Insulin-Induced Oxidative Neuronal Injury in Cortical Culture: Mediation by Induced N-Methyl-D-aspartate Receptors

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Summary
While effectively attenuating neuronal apoptosis in mouse cortical culture, insulin paradoxically induced neuronal necrosis with 48 h of exposure. The insulin neurotoxicity was blocked by an antioxidant but not by caspase inhibitors. Exposure to insulin led to tyrosine phosphorylation of the insulin receptor and the insulin-like growth factor-1 (IGF-1) receptor and activation of protein kinase C (PKC) and phosphoinositide 3-kinase (PI3-kinase). Inhibitors of tyrosine kinase and PKC, but not PI3-kinase, attenuated the insulin neurotoxicity. Conversely, the inhibitor of PI3-kinase but not PKC reversed the antiapoptotic effect of insulin. Suggesting that the gene activity–dependent emergence of excitotoxicity contributed to insulin neurotoxicity, macromolecule synthesis inhibitors and N-methyl-D-aspartate (NMDA) antagonists blocked it. Consistently, exposure to insulin increased the level of the NR2A subunit of the NMDA receptor without much altering NR1 or NR2B levels. The present study suggests that insulin can be both neuroprotective and neurotoxic in the same cell system but by way of different signaling cascades.

INTRODUCTION
Although the main function of insulin is to regulate glucose metabolism (1, 2), it also has additional biological effects. For example, insulin has neurotrophic effects in the central nervous system (CNS)3, increasing neurite outgrowth (3, 4) and preventing neuronal apoptosis (5–7). These effects on central neurons are likely mediated by the receptors for insulin or for insulin-like growth factor-1 (IGF-1), or both, containing tyrosine kinase activity (8–10). Some patients with type II diabetes maintain increased insulin levels but still require very high doses of insulin because of their insulin resistance (11, 12). In those patients, insulin concentrations in the CNS may reach significantly high levels (13, 14), which may exert long-term trophic effects on central neurons. Consequences of the prolonged neurotrophic drive by insulin, however, are largely unknown.

Recently, we have shown that certain neurotrophic factors such as IGF-1 and neurotrophins exacerbate oxidative neuronal injury in cortical culture (15, 16). Because insulin may activate signaling cascades similar to IGF-1 (17–19), it may also share the harmful effect on neurons. Hence, examination of the potential prooxidative effect of insulin on central neurons may prove to be clinically relevant. To do that, in the present study, we examined potential neurotoxic effect of insulin in cortical culture.

EXPERIMENTAL PROCEDURES
Cortical Cell Cultures and Assessment of Neuronal Injury. Mixed cortical cell cultures, containing both neurons and glial cells, were prepared as described previously (20) from fetal mice at 14–16 days gestation and were used for experiments after 10 days in vitro. Overall cell injury was estimated first by

3Abbreviations: CHX, cycloheximide; CNQX, 6-cyano-7-nitroquinoxaline -2,3-dione; CNS, central nervous system; IGF, insulin-like growth factor; LDH, lactate dehydrogenase; NMDA, N-methyl-D-aspartate; NR, NMDA receptors; PI3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C; STSP, staurosporine.
examining of the cultures with a phase-contrast microscope. Additionally, some cultures were stained with the DNA fluorescent dye, propidium iodide, to identify dead neurons. In most experiments, overall neuronal cell injury was quantitatively assessed by measuring the amount of lactate dehydrogenase (LDH) released by damaged or destroyed cells into the bathing medium at each time point after exposure to drugs. In some cases, transmission electron microscopy was done as previously described (22) to examine the ultrastructural changes of insulin neurotoxicity.

**Immunoprecipitation and Western Blotting.** After rinsing, cells were suspended in lysis buffer and centrifuged. Aliquots of supernatant (containing 500 µg of protein) were incubated with an antibody for 2 h and then precipitated on Protein A–agarose beads at 4 °C for 1 h. The immunoprecipitates were then washed 3 times with lysis buffer. For Western blots, equal amounts of protein from each sample were electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were then incubated with a respective antibody overnight at 4 °C and visualized by the enhanced chemiluminescence (Amersham Life Science) method.

**Assays for Phosphoinositide 3-Kinase (PI3-Kinase) and Membrane-Associated Protein Kinase C (PKC) Activity.** PI3-kinase activity was assayed according to the protocol of Jackson et al. (23). The PKC activity in the cell membrane was measured generally according to the procedure of Chakravarthy et al. (24, 25).

**Measurement of Reactive Oxygen Species.** Levels of ROS in cells were measured using the fluorescent probe 2,7-dichlorofluorescein diacetate (DCF) (25, 26). Membrane liperoxides were measured according to the method of Ohkawa et al. (27).

