

Short Communication

Effects of 17β -Estradiol on Intracellular Calcium Changes and Neuronal Survival After Mechanical Strain Injury in Neuronal–Glial Cultures

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ABSTRACT The neuroprotective effects of 17β -estradiol (E2) were investigated using an in vitro model of traumatic brain injury in which cortical neuronal cultures were subjected to mechanical strain-injury. The rise in intracellular calcium ($[Ca^{2+}]_i$) following neuronal injury was reduced by addition of 10 or 100 nM E2 to the cultures immediately following injury. Neuronal damage was measured 24 h after injury by propidium iodide uptake and cell viability by carboxyfluorescein diacetate uptake. Addition of 1, 10, or 100 nM E2 to cell cultures immediately following injury decreased neuronal damage and increased neuronal viability compared to vehicle-treated neurons. These results demonstrate the neuroprotective activity of E2 in an in vitro model of neuronal injury, and suggest that such effects may be related to the ability of E2 to modulate $[Ca^{2+}]_i$. **Synapse 60:406–410, 2006.** © 2006 Wiley-Liss, Inc.

The neuroprotective role of 17β -estradiol (E2) against apoptotic and necrotic neuronal death induced by chemical toxins and by ischemia, both in vivo and in vitro, has become a promising area of research (Alkayed et al., 2001; Green and Simpkins, 2000; Jover et al., 2002). The cellular mechanisms of E2 neuroprotection against these insults are not completely understood, but may be associated with inhibition of neuronal calcium currents and subsequent reduction in $[Ca^{2+}]_i$ (Chaban et al., 2003; Chen et al., 1998; Kurata et al., 2001; Mermelstein et al., 1996). Additional possible mechanisms include improved cerebral blood flow (Pelligrino et al., 1998), antioxidant activity (Stein, 2001), and induction of antiapoptotic transcriptional mechanisms (Stein, 2001; Wise et al., 2001). However, relatively few studies have directly examined the possible neuroprotective effects of E2 in traumatic brain injury, and no previous studies have examined the ability of E2 to prevent or reduce the damaging rise in intracellular calcium levels that occurs following brain trauma. Therefore, the present study was carried out to examine the effects of E2 on neuronal intracellular calcium levels and cell viability

following mechanical strain injury in an in vitro model of traumatic brain injury. The results support previous findings that indicate neuroprotective activity by E2 and the possibility that such effects may be mediated by E2 effects on calcium homeostasis after injury.

Mixed gender neuronal–glial cell cultures were prepared from cortices from 1- to 2-day-old male and female Sprague-Dawley rat pups as described previously (Floyd et al., 2005). Briefly, an astrocyte monolayer was cultured for 14 days to confluency, and then replated onto collagen-coated 25 mm diameter Flex-Plate[®] wells for 7 days (Flexcell International, Hills-

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borough, NC). Then, a neuronal layer was plated onto the astrocyte monolayer, and the antimetabolic agent 5-fluoro-2'-deoxyuridine (FUdR; 5 μ l/ml) was added to the culture medium to suppress further glial proliferation and enhance development of the neuronal layer. These mixed neuronal/astroglial cultures were used in neuronal injury studies at 11–14 days in vitro (DIV) after neuronal plating.

Water soluble E2 was prepared in sterile water (10 μ M, 17 β -estradiol, cat# E4389, SIGMA, St Louis, MO), and added to the cell culture wells to achieve final concentrations of 1, 10, or 100 nM. Distilled water was used as a vehicle control. Drug treatments were randomly assigned to individual FlexPlate[®] culture wells, with a minimum of three wells examined under each treatment condition. Mechanical strain injury was produced using the Model 94A Cell Injury System (Bioengineering Facility, BA Commonwealth Univ., Richmond, VA) as described in previous studies (Ellis et al., 1995; Floyd et al., 2005). Briefly, the media in the cell culture well were replaced with warm (37°C) 0.1 M PBS (phosphate buffer saline) supplemented with 30% glucose. Then the cultured neurons growing on the FlexPlate silastic membrane were rapidly and transiently deformed by a calibrated 50-ms pressure pulse of compressed gas introduced into the well. This pressure pulse was selected to deform the membrane by 5.5 mm in order to produce approximately a 40% neuronal loss (Floyd et al., 2001; Weber et al., 1999). This in vitro cell injury procedure was designed to model neuronal injury that occurs in humans after rotational acceleration/deceleration brain injury (Ellis et al., 1995; Meaney et al., 1995).

