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Neuronal necrosis inhibition by insulin through protein kinase C-activation

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Running title: PKC-mediated neuronal necrosis inhibition by insulin

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Abbreviations: PI, propidium iodide; TEM, transmission electron microscopy; BDNF, brain-derived neurotrophic factor; IGF-I, insulin-like growth factor-I; bFGF, basic fibroblast growth factor; NGF, nerve growth factor; PKC, protein kinase C; PI3-K, phosphatidyl inositol-3-OH-kinase; MAPKK, mitogen-activated protein kinase kinase; ERK, extracellular signal-activated protein kinase; PLC, phospholipase C; Ara-C, cytosine β-D-arabinofranoside; CNS, central nervous system.

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ABSTRACT

In the serum-free culture of rat embryonic neurons, most of neurons rapidly died by necrosis, which was revealed by propidium iodide (PI)-positive staining as early as 3 h after the start of culture, and by marked membrane disruption and mitochondrial swelling in transmission electron microscopic (TEM) analysis. However, neither nuclear condensation/fragmentation stained with Hoechst 33342 nor activated caspase-3-like immunoreactivity was observed. In the serum-deprived culture, on the other hand, neurons showed apoptotic features such as caspase-3 activation and nuclear damages in TEM analysis. Insulin at relatively higher concentrations up to 100 μg/ml ameliorated the rapid decrease in survival activity measured with WST-8 assay and PI-staining in the serum-free culture, despite that brain-derived neurotrophic factor (BDNF) and insulin-like growth factor-I (IGF-I) had no survival effect even at concentrations up to 100 μg/ml. Insulin-induced survival effects were abolished by protein kinase C (PKC) inhibitor, calphostin C, but not by phosphatidyl inositol-3-OH-kinase (PI3-K) inhibitor, wortmannin, or mitogen-activated protein kinase kinase (MAPKK) inhibitors, PD98059 or U0126. Insulin significantly stimulated the PKC activity in cell lysates and suppressed the mitochondrial swelling and membrane disruption in TEM analysis in a calphostin C-reversible manner. All these findings suggest that insulin inhibited the neuronal necrosis resistant to known neurotrophic factors under the serum-free culture through PKC mechanisms.

We have previously found that cortical neurons showed a cell density-dependent survival in the serum-free culture without supplements (Fujita et al., 2001). Further characterization of the cell death under such condition revealed that neurons in the low-density culture die by necrosis, while those in the high-density culture by apoptosis. The former necrotic cell death, however, was resistant to various neurotrophic factors, such as BDNF, basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) (Hamabe et al., 2000; Fujita et al., 2001).

Necrosis as well as apoptosis has been identified as major modes of cell death within the central nervous system (CNS), and both cell death modes contribute to cell loss and related functional deficits after acute focal brain ischemia (Colicos and Dash, 1996; Fox et al., 1998; Dirnagl et al., 1999). Following focal brain ischemia/reperfusion, neuronal cells in the ischemic core die by necrosis, which are associated with inflammatory reactions and usually impacts large group of adjacent cells, followed by apoptosis in the penumbra. Compared with apoptosis, the molecular events in necrosis are rather undefined. The inhibition or targeting deletion of several proapoptotic molecules, such as Bax or caspases, however, did not completely rescue the retinal damage or ischemic brain injury (Hara et al., 1997; Friedlander et al., 1997; Mosinger et al., 1998). The inhibition of initial necrosis steps could be an important issue for complete rescue from brain injury.

Insulin as well as other growth-promoting factors, such as putrescine, transferrin, selenium and progesterone has been widely used for serum-free culture of various kinds of cells including neurons. Previous studies revealed that only insulin is enough to keep neurons survived for a longer period, but the concentration required for

this purpose is much higher than that used for conventional growth-promotion of cells in the serum-deprivation (Snyder and Kim, 1980; Huck, 1983; Skaper et al., 1984; Salamanca and Mathers, 1987). However, little is known of the mechanisms underlying this survival activity of insulin for serum-free culture. In the course of characterization, we found insulin inhibits the necrosis of cortical neurons in the serum-free and low-density culture. Here we attempted to characterize the mechanisms underlying this action.

Materials and Methods

Cell culture. Primary culture of the cerebral cortex from 17 d of embryonic rats was performed according to the previously reported protocol (Sasaki et al., 1998; Hamabe et al., 2000; Fujita et al., 2001). In brief, cortical tissues were minced into pieces in sterile PBS and dissociated with 0.25% trypsin (Life Technologies, NY, USA) and 0.01% DNase I (Sigma-Aldrich, St. Lois, MO) in PBS (pH 7.4) for 12 min at 37°C. The reaction was terminated by adding 0.25% soybean trypsin inhibitor (Sigma-Aldrich, St. Lois, MO). For serum-free culture, the cell suspension was centrifuged at 1,000 x g for 5 min, and the pellet was resuspended in a serum-free D/F medium (1:1 Dulbecco's modified Eagle's medium / Ham's F-12 medium). Dissociated neurons were seeded at a density of 1 x 10^5 cells/cm^2 onto 96-wells plate for WST-8 assay, 24-wells plate for trypan blue exclusion test, 6-wells plate for ATP measurements, and 8-wells of Lab-Tek™ chamber for morphological assessment, which had been all coated with poly-DL-ornithine (Sigma-Aldrich, St. Lois, MO), and cultured at 37˚C in 5%-CO_2 atmosphere for indicated periods. For serum-deprived culture, the pellet was resuspended in a serum-containing D/F medium [1:1 Dulbecco's modified Eagle's medium / Ham's F-12 medium (Life Technologies, NY, USA) plus 5% FBS, 5% HS and 1% 2-Mercaptoethanol (Life Technologies, NY, USA)]. Dissociated neurons were seeded at a density of 1 x 10^5 cells/cm^2 onto 96-wells plate for WST-8 assay, 6-wells plate for ATP measurements, and 8-wells of Lab-Tek™ chamber for morphological assessment, which had been all coated with poly-DL-ornithine, and cultured at 37˚C in 5%-CO_2 atmosphere. Cytosine β-D-arabinofranoside (Ara-C) (Sigma-Aldrich, St. Lois, MO) at 1 μM was added to the

culture 24 h after seeding, followed by another 48 h culture. The culture medium was then replaced by fresh D/F medium deficient of serum, and further cultured at 37˚C in 5%-CO_2 atmosphere for indicated periods.

WST-8 assay. The viability of cells was assessed by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) reduction assay kit (Dojin Lab. Tokyo, Japan) according to the manufacturer's instructions. WST-8 was added to the culture and incubated for 3 h at 37˚C prior to the colorimetry. Percentage of WST-8 reduction activity was represented as the ratio of activity at different time points to that in the beginning of culture.

Trypan blue exclusion test. To assess the neuronal cell death, equal volume of 0.4% trypan blue (Wako Pure Chemicals, Richmond, VA) in saline was added to the culture, followed by twice wash with ice-cold PBS and fixation with 4% paraformaldehyde (PFA) in PBS for 30 min at 25˚C. Results were represented by the percentage of stained cells among the100 to 500 cells each in five separate fields.

Hoechst 33342-staining and PI-staining assay. Apoptotic or necrotic cell-death was characterized by use of Hoechst 33342 (Molecular Probes. Eugene, OR) and propidium-iodide (PI; Sigma-Aldrich. St. Lois, MO) -double staining. Twelve hour after seeding, cells were stained with 10 μg/ml Hoechst 33342 and 10 μg/ml PI for 30 min at 37°C. After washing with PBS twice, cells were fixed with 4% PFA in PBS for 30 min at 25°C. Cells were mounted with Aqua Poly/Mount (Polysciences, Inc., Warrington, PA), and imaged on an AxioVision 3.0 (Carl Zeiss; Tokyo, Japan) attached to a fluorescence microscopy (BX50; OLYMPUS; Tokyo, Japan). The nuclei stained

with Hoechst 33342 which were markedly bright and small or divided into several homologous chromatin clumps were defined to be condensed or fragmented nuclei, respectively. The percentage of condensed or fragmented nuclei or PI-positive nuclei were expressed as the ratio of total nuclei of 100 to 500 cells each in five separate fields.

Immunocytochemistry of active caspase-3. Cortical cells on 8-wells Lab-Tek™ chamber were fixed with 4% PFA in PBS for 30 min at 25°C, followed by permeabilization using 50% methanol and 100% methanol each for 5 min at 25°C. Cells were then rinsed twice with PBS and preincubated for 1 h at 25°C in blocking buffer (2% bovine serum albumin, 0.1% Triton X-100 in PBS). Cells were incubated overnight at 4°C in blocking buffer containing rabbit anti-cleaved caspase-3 antibody (1:50; Cell Signaling, Tokyo, Japan), rinsed in PBS and incubated for 2 h at 25˚C with FITC-conjugated anti-rabbit immunoglobulin (IgG) (1:200; Cappel, Aurora). Immunolabeled cells were mounted with Aqua Poly/Mount (Polysciences, Inc., Warrington, PA), and imaged on an AxioVision 3.0 (Carl Zeiss; Tokyo, Japan) attached to a fluorescence microscopy (BX50; OLYMPUS; Tokyo, Japan).