**RESULTS**

A nonselective protein kinase inhibitor staurosporine (STSP, 100 nM) induced neuronal apoptosis in cortical culture with 24 h exposure, as previously described (28) (Fig. 1A). Addition of insulin (100 ng/ml) markedly attenuated this apoptosis (Fig. 1B). Unexpectedly, however, continuous exposure to insulin induced extensive neuronal death. Neurons that were exposed for 20 h to 100 ng/ml insulin exhibited increases in cell body sizes (compare Fig. 1D with Fig. 1C). Continued exposure to insulin for 48 h resulted in widespread neuronal death (Fig. 1E) accompanied by disruption of cell membrane and swelling of cell bodies and intracellular organelles (Fig. 1G), all consistent with necrosis-type death. Assay of LDH release revealed that insulin induced concentration- and exposure duration–dependent neuronal death (Fig. 1H).

Suggesting that insulin neurotoxicity is caused by increased oxidative stress in cultured cortical neurons, both lipid peroxidation (Fig. 2A) and DCF fluorescence in neurons (Fig. 2, Continued)

**Figure 1.** Insulin attenuates neuronal apoptosis but induces necrosis. (A, B) Fluorescence photomicrographs of cortical cultures stained with propidium iodide after 24 h exposure to staurosporine (100 nM) alone (A) or with addition of 100 ng/ml insulin (B). (C–E) Phase-contrast photomicrographs of cortical cultures after sham wash (C) or after 20 (D) or 48 h (E) of exposure to 100 ng/ml insulin. Scale bar, 100 µm. (Continued)
Figure 1. (Continued) (F, G) Electron microscopic photos of cortical neurons without (F) or with 34 h exposure to 100 ng/ml insulin (G). Disruption of cell membrane, beginning of chromatin condensation, and swollen mitochondria (thick arrow), but intact nuclear membrane (thin arrow) were noted. N, nucleus. Scale bar, 2 μm. (H) Percentage of LDH release (mean ± SEM, n = 4) in cortical cultures after 20, 34, or 48 h of exposure to indicated concentrations of insulin. All LDH values were scaled to the mean value in sister cultures exposed to 300 μM NMDA (=100%), where near-complete neuronal death resulted.

B–D) were increased in cortical cultures after 24 h exposure to 100 ng/ml insulin. Furthermore, addition of an antioxidant trolox (100 μM) completely blocked both free radical generation and neurotoxicity induced by insulin (Fig. 2, A, D, E). In contrast, the caspase inhibitors z-VAD and z-DEVD did not attenuate insulin neurotoxicity (Fig. 2E).

We examined the possibility that the receptors for insulin or for IGF-1 (or both) mediate the insulin neurotoxicity. Western blot analysis and immunocytochemistry (Fig. 3A) revealed that cultured cortical neurons expressed both receptors. Furthermore, both receptors were phosphorylated at tyrosine residues after insulin exposure (Fig. 3A). Exposure of cortical cultures to insulin increased the activity of PI3-kinase and PKC (Fig. 3, B and C). Addition of tyrosine kinase inhibitor or selective inhibitors of PKC attenuated insulin neurotoxicity (Fig. 3D). Indicating that the late PKC activation was critical, GF109203X added 24 h after the onset of insulin exposure also markedly attenuated insulin neurotoxicity (data not shown). On the other hand, wortmannin, a PI3-kinase inhibitor, did not attenuate insulin neurotoxicity (Fig. 3D). In contrast, the protective effect of insulin against STSP-induced neuronal apoptosis was completely reversed by wortmannin but not by GF109203X (Fig. 3E). Suggesting...
the role of excitotoxicity, addition of MK-801, a selective
N-methyl-D-aspartate (NMDA) antagonist, prevented insulin
neurotoxicity, whereas selective antagonists of α-amino-3-hy-
droxy-5-methyl-4-isoxazolepropionionic acid and kainate, 6-
cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6-nitro-7-sul-
phanoylbenzo[f]quinoxaline-2,3-dione (NBQX) did not (Fig.
3F). Furthermore, addition of actinomycin-D or cycloheximide
(CHX) completely blocked the insulin neurotoxicity (Fig. 3F).

Because in our cortical culture excitotoxicity per se, regardless
of its fulminance, is not sensitive to these macromolecule syn-
thesis inhibitors (29), we examined the possibility that the NMDA
receptors (NR) were induced by insulin exposure. Consistent
with this idea, Western blot analysis revealed that the NR2A
subunit was selectively induced at 24 h after the onset of insulin
exposure, which was blocked by the addition of CHX (Fig. 3G).
Insulin exposure did not alter NR1 or NR2B levels (Fig. 3G).

