the anti-apoptotic protein Bcl-x(L), leading to block Bcl-x(L)-dependent cell survival pathway [3]. Furthermore, dephosphorylated forkhead transcription factors translocates into the nucleus, where they initiate a program of gene expression such as Fas ligand. Fas ligand activates the cell surface Fas protein, activates a caspase cascade, and induces cell death [2]. Consequently, the phosphorylation of Bad, FKHR, and FKHRL1 is critical for the suppression of apoptosis.

Estradiol was reported to induce the phosphorylation of Akt, noting that the PI3-K/Akt survival pathway mediates the neuroprotective effects of estradiol against brain injury [13]. Although several studies have demonstrated the neuroprotective effect of estradiol, little data are available regarding the activation of Akt and its downstream targets, in HT22 cells with glutamate toxicity. Glutamate excitotoxicity is characteristic of numerous neurodegenerative disease, and then is a frequently utilized cell death model in neuroprotective studies [1] Thus, this study examined the neuroprotective effect of 17β-estradiol against glutamate toxicity and investigated the role of 17β-estradiol in the mediation of anti-apoptotic signaling through the activation of Akt and its downstream targets.

MATERIALS AND METHODS

Cell culture and treatment: HT22 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, without L-glutamine), supplemented with 10% fetal bovine serum, penicillin (100 unit/ml), and streptomycin (100 μg/ml) (Gibco BRL, Gaithersburg, MD, U.S.A.) at 37°C in a 5% CO_2 atmosphere. HT22 cells were seeded onto 96-well plate at 5,000 cells per 100 μl growth media in each well and grown overnight prior to initiation of any experimental treatments. For Western blot analysis, HT22 cells were seeded on 60-mm culture dishes at 100,000 cells per dish. Cell density was maintained 70% or less confluency as described previously [5]. Glutamate (Sigma, St. Louis, MO, U.S.A.) was diluted to a final concentration of 5 mM in culture medium, and cells were exposed for 24 hr. 17β-estradiol was initially dissolved in 95% ethanol at a concentration of 1 mM and diluted to the appropriate concentration (1 μM) in culture medium. Ethanol was used at a final concentration of 0.1% as a vehicle control. This concentration of ethanol had no effect on cell viability or glutamate toxicity. Exposure to 17β-estradiol was initiated

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15 min before glutamate addition. A dose of estrogen (1 μM) protected HT22 cells from glutamate toxicity [7]. Cell viability was estimated by measuring metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), as described previously [11]. Briefly, MTT solution (5 mg/m*l*) was added to each well of a 96-well plate, and cells were maintained for 4 hr at 37°C. After removal of the MTT solution, 1 m*l* of solubilization solution containing 50% dimethylformamide, and 20% sodium dodecyl sulfate (pH 4.8) was added. After an overnight incubation, absorption valves at 570 nm were measured. Cell viability was expressed as percentage of neuroprotection vs. controls set at 100%.

TUNEL histochemistry: TUNEL histochemistry was performed using the DNA Fragmentation Detection Kit (Oncogene Research Products, Cambrige, MA, U.S.A.). The cells were treated proteinase K (20 μg/m*l*) for 2 min and blocked with 0.3% hydrogen peroxide in methyl alcohol for 3 min. After washes in PBS, cells were incubated with equilibration buffer followed by TdT enzyme in a humidified chamber at 37°C for 1 hr, and then a stop/wash buffer was applied for 30 min at 37°C. Finally, cells were incubated with peroxidase labeled anti-digoxigenin antibody for 30 min at room temperature. Positive cells were visualized using a diaminobenzidine substrate (Sigma chemical Co., St. Louis, MO, U.S.A.) and counterstained with 1% cresyl violet.