Changes in levels of [Ca²⁺]_i in cultured cortical neurons were measured before and after mild cell injury using the ratiometric fluorescent calcium indicator, Fura-2-AM. The protocol was adapted from previous studies (Floyd et al., 2005). Briefly, cultures were washed in DPBS and then incubated with 1 ml of 5 μ M Fura2-AM (Molecular Probes Inc., Eugene, OR) in DPBS at 37°C for 1 h. After incubation, cultures were washed once with DPBS and media replaced with 1 ml of DPBS supplemented with 30% glucose.

Imaging was carried out using a Nikon E-600 fluorescence microscope (Nikon E-600, Tokyo, Japan), TILL Photonics monochromator (Grafelfing, Germany), and an Orca II-ER CCD camera (Hamamatsu Photonics, Japan). Preinjury [Ca²⁺]_i baselines were established, and then imaged every 5 s for 10 min after strain injury. Changes in [Ca²⁺]_i following injury and drug treatment were expressed as percent of preinjury baseline. Data were presented as mean \pm SEM. Data were analyzed by ANOVA, followed by Duncan post hoc group comparisons (version 11.5, SPSS, Inc. Chicago, IL). Each experimental condition was repeated three times.

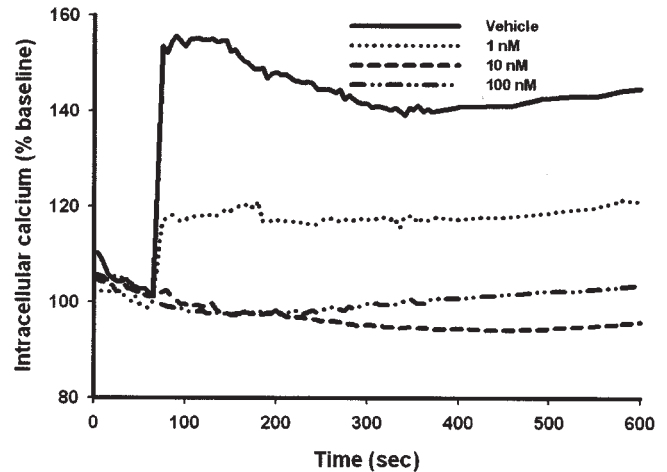


Fig. 1. Representative calcium imaging traces demonstrating the pattern of changes in [Ca²⁺]_i following neuronal injury and treatment with vehicle or E2. The rise in [Ca²⁺]_i following strain injury in vehicle-neurons was immediate and stable across the 10 min of imaging.

In separate culture wells, neuronal damage was measured 24 h after injury by counting the numbers of neurons stained with propidium iodide (PI), while neuronal viability was indicated by the numbers of neurons taking up carboxyfluorescein diacetate (CFDA) (Molecular Probes Inc., Eugene, OR). Briefly, 5 μ l of PI and CFDA, prepared from stock solutions as previously described (Floyd et al., 2005), were added to each culture well for 15 min. Stained neurons were observed at 200 \times by laser confocal microscopy (Zeiss LSM 510, Carl Zeiss AG, Germany), at excitation wavelengths of 563 nm for PI and 488 nm for CFDA. Stained neurons were counted in five randomly selected views (South, North, West, East, and Center of the well) of each well. The percentages of PI- and CFDA-positive neurons of the total number of neurons counted (i.e., CFDA + PI) were calculated. Neurons were differentiated from astrocytes based on general morphology, including an ovoid, pyramidal or fusiform soma, and multiple neurite outgrowths, as well as their position in the Z-plane on the astrocyte bed.

Figure 1 shows a representative set of calcium imaging traces showing the large increase in [Ca²⁺]_i in vehicle-treated control cultures following strain injury. Neurons treated with 10 nM or 100 nM E2 showed only small increases in peak [Ca²⁺]_i, that rapidly returned to baseline. The effects of 1 nM E2 on the rise in [Ca²⁺]_i were of much lower magnitude. Figure 2 summarizes the effects of strain injury and postinjury addition of vehicle or E2 to neuronal/glial cultures on peak levels of neuronal [Ca²⁺]_i. Addition of E2 did not significantly alter levels of [Ca²⁺]_i in the absence of injury at any concentration ($F_{3,12} = 1.59$, $P > 0.24$), although there was an apparent nonsignificant decrease in [Ca²⁺]_i following treatment with

100 nM E2. Following strain injury, there was a large and immediate increase in levels of $[Ca^{2+}]_i$ in vehicle-treated neurons that was sustained for the duration of the calcium imaging period (i.e., 10 min postinjury). Addition of E2 at 10 nM and 100 nM, but not at 1 nM, immediately after injury significantly ($F_{3,12} = 3.60$, $P < 0.05$) decreased peak $[Ca^{2+}]_i$ levels after strain injury when compared to vehicle treatment ($P < 0.05$).