Transmission electron microscopy. Cultured cortical neurons were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at 25˚C. The fixed cortical neurons were postfixed with 1% osmium tetroxide for 1 h at 25˚C, dehydrated in graded alcohol series, and embedded in Epon812. Ultrathin sections (80 nm thick) were cut with an Ultracut S (Leica, Austria), and then stained with uranyl acetate and lead citrate for 30 and 5 min, respectively. The stained sections were observed under an electron microscope (JEM-1210; JEOL, Tokyo, Japan).

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Measurement of intracellular ATP levels. Intracellular ATP was extracted from cells in the exponential phase of growth and measured by the luciferin/luciferase method, using ATP-Determination Kit (Molecular Probes. Eugene, OR) (Ha and Snyder, 1999). Cells were seeded onto 6-wells plate at the density of 1 x 10^5 cells/cm^2 and cultured for indicated periods. The entire cells including any floating cells were subjected to the assay. The reaction buffer (200 μl) containing 0.5 μM luciferin, 1.25 μg/ml luciferase and 1 mM DL-dithiothreitol (DTT) was mixed with cell lysates (20 μl). Luminescence was analyzed using LUMAT LB 9507 from EG&G berthold (Bad Wildbad, Germany).

2-Deoxy-D- [^{3}H] glucose uptake. 2-Deoxy-D- [^{3}H] glucose ([^{3}H]-2-DG; NEN, Boston, MA) uptake was measured using modified Koivisto's methods (Koivisto et al., 1991). Briefly, [^{3}H]-2-DG (1 μCi/well, 10 nM) was added to the 6-wells plate in the beginning of culture and incubated for 2 h at 37˚C in 5%-CO_2 atmosphere. Uptake of [^{3}H]-2-DG was terminated by a rapid removal of medium, followed by twice washes with ice-cold PBS. The cells were lysed in 100 μl of 0.5 M NaOH, followed by neutralization with 0.5 M HCl. The radioactivity of collected cell lysates was determined by a liquid-scintillation counter.

Measurement of PKC-activity. Cultured cells were harvested using a solution containing 0.3% β-mercaptoethanol, 5 mM EDTA, 10 mM EGTA, 50 mg/ml phenyl methyl sulfonyl fluoride, 10 mM benzamidine and 50 mM Tris-HCl (pH 7.5), homogenized on ice and added to equal volume of glycerol. PKC assay was performed using Protein Kinase C enzyme assay system (Amersham Pharmacia Biotech, Piscataway, NJ), according to the manufacturer's instruction with modifications.

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Briefly, samples [S] were incubated with the substrate peptide to be phosphorylated by PKC in 50 mM Tris-HCl (pH 7.5), 30 mM DTT and [γ-^{32}P]ATP in magnesium ATP buffer (1.2 mM ATP, 30 mM Hepes and 72 mM $MgCl_2$, pH 7.4) for 15 min at 37°C. Maximum PKC activation [Max] was obtained by further addition of 0.3 mg/ml La-phosphatidyl-L-serine, 24 μg/ml phorbol 12-myristate 13-acetate and 12 mM calcium acetate in 50 mM Tris-HCl (pH 7.5). Background activation [B] was obtained by omitting substrate peptide from [S] reaction mixture. After addition of stop solution, the reaction mixture was transferred onto peptide binding paper. These papers were washed twice with 5%-acetic acid and the incorporation of ^{32}P into peptide was determined by a liquid scintillation counter. PKC activation was calculated by the following equation; PKC activation (%) = ([S]-[B])/([Max]-[B]) x 100.

Western blot analysis. Cells were lysed in SDS-sample buffer (50mM Tris-HCl, pH6.8, 2% SDS and 10% glycerol) immediately after the stimulation with or without insulin or BDNF. Cell lysates were boiled and reduced with 10 mM of DTT. Samples and molecular weight standards (Invitrogen™, USA) were electrophoresed in 10% of SDS-PAGE acrylamide gels and transferred onto PVDF membranes (MILLIPORE, Tokyo, Japan). The membranes were blocked for 2 h at 25°C with 5% skimmed milk in TBS containing 0.1% Tween 20 for Akt, ERK and phosphorylated ERK1/2 and with 1% BSA in TBS containing 0.05% Tween 20 for phosphorylated Akt. Proteins were detected with rabbit anti-phospho-Akt (1:200; Promega, Tokyo, Japan), rabbit anti-phospho-ERK1/2 (1:200; New England Biolabs Inc., Tokyo, Japan), rabbit anti-Akt (1:200; New England Biolabs Inc., Tokyo, Japan) or rabbit anti-ERK1/2 antibody (1:200; New England Biolabs Inc., Tokyo, Japan) overnight at 4°C. The blots

were then incubated for 2 h at 25°C with anti-rabbit antibody conjugated with horseradish peroxidase. All visualization of immunoreactive bands was performed by the Light Capture™ (AE-6960/C/FC, ATTO, Tokyo, Japan) with an enhanced chemiluminescent substrate for the detection of horseradish peroxidase, Super Signaling Substrate (PIERCE, Rockford, IL). As this Light Capture™ can repeatedly expose at intervals of 1min and automatically superimpose these signals to obtain high contrast, we can detect enhanced signals with less background signals.

Statistical analysis. The data was analyzed using Student's *t*-test after multiple comparisons of ANOVA. The criterion of significance was set at $p<0.05$. All results are expressed as the mean ± S.E.M.

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Results

Distinct characteristics of neuronal death modes caused in the serum-free and serum-deprived culture. Cortical neurons of embryonic (E17) rats were seeded at a density of 1 x 10^5 cells / cm^2. The population of bromodeoxyuridine (BrdU)-positive cells was below 5%, indicating that the cells were mostly neurons (data not shown). Although neurons looked healthy in the serum-containing culture [serum (+)], and have short and long neurites at 12 h and 3 days, respectively after the start of culture (Fig. 1Aa, c), neurons in the serum-free culture [serum (-)] lost normal thickness and neurites 12 h after the start of culture (Fig. 1Ab). On the other hand, in the serum-deprived culture, neurons looked slightly condensed and lost neurites 12 h after serum-deprivation (Fig. 1Ad). PI was used for detection of the plasma membrane damage (Bal-Price and Brown, 2000). Most of neurons were PI-positive in the serum-free culture (68.8%; Fig. 1Af and B), while little in the serum-containing (1.7%; Fig. 1Ae and B, 2.3%; Fig. 1Ag and B) and serum-deprived culture (9.4%; Fig. 1Ah and B). Hoechst 33342 was used as an apoptosis marker, which detects apoptotic nuclei with condensed and/or fragmented DNA. Significant intense Hoechst 33342-stained neurons were observed in serum-deprived culture (75.2%; Fig. 1Al and B), but not in serum-containing (4.9%; Fig. 1 Ai and B, 5.6%; Fig. 1Ak and B) and serum-free culture (10.0%; Fig. 1Aj and B). The conversion of procaspase-3 to active caspase-3 is generally accepted to be the most reliable indicator of apoptosis (Green et al., 1998). As shown in Fig. 1Ap, significant active caspase-3 signal was observed in serum-deprived culture (57.9%; Fig. 1Ap, and B), but not in serum-containing (1.4%; Fig. 1Am and B, 3.3%; Fig. 1Ao and B) and serum-free culture (6.6%; Fig. 1An and

B). There were no significant differences on PI-, Hoechst 33342- and active caspase-3-staining between neurons in serum-containing culture at 12 h after the start of culture and neurons in the same condition at 3 days after the start of culture (Fig. 1A).

As shown in Fig. 2A, most neurons in serum-free culture rapidly became PI-positive by 3 h after the start of the culture. Density of PI-positive neurons reached plateau at 12 - 24 h. On the other hand, approximately 10% of Hoechst 33342-positive neurons were found throughout the time course up to 24 h (Fig. 2A). In the serum-deprived culture, the population of significant Hoechst 33342-positive was gradually increased from 6 h, and reached to 93.6% at 36 h. PI-positive neurons were as low as 1.9% to 5.2% throughout the time course up to 36 h (Fig. 2B).

TEM analysis of neurons in the serum-free and serum-deprived culture. In the TEM analysis, the neuron 12 h after the start of serum-containing culture looked intact in the nucleus and mitochondria (Fig. 3Aa, b), the neuron in serum-free culture, on the other hand, had disrupted plasma membranes, grossly swollen mitochondria which had no clear cristae structures (Fig. 3Bb), and decreased electron density in the cytoplasm (Fig. 3Ba, b), as previously reported (Fujita and Ueda, 2003). Good contrast was observed with the neuron in the serum-deprived culture at 12 h after deprivation, which had intact mitochondria without swollen (Fig. 3Cb) but marked nuclear fragmentation with some condensed parts (Fig. 3Ca).

Insulin-induced neuroprotection in the serum-free culture. The viability of neurons measured with WST-8 assay, a marker of mitochondrial reduction activity, decreased to 28.2 % of the control (0 time) at 12 h after the start of culture in

serum-free condition. When insulin at concentrations of 0.1 to 100 μg/ml was added to the culture in the beginning of culture, the survival activity was concentration-dependently increased (Fig. 4A). Most neurons with insulin at 100 μg/ml were appeared healthy and had almost normal thick under the phase contrast microscope (data not shown). However, neither BDNF nor IGF-I up to 100 μg/ml showed significant increase (Fig. 4A). These neurotrophic factors (insulin, BDNF and IGF-I) however, protected neuronal death in the serum-deprived culture at as low as 100 ng/ml (data not shown) as elsewhere reported (Carlson et al., 1999; Hetman et al., 1999). In the trypan blue exclusion test, another indicator of survival activity, significant survival activity of insulin and recombinant insulin at 100 μg/ml was also observed (Fig. 4B).