Western blot analysis: Cells were collected and lysed in buffer (1% Triton X-100, 1 mM EDTA in 1 × PBS (pH 7.4)) containing 10 μM leupeptin and 200 μM phenylmethylsulfonyl fluoride. The protein concentration of each lysate was determined using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's protocol. Thirty micrograms of total protein was applied to each lane on to 10% SDS–polyacrylamide gels (SDS-PAGE). After electrophoresis and immunoblotting, the poly-vinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, U.S.A.) were washed in Tris-buffered saline containing 0.1% Tween-20 (TBST) and then incubated with the following antibodies: anti-Akt, anti-phospho-Akt(Ser473), anti-Bad, and anti-phospho-Bad(Ser136), anti-FKHR, anti-phospho-FKHR(Ser256), anti-FKHRL1, anti-phospho-FKHRL1(Thr32) antibodies (diluted 1:1000, Cell Signaling Technology, Beverly, MA, U.S.A.) as primary antibody. All primary antibodies were used polyclonal rabbit IgG. And the membrane was incubated with secondary antibody (1:5000, Pierce, Rockford, IL, U.S.A.), and the ECL Western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) according to the manufacturer's protocol was used for detection. The intensity analysis was carried out using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA, U.S.A.) and SigmaPlot 4.0 (SPSS Inc., Point Richmond, CA, U.S.A.). The results are the mean of five independent experiments.

Data analysis: All data are expressed as mean ± S.E.M. The results in each group were compared by one-way analysis of variance (ANOVA) followed by the post hoc Dunnet test when the F value showed significant differences at $P<0.05$.

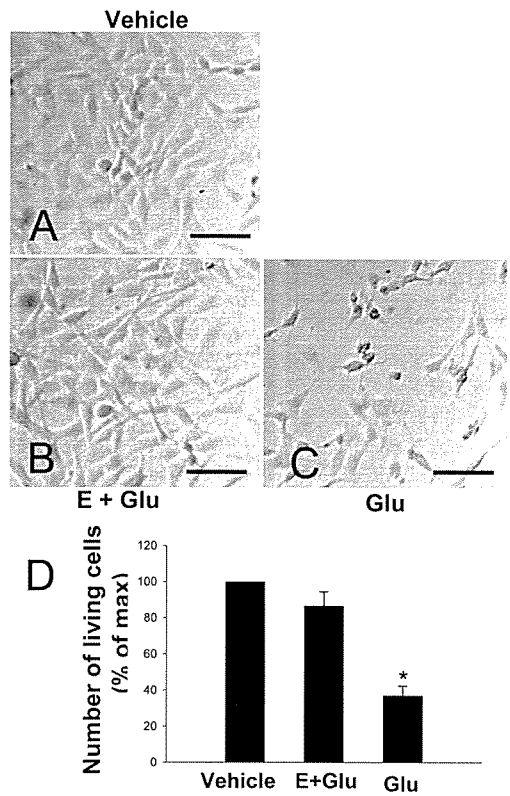

Fig. 1. Neuroprotective effect of estradiol against glutamate toxicity in HT22 cells. Glutamate (5 mM, Glu) exposed for 24 hr and 17β-estradiol (E) treated at 15 min before glutamate exposure. (A-C) Representative photomicrographs of the HT22 cells. Photomicrographs show phase-contrast images of representative fields of cells. Scale bars = 100 μm. (D) Cellular viability was assessed using the MTT assay. Cell survival was expressed as percentage of neuroprotection vs. controls set at 100%.

RESULTS

Figure 1A-C showed the neuroprotective effect of 17β-estradiol against glutamate toxicity. HT22 cells were sensitive to glutamate exposure, in which greater than 50% of cells were killed. However, pretreatment with 17β-estradiol (1 μM) protected HT22 cells from glutamate toxicity. The number of living cells was 87.55% in presence of 17β-estradiol, whereas it was 37.65% in only glutamate-treated group (Fig. 1D). TUNEL staining showed that pretreatment with 17β-estradiol significantly decreased the glutamate-induced cell death. TUNEL positive cells significantly increased in only glutamate-treated group, whereas it was maintained as the levels of control in the presence of 17β-estradiol (Fig. 2A-C). We investigated the activation of Akt, Bad, FKHR, and FKHRL1 by phosphorylation using Western blot analysis. Glutamate exposure induced a decrease of pAkt level,