Figure 2. Oxidative stress is the main mechanism of insulin neurotoxicity. (A) Increase in thiobarbituric acid reactive substance
(TBARS) concentrations in sham wash control cultures (= 1), or in cultures after 36 h exposure to 100 ng/ml insulin alone or to
insulin plus 100 μM trolox. *, significantly different from sham wash results; #, significantly different from insulin alone results
(P < 0.05, two-tailed t-test). (B–D) Fluorescence photomicrographs of DCF-stained cortical cultures: sham wash controls (B), or
after 20 h of exposure to 100 ng/ml insulin alone (C) or to insulin plus 100 μM trolox (D). Scale bar, 100 μm. (E) Percentage
of LDH release (mean ± SEM, n = 4) in cortical cultures after 48 h of exposure to 100 ng/ml insulin alone or with addition of
100 μM each of trolox, z-VAD, or z-DEVD. *, significantly different from insulin alone (P < 0.05, two-tailed t-test).
DISCUSSION
The present study has demonstrated that insulin can induce neuronal injury in cortical cultures. Because the insulin toxicity was seen in the same neurons at concentration range where it blocked their apoptosis, it cannot be attributed simply to marginal effects occurring in a particular cell type or at unusually high concentrations. The insulin neurotoxicity occurred mainly by caspase-independent necrosis, accompanied by cell body swelling, membrane disruption, and organelle damage. Insulin-induced neuronal necrosis seemed to occur mainly by the oxidative injury mechanism. Supporting this, insulin-induced free radical generation and lipid peroxidation preceded neuronal death.

Figure 3. Insulin (INS), in comparison with the sham wash control (BL), activates the insulin receptors and the IGF-1 receptors and induces NR2A. (A) The upper panels show Western blots for β subunits of the IGF-1 receptor (IGF-1 Rβ) and the insulin receptor (insulin Rβ). The lower panels show Western blots of the fraction immunoprecipitated with anti-IGF-1 Rβ or anti-insulin Rβ antibody, which were blotted with an anti-phosphotyrosine antibody. Photographs show cortical cultures immunocytochemically stained for insulin (right) and IGF-1 (left) receptor α subunits. Scale bar, 100 μm. Arrows denote astrocytic nuclei. (B) Increases in PKC activity in the membrane fraction (mean ± SEM, n = 3) after exposure for the indicated times to 100 ng/ml insulin, in comparison with a control (= 1). Addition of GF109203X (+GFX), 3 μM, completely blocked the late PKC increases. *, significantly different from control results; #, significantly different from results with insulin alone (P < 0.05, two-tailed t-test). (C) PI3-kinase activity. Blots show radioactivity of phosphatidylinositol-4,5-bisphosphate (PIP2). Insulin exposure for 30 min induced marked increase in the radioactivity. Wortmannin (1 μM) effectively reduced insulin-induced PI3-kinase activity. (D) LDH release (mean ± SEM, n = 4) in cortical cultures after 48 h of exposure to 100 ng/ml insulin alone or with addition of genistein (30 μM), GF109203X (3 μM), or wortmannin (1 μM). *, significant difference from results with insulin alone (P < 0.05, two-tailed t-test). (E) LDH release (mean ± SEM, n = 3) in cortical cultures after 24 h of exposure to 100 nM STSP alone or in the presence of 100 ng/ml insulin without or with indicated kinase inhibitors. *, significantly different from results with staurosporine; #, significantly different from results with insulin alone (P < 0.05, two-tailed t-test); ++ denotes insulin plus inhibitors in addition to staurosporine (STSP). (Continued)
defined by cell membrane disruption and LDH release. Furthermore, the antioxidant trolox markedly attenuated insulin neurotoxicity.

This neurotoxicity may be mediated by insulin receptors, IGF-1 receptors, or both. Cultured cortical neurons express both of these receptors (19, 30). Furthermore, exposure to insulin resulted in autophosphorylation of tyrosine residues of both receptors. Suggesting the involvement of receptor tyrosine kinase, an inhibitor of tyrosine kinase, genistein, attenuated insulin neurotoxicity. Regarding more downstream events, PKC and PI3-kinase were activated. Of these two, PKC mediated most of insulin neurotoxicity, whereas PI3-kinase mediated the neuroprotective effect against apoptosis.

The NR has high calcium permeability (31) and thus has been implicated as the major mediator of calcium overload neurotoxicity (32, 33). This appears also to be the case in insulin neurotoxicity but in a rather dynamic way. Exposure to insulin selectively increased the expression of NR2A subunits without altering NR1 or NR2B levels. Consistent with the idea that the increased NMDA receptors, particularly NR2A, contribute to the neuronal death, a selective NMDA antagonist MK-801 largely attenuated the insulin neurotoxicity.

The present study has demonstrated for the first time the potentially harmful effect of insulin on central neurons. Although further studies may be needed to show the relevance of this finding in humans, administration of large doses of insulin in insulin-resistant diabetics, who might already have a leaky blood–brain barrier (13, 14), may paradoxically contribute to further oxidative neuronal injury over time.

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REFERENCES