Insulin-induced neuroprotection was also observed when the PI-staining was evaluated. As mentioned above, PI-staining was used for evaluation of membrane disruption, a death (necrosis) marker. Insulin and recombinant insulin at a concentration of 100 μg/ml significantly inhibited the PI-staining (Fig. 4Ca-c and D). The population of PI-positive cells in the serum-free culture was 70.8% of 500-600 cells assessed at 12 h after seeding (Fig. 4D). As shown in Fig. 4D, insulin (100 μg/ml) reduced the PI-staining by approximately 50%. However, insulin and recombinant insulin had no effect on Hoechst 33342-staining (Fig. 4Cd-f and D).

Lack of effect of insulin on glucose uptake and cellular ATP levels. In order to examine whether the potential cellular mechanisms related to energy failure are involved in the insulin-induced neuroprotection in the serum-free culture, [^{3}H]-2-DG (2-DG) uptake and cellular ATP levels were assessed (Fig. 5A and B). In the serum-free

culture, 2-DG uptake within 2 h after the start of culture was decreased to as low as 33.5% of the serum-containing culture. Insulin did not affect 2-DG uptake at concentrations up to 100 μg/ml (Fig. 5A). On the other hand, cellular ATP levels in the serum-free culture also rapidly decreased, while there was no decrease in ATP levels in the presence of serum, as shown in Fig. 5B. Insulin at 100 μg/ml did not affect the cellular ATP levels (Fig. 5B).

Insulin-induced neuroprotection in the serum-free culture through PKC mechanisms. The insulin-induced survival activity measured with WST-8 assay was significantly inhibited by PKC inhibitors, calphostin C or GF109203X at 1 μM, tyrosine kinase inhibitors, herbimycin A (10 μM) or genistein (1 μM) and phospholipase C (PLC) inhibitor U73122 (1 μM), 12 h after the start of culture. However, PI3-K inhibitor, wortmannin (1 μM), MAPKK inhibitors, PD98059 or U0126 at 1 μM and an inactive analog of U73122, U73343 (1 μM), failed to inhibit the insulin-induced neuroprotection (Fig. 6A). These inhibitors had no effect on the basal survival activity of neurons in the serum-free culture (data not shown). As shown in Fig. 6Ba, insulin at as high as 100 μg/ml significantly increased the PKC activity by 13% of maximum activation induced by excess amounts of phorbol 12-myristate 13-acetate. This effect was also significantly inhibited by 1 μM of calphostin C, 3 μM of herbimycin A and 1 μM of U73122 at 2 h after the start of culture, while 1 μM of U73343 and 1 μM of wortmannin showed no effects (Fig. 6Bb). Similar results were also observed 12 h after the start of culture (data not shown). As shown in Fig. 6C, insulin-induced decrease in the population of PI-positive neurons was also blocked by 1 μM of calphostin C, 3 μM of herbimycin A and 1 μM of U73122, but not affected by

1μM of U73343 and 1μM of wortmannin.

Effects of insulin and BDNF on the phosphorylation of Akt and ERK1/2. As shown in Fig. 7A, the phosphorylation of Akt but not ERK1/2 was enhanced by insulin or BDNF at 100 μg/ml in the serum-free culture. In the serum-deprived culture, on the other hand, the application of insulin or BDNF at as low as 100 ng/ml induced activation of both of Akt and ERK1/2 (Fig. 7B), as elsewhere reported (Ryu et al., 1999; Hetman et al., 1999).

PKC-involvement in the insulin-induced inhibition of necrosis in TEM analysis. The mitochondrial swelling and loss of electron density in the cytoplasm due to membrane disruption in the serum-free culture as shown in Fig. 3, were completely abolished by the treatment with insulin (100 μg/ml), in a calphostin C (1 μM) – reversible manner (Fig. 8Aa, b and Ba, b). As shown in Fig. 8Ca and Cb, wortmannin (1μM) did not affect the insulin-induced necrosis-inhibition.

Discussion

Necrosis is characterized as a more passive process due to energy failure, which leads to loss of ionic homeostasis, membrane disruption and mitochondrial swelling. Compared to the studies of apoptosis, little is known on the mechanisms for necrosis, which may give us the strategies to develop anti-necrotic drugs. In the previous studies, we developed a new approach to cause neuronal necrosis by performing serum-free culture of cerebral cortex without any supplements (Fujita et al., 2001). We have proposed that necrosis might be caused by rapid decreases in glucose uptake and cellular ATP levels (Fujita and Ueda, 2003), which might be related to the disappearance of glucose transporters (GLUT1 and GLUT4) from plasma membranes (Fujita and Ueda, in preparation). From these findings, we initiated the experiments to examine whether the neuronal necrosis might be protected by insulin, which translocates glucose transporters to the plasma membrane of peripheral cells such as muscle cells or adipocytes (Pessin and Saltiel, 2000; Saltiel, 2001; Bose et al., 2002). As expected, insulin showed marked neuroprotective actions (Fig. 4). However, this peptide did not ameliorate the rapid decrease in glucose uptake and cellular ATP levels (Fig. 5A and B). These findings suggest that the neuroprotective actions of insulin in this culture system are supposed to use different cellular mechanisms.

In the present study, we characterized necrosis by use of selective staining with PI or Hoechst 33342 as well as TEM analysis. In the serum-free culture, most neurons are PI-positive, but very few are intensely stained with Hoechst 33342 or anti-active caspase-3 antibody. Good contrast is observed with neurons in the serum-deprived culture, which are intensely stained with Hoechst 33342. As previously

reported (Raff, 1998; Hetman et al., 2000; Barber et al., 2001), serum-deprivation is one of pro-apoptotic stress. Differentiated cells including neurons in the CNS require the presence of survival factors to support the intrinsic cell death machinery and thereby avoid apoptosis. From the time course study, the PI-staining was mostly completed at 3 h after the start of serum-free culture, while the Hoechst 33342-staining showing apoptosis was gradually increased as the time till 36 h after serum-deprivation (Fig. 2B). Thus, it is evident that PI- and Hoechst 33342-staining are good tools to characterize necrosis and apoptosis, respectively. The validity of this view was clearly proved by TEM analysis. Neurons in the serum-free culture showed loss of electron density in the plasma due to membrane disruption and mitochondrial swelling (Fig. 3Ba, b). The former phenomena may link to the intracellular penetration of hydrophilic PI (Bal-Price and Brown, 2000). On the other hand, neurons in the serum-deprived culture showed intact mitochondria and intact plasma membrane, but nuclear fragmentation (Fig. 3Ca, b).

As shown in Fig. 4A, insulin increased the survival activity in a concentration-dependent manner, with the effective concentrations are as high as 10 and 100 μg/ml, which is approximately 1,000-times higher than the concentrations required or anti-apoptotic actions in different culture systems (Carlson et al., 1999; Hetman et al., 1999; Barber et al., 2001). However, it is unlikely that insulin exerts such anti-necrotic actions through IGF-I receptor, which has low-affinity to insulin, since IGF-I at as high as 100 μg/ml had no significant effect (Fig. 4A). It is also unlikely that small amounts of unidentified molecules contained in the purified insulin (derived from bovine) are in charge of this action, since the recombinant peptide

showed neuroprotective and anti-necrotic actions (Fig. 4B, C and D).

It is well known that various insulin actions are closely related to the cellular protein kinase activities. Recent reports suggest that glucose uptake by insulin is closely related to the phosphorylation of glucose transporters by PKC (Pessin and Saltiel, 2000, Watson and Pessin, 2001), while its cell growth or survival activity against apoptosis is to PI3-K, Akt or ERK1/2 (Barber et al., 2001, Watson and Pessin, 2001). In the present study, however, the neuroprotective activity of insulin was only inhibited by tyrosine kinase inhibitors, PKC inhibitors, PLC inhibitor, but not by PI3-K inhibitor and MAPKK inhibitors, which are known to block insulin-induced signaling related to anti-apoptotic actions (Gauthier et al., 2001; Johnson and Denton, 2003). As the insulin-induced PKC activation was blocked by herbimycin A and U73122, it is suggested that the neuroprotection is mediated by tyrosine kinase and PLC activation followed by PKC activation, which are also as known downstream mechanisms of insulin receptor (Slaaby et al., 2000; Lorenzo et al., 2002, Eichhorn et al., 2002). The involvement of tyrosine kinase, PLC and PKC mechanisms in the insulin actions were confirmed in the inhibition of PI-staining, as a necrosis marker (Fig. 6C). The lack of action by PI3-K and MAPKK inhibitors may be attributed to the fact that the neuronal death occurred in the serum-free culture is characterized to be necrosis resistant to known neurotrophic factors, such as BDNF or IGF-I (Fig. 4A). Although the reason of necessity of high concentration of insulin remains to be determined, it might be related to the unique findings that insulin-induced survival activity was selectively inhibited by PLC and PKC inhibitors, but not by inhibitors of PI3-K and MAPKK in the serum-free culture system. Indeed, we observed that high concentration of insulin was

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also required for the PKC activation in this system (Fig. 6Ba).