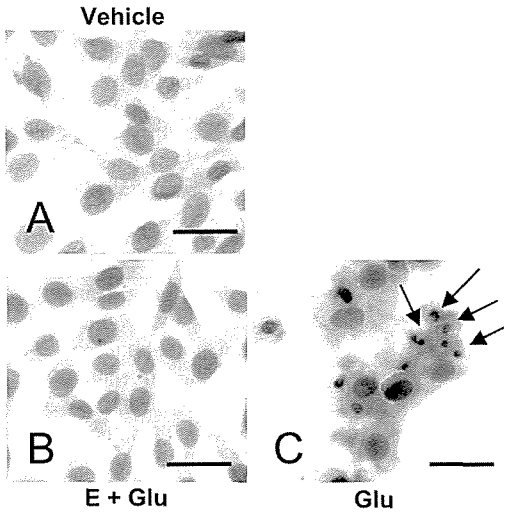

Fig. 2. Photographs of TUNEL staining in HT22 cells. Glutamate (5 mM, Glu) exposed for 24 hr and 17β-estradiol (E) treated at 15 min before glutamate exposure. Positive cells of TUNEL staining were markedly decreased in pre-treated cells with 17β-estradiol. Arrows indicate positive cells. Scale bars = 20 μm.

and pretreatment with 17β-estradiol prevented glutamate-induced down-regulation of pAkt. The level of pAkt was 0.93 ± 0.15 and 0.52 ± 0.10 in pre-treated group with 17β-estradiol and only glutamate-treated group, respectively (Fig. 3A). Also, 17β-estradiol prevented glutamate-induced decline of pBad level. The level of pBad was 1.02 ± 0.15 in presence of 17β-estradiol, whereas it was 0.58 ± 0.09 in only glutamate-treated group (Fig. 3B). Furthermore, the levels of pFKHR and pFKHRL1 were 1.12 ± 0.11 and 1.13 ± 0.12 in pre-treated group with 17β-estradiol, and the levels of these were 0.55 ± 0.15 and 0.41 ± 0.10 in only glutamate-treated group, respectively (Fig. 4A and 4B).

DISCUSSION

Estradiol is known to exert neuroprotective effects against glutamate-induced toxicity and oxidative stress [4, 14]. Also, glutamate-induced apoptosis in HT22 neuronal cell is inhibited by estradiol through the down-regulation of caspase-3 and prevention of mitochondrial cytochrome c release [14]. The present study focused on the neuroprotective effect of 17β-estradiol through activation of Akt and its downstream target. This study showed that pretreatment with 17β-estradiol increased the number of living cells and significantly decreased the number of TUNEL positive cells, compared to that of only glutamate-treated cells. These findings have clearly confirmed that 17β-estradiol mediates the neuroprotective effect through the inhibition of cell death.

The PI3K/Akt signal pathway is important for suppressing cell death and promoting cell survival [3]. Moreover, it was demonstrated that the neuroprotective effects of estradiol against brain injury were mediated through the phosphorylation of Akt [13]. This study focused on the activation of Akt downstream targets through the use of 17β-estradiol in glutamate-induced cell death. This study showed that the level of pAkt decreases in glutamate exposure, and 17β-estradiol prevents a down-regulation of pAkt level, thereby protecting against glutamate-induced cell death. In the absence of 17β-estradiol, the level of pBad significantly decreased in only glutamate-treated cells. However, pretreatment of 17β-estradiol prevents the glutamate-induced decline of pBad in HT22 cells. This study demonstrated that 17β-estradiol prevents the glutamate toxicity-induced decrease of Akt activation and Bad phosphorylation. It is known that the activated Akt leads to the phosphorylation and inactivation of the pro-apoptotic bcl-2 family protein, Bad. pBad prevents the binding Bad and bcl-2, and pFKHR and pFKHRL1 block the translocation of FKHR and FKHRL1 into the nucleus. Thus, the phosphorylation of Bad and forkhead transcription factors is necessary for