As shown in Figure 3A, after mechanical strain injury, the percentage of CFDA-positive viable neurons was reduced to approximately 66% of the total number of neurons counted in vehicle-treated cultures (i.e., CFDA + PI stained neurons). In contrast, approximately 90% of the neurons were CFDA-positive in wells treated with E2 immediate postinjury.

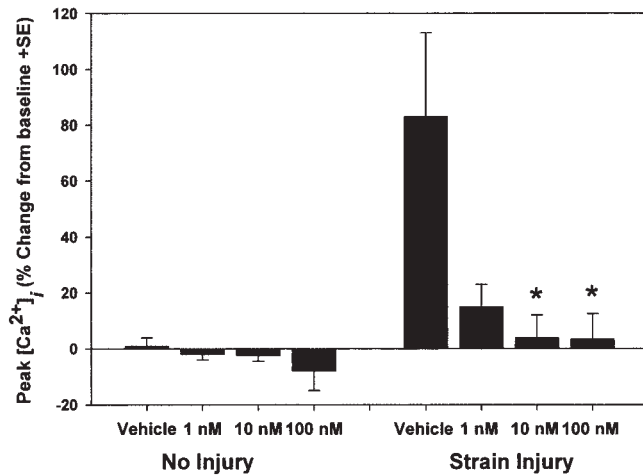


Fig. 2. Peak intracellular neuronal calcium levels ($[Ca^{2+}]_i$) following mechanical strain injury (% change from baseline). Injury produced a large increase in $[Ca^{2+}]_i$ compared to preinjury baseline. Exposure to 10 or 100 nM E2 immediately after injury significantly reduced peak $[Ca^{2+}]_i$ compared to vehicle-treated controls, while effects of 1 nM were not significant. * $P < 0.05$ vs. vehicle.

This difference between E2 treated and vehicle-treated neurons was statistically significant ($F_{3,8} = 9.9$, $P < 0.005$) at each of the three concentrations tested ($P < 0.05$ each). Figure 3B presents the complementary data for the percentage of PI-stained injured neurons, where it can be seen that approximately 36% of injured neurons in the vehicle-treated group showed PI staining. Treatment with E2 significantly reduced the percentage of injured neurons stained with PI to approximately 10% or less compared to the vehicle control for each of the three concentrations of E2 tested.

Neuroprotection was found with exposure of mechanically injured neurons immediately after injury to E2 concentrations as low as 1 nM. This is consistent with previous reports that even sub-nM concentrations of E2 can be neuroprotective against glutamate excitotoxicity in hippocampal cultures (Ba et al., 2004; Wu et al., 2005). Similar neuroprotective effects of E2 have been reported in several previous studies on models of chemical and ischemic induced neuronal death (Chen et al., 1998; Culmsee et al., 1999; Harms et al., 2001; Huang et al., 2004; Wang et al., 1999; Wise et al., 2000). The fact that 1 nM E2 was neuroprotective, but was only partially effective in reducing the rise in $[Ca^{2+}]_i$ after cell injury indicates that a threshold may exist for calcium toxicity as previously suggested (Yu et al., 2001).

The mechanisms by which estrogen exerts this protective activity are unclear (Green and Simpkins, 2000; Lee and McEwen, 2001), but may be due to activation of nuclear estrogen receptors, modulation of apoptotic cascades (Stoltzner et al., 2001; Wise et al., 2000), antioxidant activity (Culmsee et al., 1999; Green and Simpkins, 2000; McEwen, 2001; Moor et al., 2004), and effects on calcium homeostasis (Ba et al., 2004; Brinton, 2001). Neuroprotection by E2 against ischemic injury (Alkayed et al., 2001; Wise et al., 2000) and against KA-induced injury may be