Calphostin C, a PKC inhibitor abolished the neuroprotective and anti-necrotic actions of insulin in WST-8 assay, PI-staining and TEM-analysis (Fig. 6A and C and Fig. 8). However, the molecular targets for PKC in relation to the anti-necrotic actions following insulin stimulation remain to be determined. Glucose transporters observed in muscle or adipocytes (Bose et al., 2002) are unlikely related, since insulin did not show significant changes in glucose uptake or in cellular ATP levels (Fig. 5A and B). The stimulation of Na^+-K^+ ATPase by insulin might be a candidate for the anti-necrotic mechanisms, since energy failure related to necrosis is coupled to the reduction of Na^+-K^+ ATPase activity, which leads to a loss of ionic homeostasis (Xiao et al., 2002), followed by mitochondrial swelling (Halestrap et al., 2002) and membrane disruption (Malis and Bonventre, 1988), and this pump is stimulated by PKC mechanisms (Chibalin et al., 2001; Sweeney et al., 2001). Molecular identification of PKC targets in relation to the insulin-induced anti-necrosis would be the next important subject.

In conclusion, we provided evidence that insulin protects neuronal necrosis resistant to known neurotrophic factors under serum-free starvation stress through PKC-activation.

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Legends for figures

Fig. 1. Distinct characteristics of neuronal death modes caused in the serum-free and serum-deprived culture. A (a-d): Phase-contrast and differential interference contrast (Nomarski) photographs of neurons cultured in the serum containing [serum (+)](a, c), serum-free [serum (-)] (b) and serum-deprived [serum-deprivation] (d) conditions. For the results with serum-deprivation, the culture medium was replaced by serum-free medium and cultured for 12 h. A (e-p): Triple-staining with PI, Hoechst 33342 and anti-active caspase-3 antibody under different conditions. A (e-h): The signal represents the PI fluorescence incorporated into nuclei. A (I-l): Intense signal represents the nuclear condensation. Arrowhead represents the nuclear fragmentation (i). A (m-p): The immunoreactive signal represents the activated caspase-3. B: Quantitative comparisons of stained cells with PI, Hoechst 33342 or anti-active caspase-3 antibody. Results (500-800 cells in each group) are expressed as the means ± S.E.M. from 3 independent experiments. $*p<0.05$ and $\#p<0.05$, compared with corresponding preparations in the serum-containing culture.

Fig. 2. Time course of separate cell death modes in serum-free and serum-deprived culture. Neurons were cultured under the serum (-) (A) or serum-deprived condition (B) for indicated periods, followed by staining with 10 μg/ml PI and 10 μg/ml Hoechst 33342 for 30 min and by fixation with 4% PFA. In these analyses, 500-800 cells in each group were counted. Results were reproduced in another set of experiments.

Fig. 3. TEM analysis of neurons under different culture conditions. Photographs with

low and high-magnification (upper and lower panels). A (a, b): Neuron in the serum (+) culture at 12 h after the start of culture. B (a, b): Neuron in serum (-) culture at 12 h after the start of culture. C (a, b): Neuron in serum-deprived culture at 12 h after deprivation. Arrowheads and arrows represent intact mitochondria and swollen mitochondria, respectively. 'N' represents nuclei.

Fig. 4. Insulin-induced neuroprotection on cortical neurons in serum-free culture. A: Survival activity measured with WST-8 assay at 12 h after the start of culture. Insulin, BDNF and IGF-I at indicated concentrations were added at the start of culture. B: Cell death was measured by trypan blue exclusion test 12 h after the start of serum-free culture. Purified bovine insulin (I) and recombinant human insulin (rI) were added to the culture medium at a concentration of 100 μg/ml immediately after the start of culture. 'C' represents control without insulin. C: Double staining with PI (Ca-c) and Hoechst 33342 (Cd-f) at 12 h after the start of serum-free culture. D: Quantitative comparison of PI and Hoechst 33342-staining. Results (500-800 cells in each group) are expressed as the means ± S.E.M. from 3 independent experiments. $*p<0.05$, compared with serum-free culture without insulin.

Fig. 5. Lack of insulin effects on glucose uptake and cellular ATP levels. A: Lack of insulin effects on [^{3}H]-2-DG uptake within 2 h from start of culture. Each data was indicated as the percentage of serum (+) culture. B: Time course of changes in cellular ATP levels in the serum (+), serum (-) and serum (-) with insulin (100 μg/ml) [serum (-) insulin (+)], respectively. Relative ATP levels were calculated as the percentages of

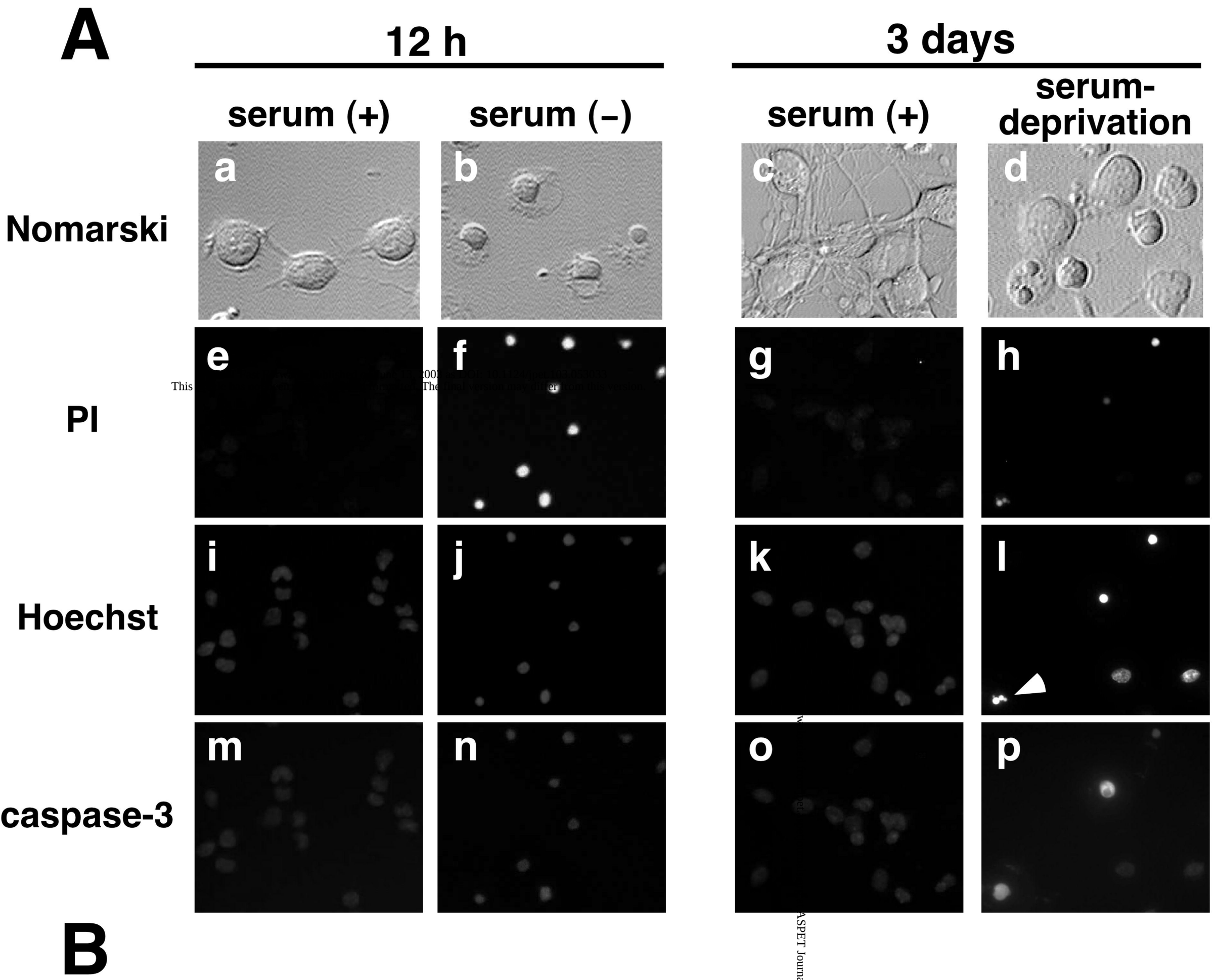
the level at 0 time. Results are expressed as the means ± S.E.M. from 3 independent experiments. ∗p<0.05, compared with serum (+) culture.

Fig. 6. PKC involvement in insulin-induced neuroprotection in the serum-free culture. A: Effects of various inhibitors on insulin (100 μg/ml)-induced survival activity. Cells were pretreated with vehicle (0.1% DMSO), PKC inhibitors (1 μM calphostin C and 1 μM GF109203X), tyrosine kinase inhibitors (10 μM herbimycin A and 1μM genistein), PLC inhibitor and its inactive analog (1 μM U73122 and 1μM U73343), PI3-K inhibitor (1 μM wortmannin) and MAPKK inhibitors (1 μM PD98059 and 1 μM U0126) for 0.5 h before the start of culture on ice, and cell survival was measured by WST-8 assay 12 h after the start of culture. B: (a) Concentration-dependent activation of PKC by insulin 2 h after the start of culture. (b) Effects of various inhibitors on insulin-induced PKC activation. Cells were pretreated with vehicle (0.1% DMSO), 1 μM calphostin C, 3 μM herbimycin A, 1 μM wortmannin, 1 μM U73122 and 1 μM U73343 for 0.5 h before the start of culture. PKC activity was measured with preparations harvested 2 h after seeding. The maximum PKC activation stimulated by 24 μg/ml phorbol 12-myristate 13-acetate was 2,500 cpm, as ^{32}P incorporation. C: Effects of various inhibitors on insulin-induced inhibition of PI-staining. The details of PI-staining and pretreatments of inhibitors are given in Fig. 4 and panel B in this figure. Results are expressed as the means ± S.E.M. from 3 independent experiments. ∗p<0.05, compared with vehicle-treated culture without insulin. #p<0.05, compared with vehicle-treated culture in the presence of 100 μg/ml insulin.