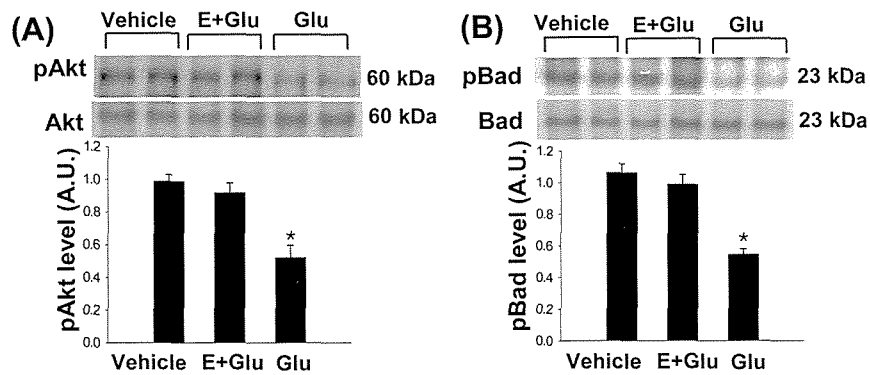

Fig. 3. Western blot analysis of phospho-Akt (A) and phospho-Bad (B) in HT22 cells. Glutamate (5 mM, Glu) exposed for 24 hr and 17β-estradiol (E) treated at 15 min before glutamate exposure. Each lane represents an individual experimental animal. Densitometric analysis is represented as an arbitrary unit (A.U.), normalized by α-tubulin. All results represent the mean ± S.E.M. from five separate data. * $P<0.05$.

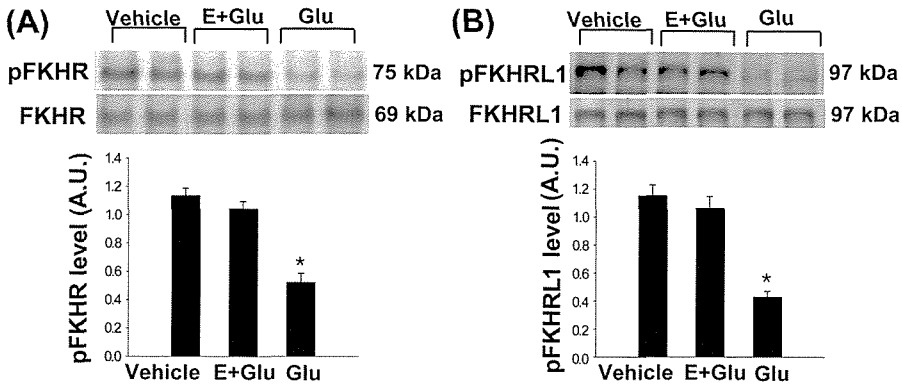

Fig. 4. Western blot analysis of phospho-FKHR (A) and phospho-FKHRL1 (B) in HT22 cells. Glutamate (5 mM, Glu) exposed for 24 hr and 17β-estradiol (E) treated at 15 min before glutamate exposure. Each lane represents an individual experimental animal. Densitometric analysis is represented as an arbitrary unit (A.U.), normalized by α-tubulin. All results represent the mean ± S.E.M. from five separate data. * $P<0.05$.

the suppression of cell death. Our results showed that 17β-estradiol prevents neuronal cell death caused by glutamate toxicity and the glutamate-induced decrease of Bad, FKHR, and FKHRL1 phosphorylation. This study demonstrates that 17β-estradiol prevents the glutamate-induced decrease of Akt activation and its downstream signaling pathway, Bad, FKHR, and FKHRL1 phosphorylation, thereby protecting against neuronal cell death.

In conclusion, these findings suggest the fact that 17β-estradiol plays a neuroprotective role by preventing the glutamate toxicity-induced decline of Akt activation and its substrates phosphorylation.

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