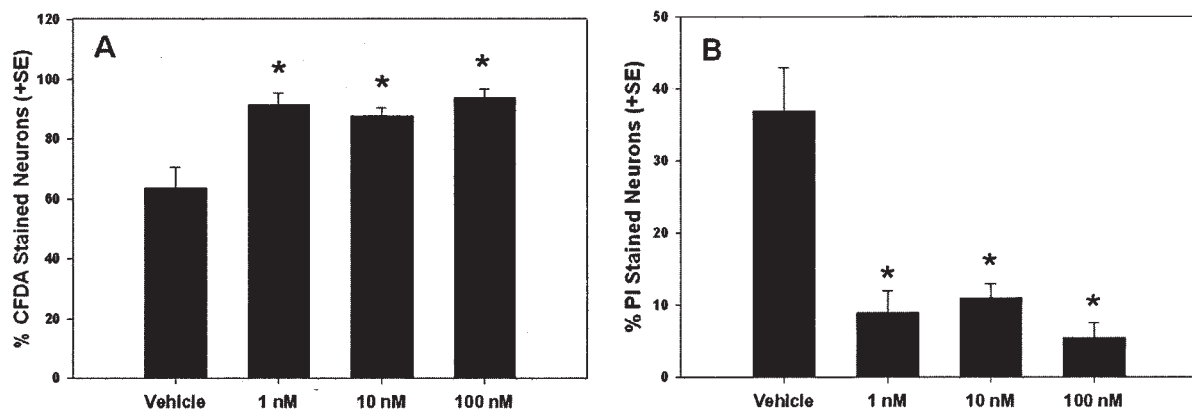


Fig. 3. Percentages (\pm SEM) of viable CFDA-stained neurons (A) and damaged PI-stained neurons (B) following mechanical strain injury. * $P < 0.05$ vs. vehicle.

genomically mediated by activation of estrogen receptors (ER), which can increase expression of the antiapoptotic protein Bcl-2 (Alkayed et al., 2001; Wise et al., 2000). Recently 100 pM E2 was found to be neuroprotective against β -amyloid protein cytotoxicity in SK-N-SH cells (Ba et al., 2004), possibly by altering the expression of α 1C and α 1D subunits of L-type voltage gated calcium channels (VGCC).

Exposure of striatal neurons to E2 has also been shown to reduce, within seconds, calcium currents carried by L-type, and less strongly by N and P-type VGCCs (Mermelstein et al., 1996). The effects appear to be mediated via G-protein activation, suggesting direct action on a cell surface ion channel or receptor. Similarly, E2 has been shown to inhibit the rise of [Ca²⁺]_i in rat hippocampal neurons induced by NMDA or K⁺ depolarization, primarily via modulation of L-type VGCCs (Kurata et al., 2001). A 5 min exposure of dorsal root ganglia neurons to 100 nM E2 was shown to block the rise in [Ca²⁺]_i evoked by ATP activation of P2X receptors, and this effect was also via inhibition of L-type VGCCs (Chaban et al., 2003). Moreover, E2 was shown to attenuate glutamate-induced calcium overload in rat hippocampal neurons via a nontranscriptional cell surface membrane receptor mechanism (Huang et al., 2004). E2 has also been shown to reduce cell death in an in vitro hippocampal slice model of global ischemia in gerbils by slowing the rate of increase in [Ca²⁺]_i and lowering the peak level of [Ca²⁺]_i following ischemia. E2 modulated the ischemia-induced [Ca²⁺]_i rise by inhibiting the influx of Ca²⁺ from the extracellular space, as well as by inhibiting the release of Ca²⁺ from intracellular Ca²⁺ stores (Chen et al., 1998). Considered together, these results demonstrate effects of E2 on nontranscriptional (i.e., nongenomic) calcium signaling pathways, and are consistent with the ability of nM levels of E2 to rapidly inhibit the increase in [Ca²⁺]_i following strain injury to rat cortical neurons. A reduction in [Ca²⁺]_i after cell injury may subsequently be neuroprotective by reducing the levels of necrosis and apoptosis caused by calcium overload (Lee et al., 2004; Weber, 2004).

In conclusion, this study demonstrates E2 has neuroprotective effects against neuronal damage by mechanical strain injury in mixed neuronal/glial primary cultures. One possible cellular mechanism of the neuroprotective actions of E2 is via reduction in the rise in [Ca²⁺]_i that occurs following mechanical injury. The acute effects of postinjury treatment with E2 on [Ca²⁺]_i were immediate, suggesting a nongenomic effect on modulation of [Ca²⁺]_i after mechanical cell injury in an in vitro model of brain trauma.

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