Fig. 7. Effects of insulin and BDNF on the phosphorylation of Akt or ERK1/2 in the serum-free culture. A: Insulin or BDNF at a concentration of 100 μg/ml was added to the serum-free cortical culture, and the cells were harvested by adding SDS-sample buffer 45 min after the start of culture for Western blot analysis. B: Serum-deprived culture of cortical neurons was performed as described under **Materials and Methods.** Insulin or BDNF at 100 μg/ml was added to the culture immediately after the replacement by serum-free medium. The cells were harvested 45 min after the serum-deprivation for Western blot analysis. Cell lysates (20-30 μg of proteins) were subjected to Western blotting with the anti-Akt, anti-ERK1/2, anti-phospho-Akt and anti-phospho-ERK1/2 antibody. All results were reproducible in another set of experiments.

Fig. 8. PKC-involvement in the insulin-induced inhibition of necrosis in TEM analysis. Neurons were cultured with insulin (100 μg/ml) in the absence (Aa, b) or presence of 1 μM calphostin C (Ba, b) and 1 μM of wortmannin (Ca, b) in the serum (-) condition for 12 h after the start of culture. Calphostin C and wortmannin was added to neurons on ice for 0.5 h before the start of culture. ‘N’ represents nuclei. White or black arrows represent intact or swollen mitochondria, respectively.

Figure 1



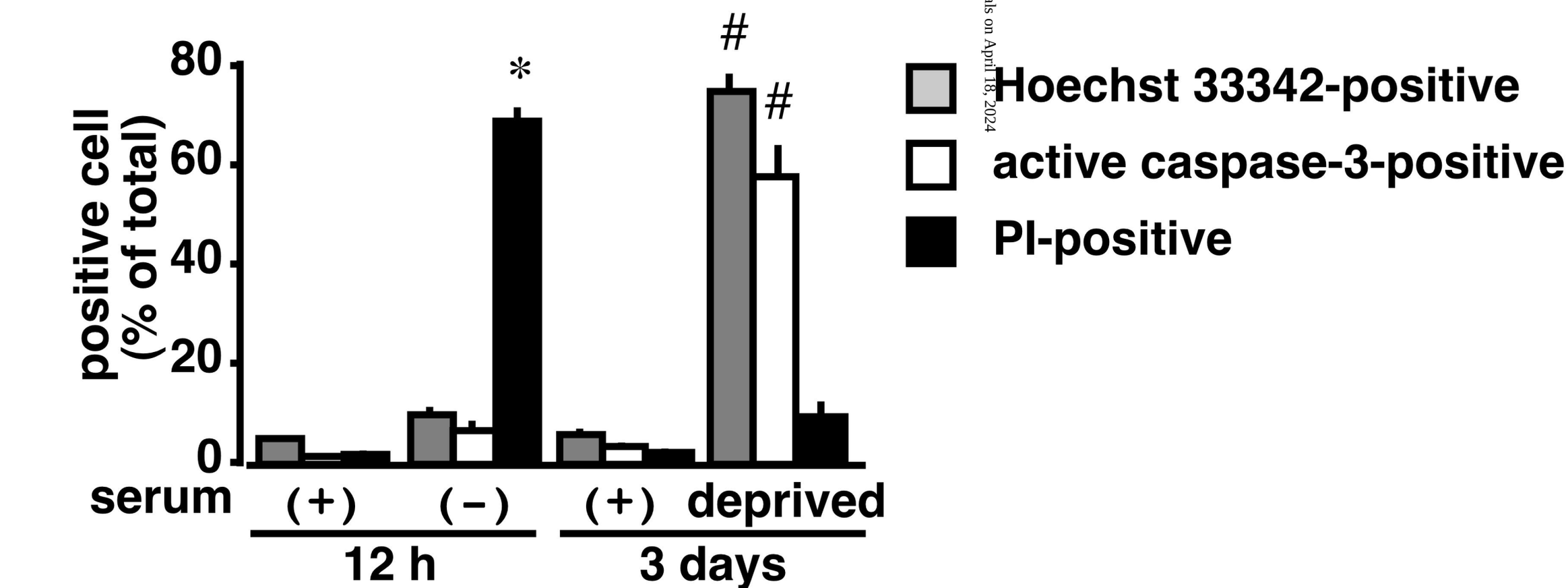


Figure 2

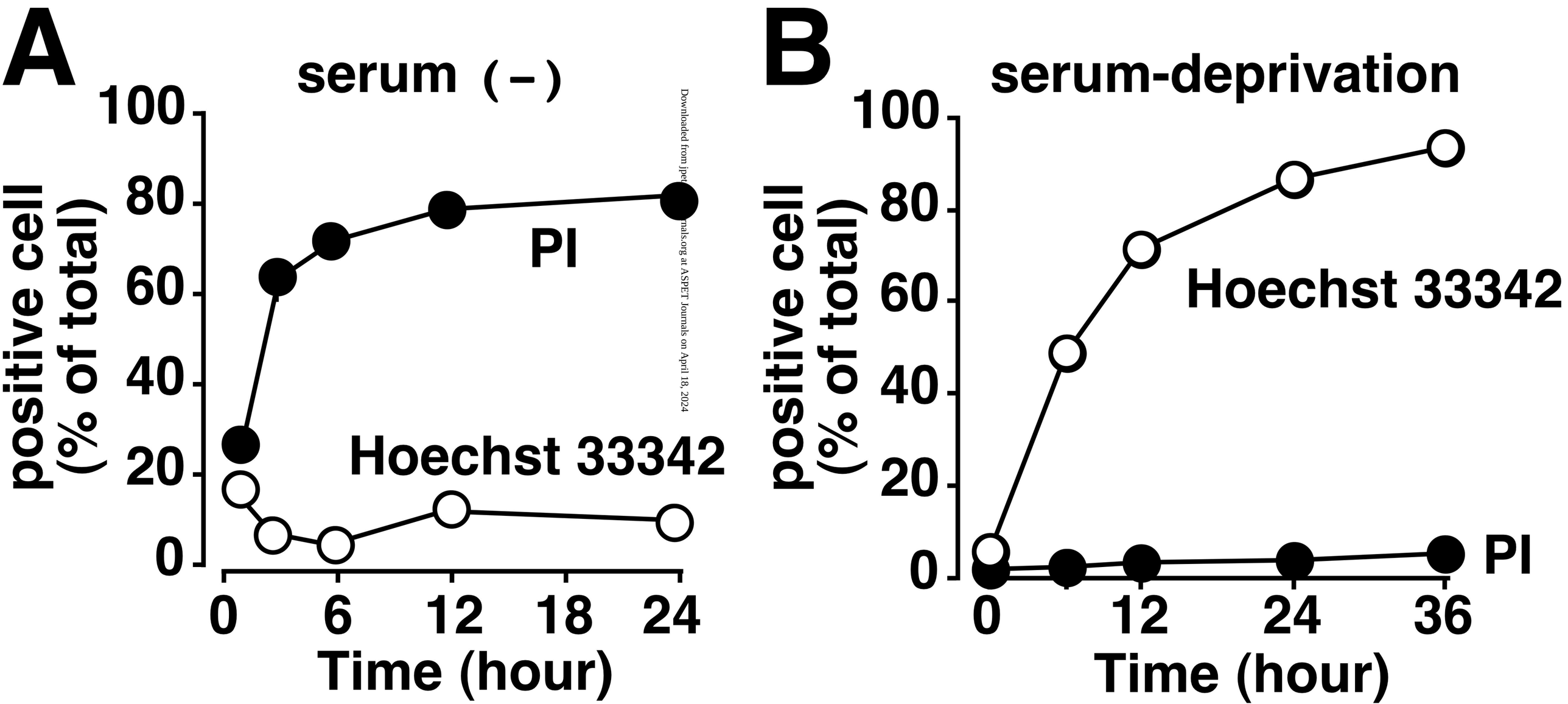


Figure 3

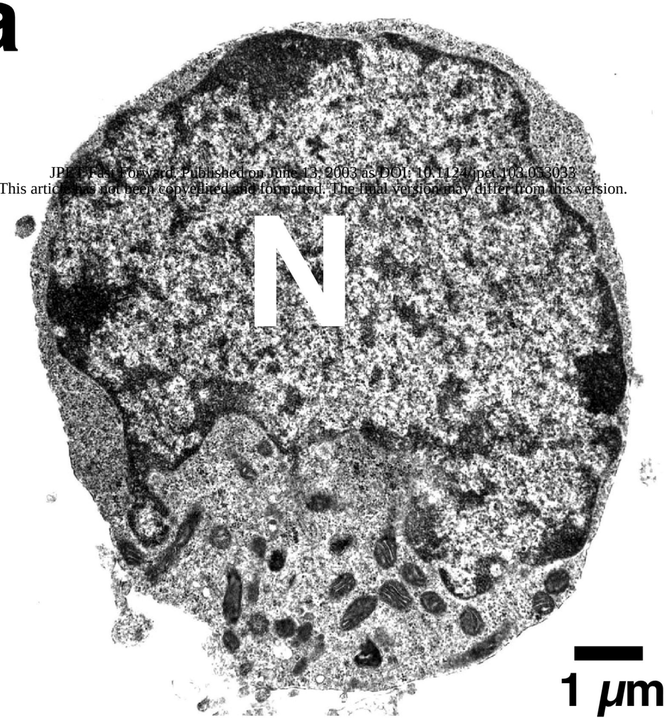
A serum (+)

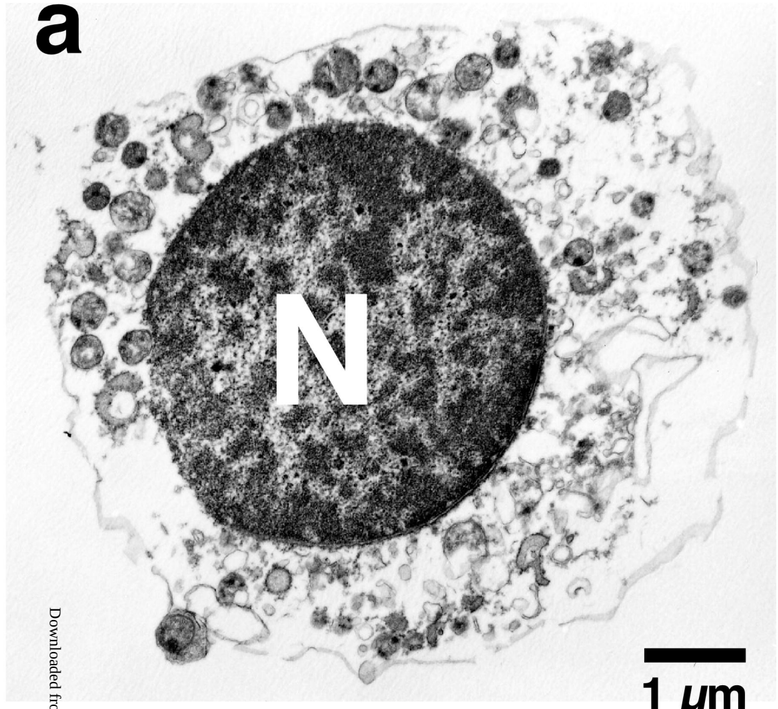
B serum (–)

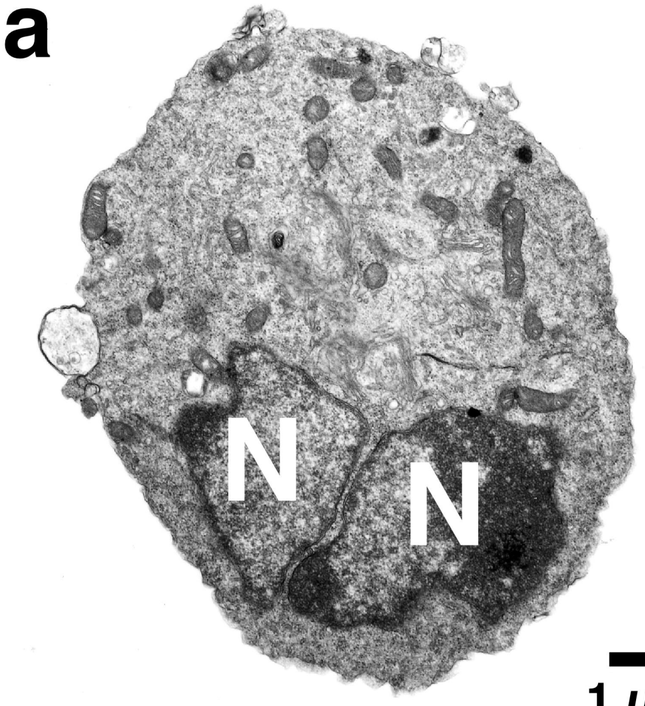
C serum-deprivation

LM

a

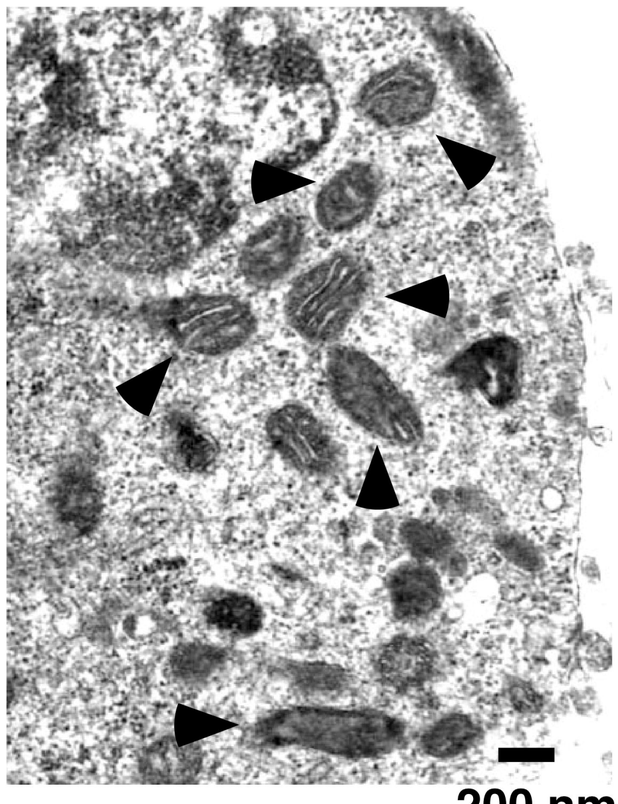


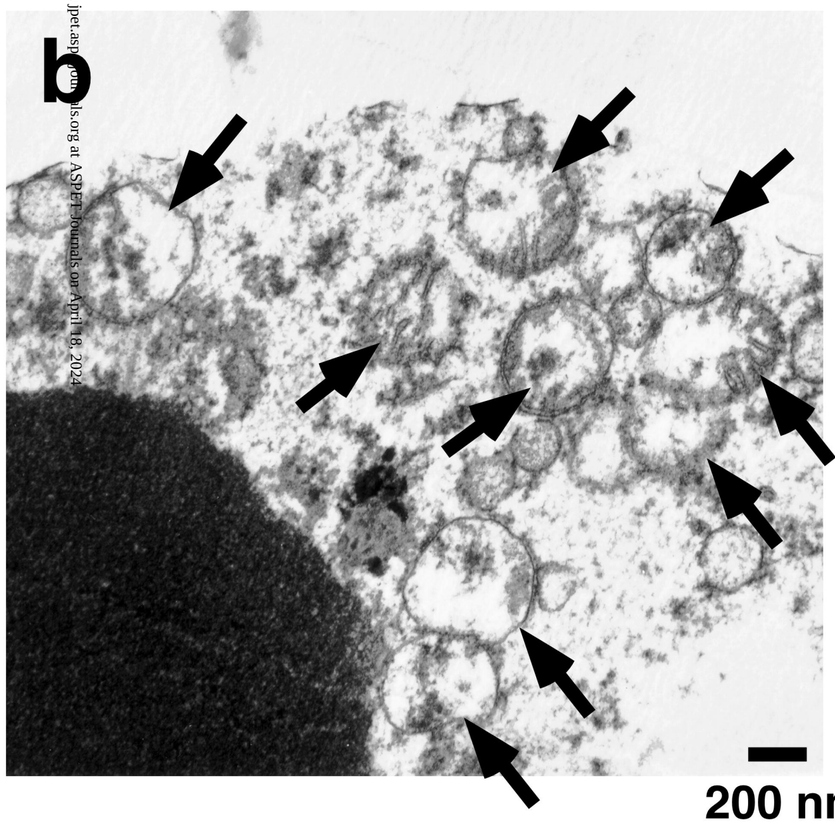




HM

b





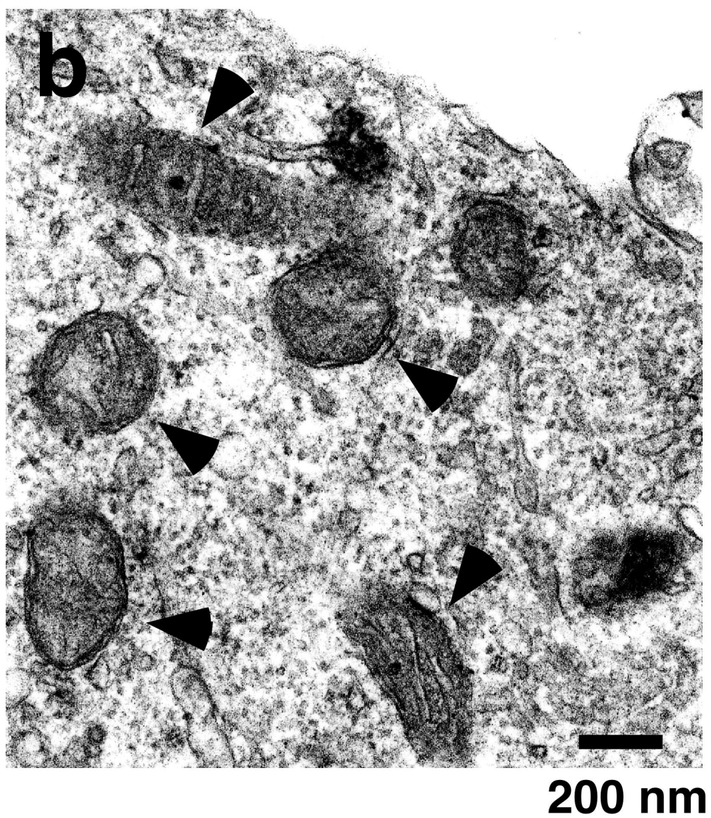


Figure 4

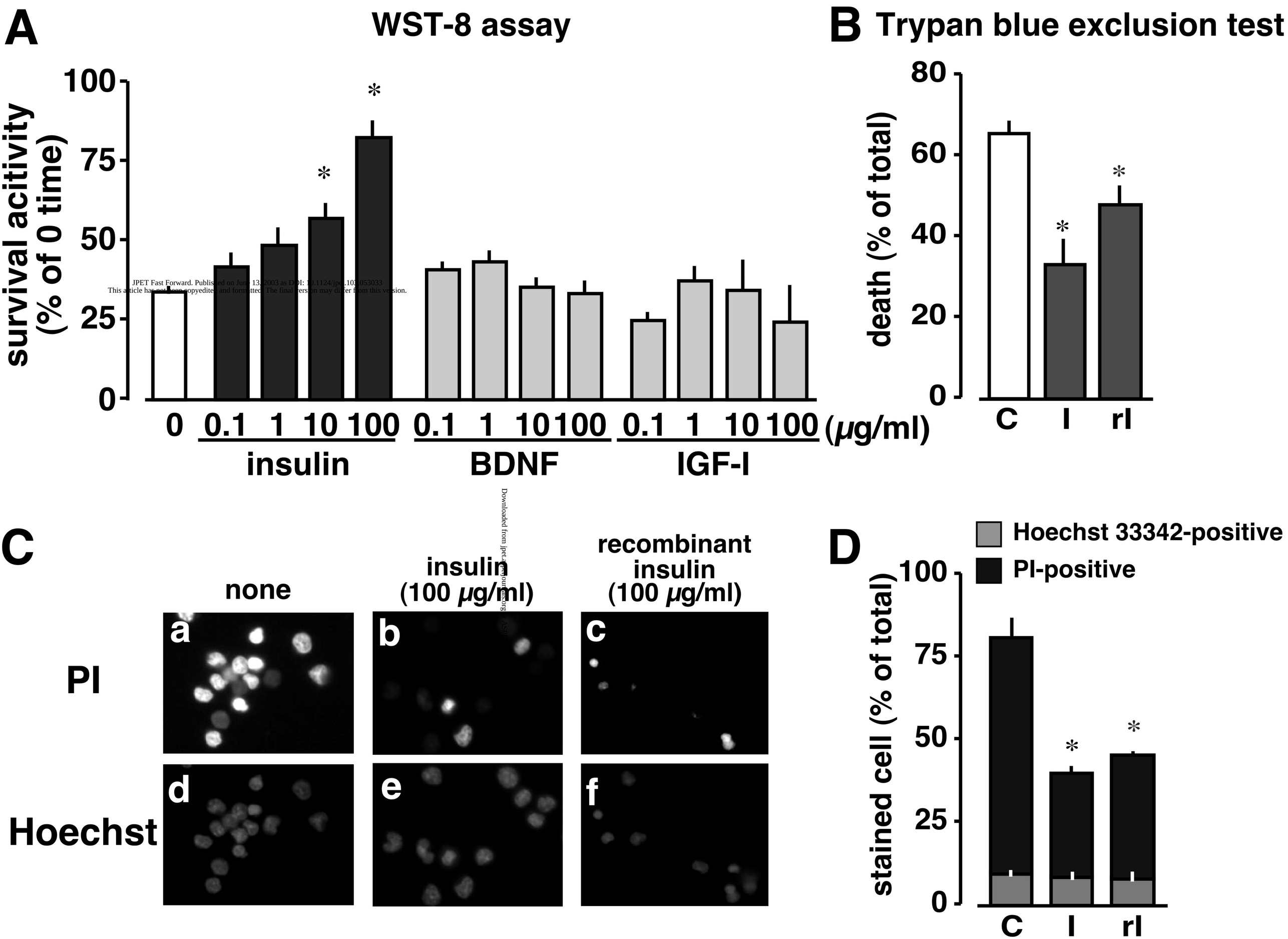
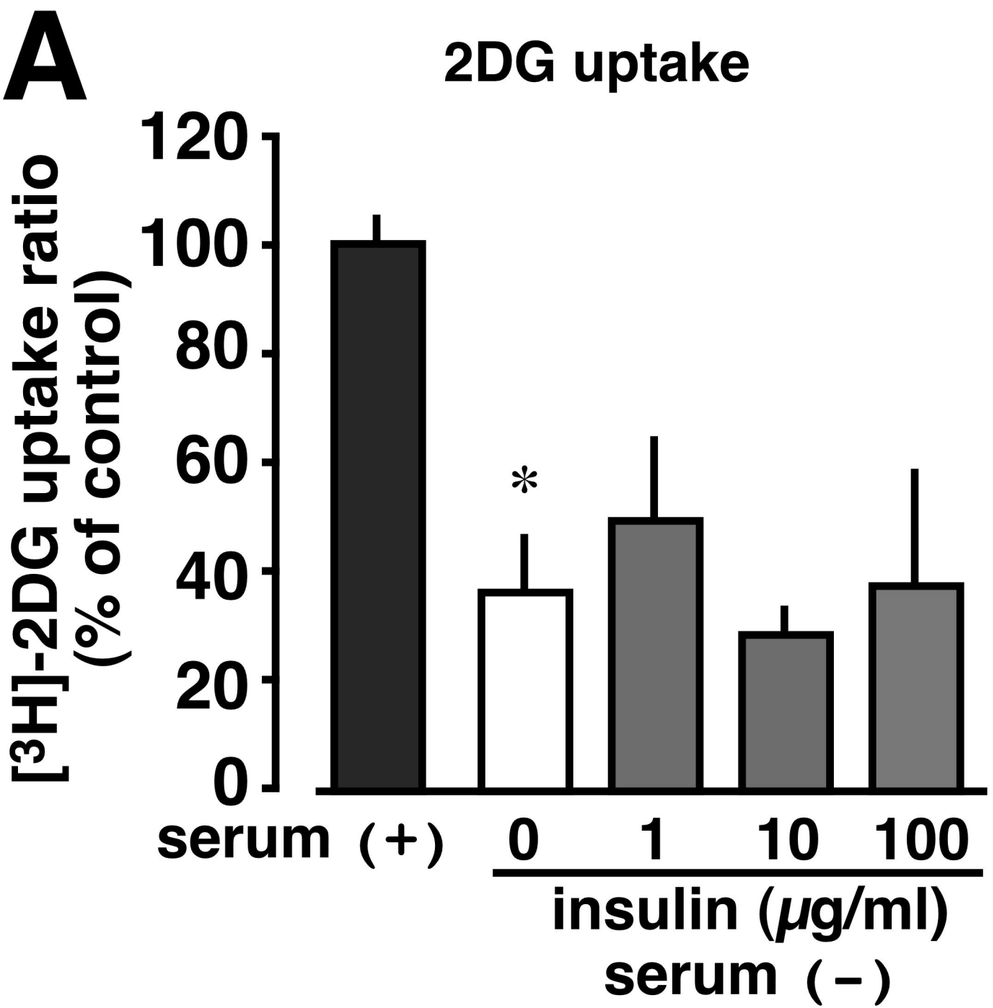


Figure 5



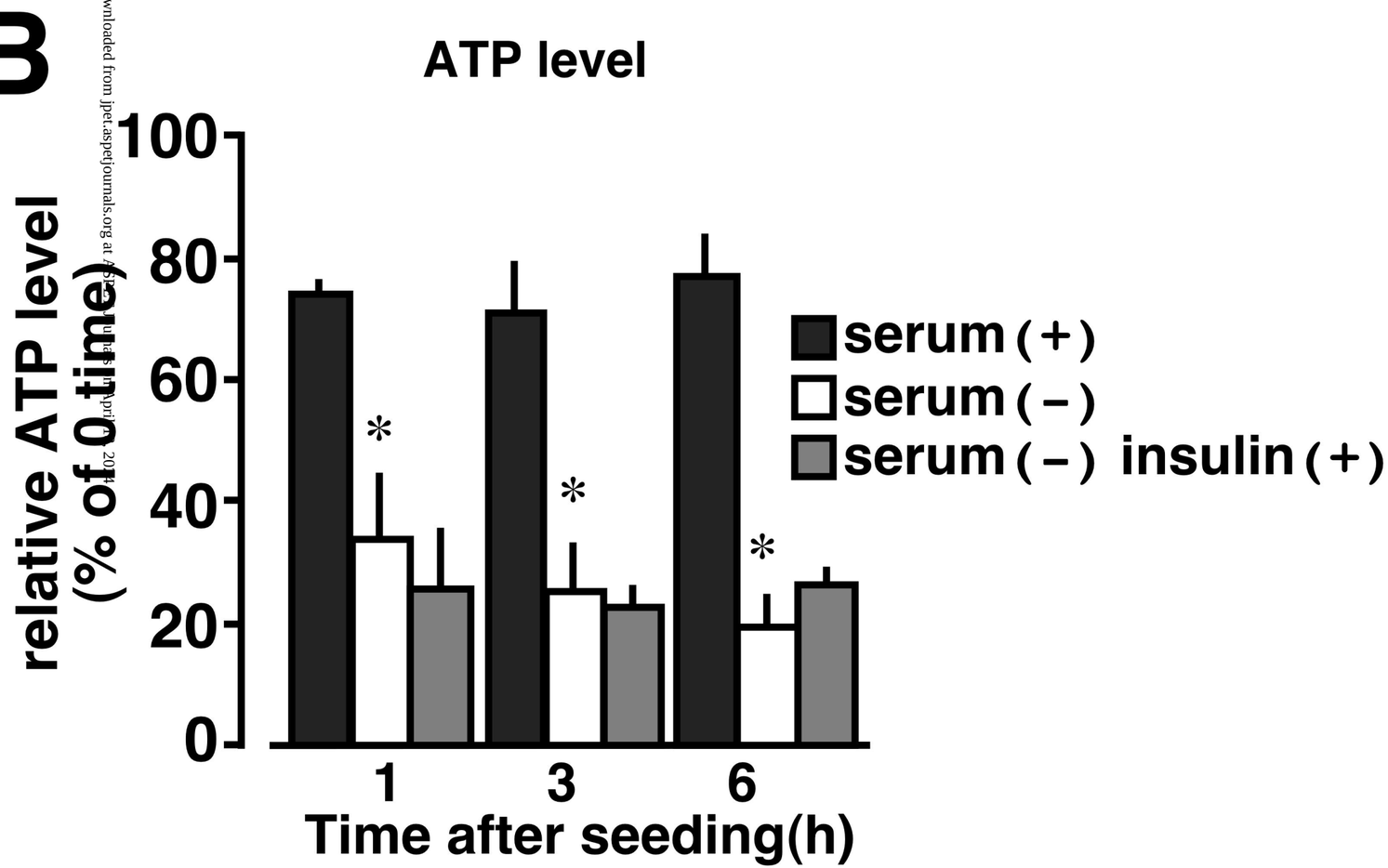


Figure 6

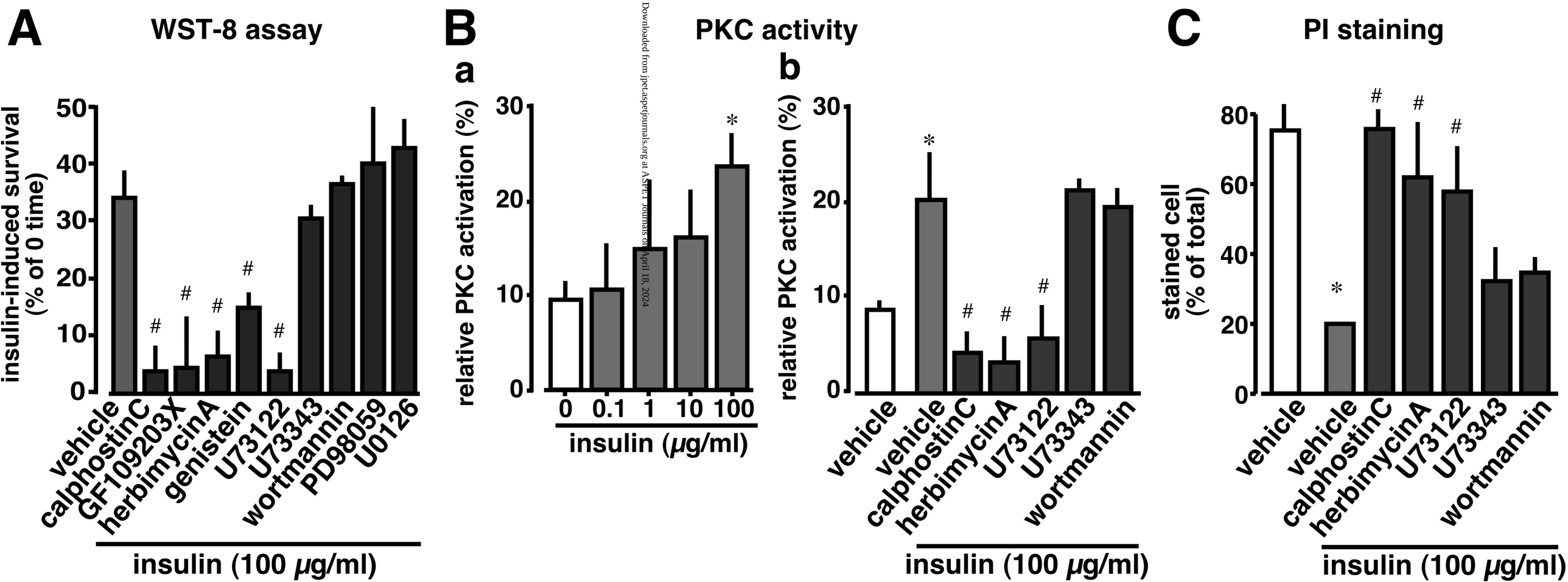


Figure 7

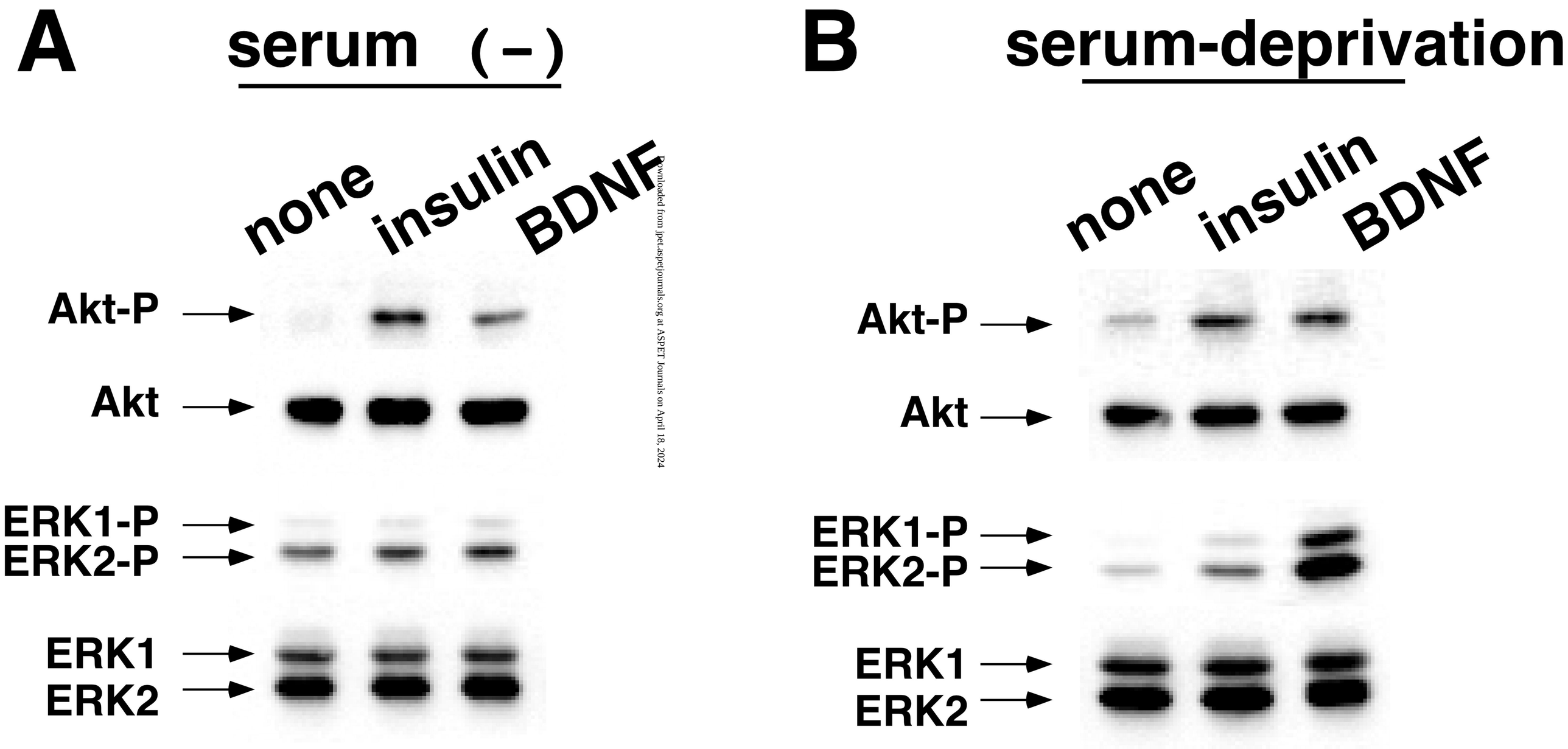


Figure 8