Insulin-like growth factor-I regulates glucose-induced mitochondrial depolarization and apoptosis in human neuroblastoma

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Received 20.12.02; revised 22.12.03; accepted 16.1.04; published online 23.4.04
Edited by ME Peter

Abstract

Neuroblastoma, a pediatric peripheral nervous system tumor, frequently contains alterations in apoptotic pathways, producing chemoresistant disease. Insulin-like growth factor (IGF) system components are highly expressed in neuroblastoma, further protecting these cells from apoptosis. This study investigates IGF-I regulation of apoptosis at the mitochondrial level. Elevated extracellular glucose causes rapid mitochondrial enlargement coupled with an increase in the mitochondrial membrane potential ($\Delta\Psi_M$) followed by mitochondrial membrane depolarization (MMD), uncoupling protein 3 (UCP3) downregulation, caspase-3 activation and decreased Bcl-2. MMD inhibition by Bongkreik acid prevents high-glucose-induced loss of UCP3 and apoptosis. Glucose exposure induces caspase-9 cleavage within 30 min, and caspase-9 inhibition prevents glucose-mediated apoptosis. IGF-I prevents caspase activation and mitochondrial events leading to apoptosis. These results suggest that elevated glucose produces early initiator caspase activation, followed by $\Delta\Psi_M$ changes, in neuroblastoma cells; in turn, IGF-I prevents apoptosis by preventing downstream caspase activation, maintaining $\Delta\Psi_M$ and regulating Bcl proteins.

Cell Death and Differentiation (2004) 11, 885–896.

doi:10.1038/sj.cdd.4401429

Published online 23 April 2004

Keywords: apoptosis; programmed cell death; neuroblastoma; mitochondria; IGF-I; UCP3

Abbreviations: IGF, insulin-like growth factor; IGF-I, insulin-like growth factor-I; IGF-II, insulin-like growth factor-II; IGF-IR, type I IGF receptor; PI3K, phosphatidylinositol 3-kinase; $\Delta\Psi_M$, mitochondrial membrane potential; PTP, permeability transition pore; ANT, adenine nucleotide transporter; VDAC, voltage-dependent anion channel; MMD, mitochondrial membrane depolarization; UCPs, uncoupling proteins; UCP3, uncoupling protein 3; UCP2, uncoupling protein 2; PI, propidium iodide; Rh123, rhodamine

123; BKA, Bongkreik acid; MEK1/2, extracellular signal-regulated kinase 1/2; MAPK, mitogen-activated protein kinase; C9DN, caspase-9 dominant negative; C8DN, caspase-8 dominant negative; FLS, forward light scatter

Introduction

Neuroblastoma is a childhood tumor of the nervous system arising through improper differentiation of the neural crest cells that normally form the adrenal medulla and the sympathetic nervous system.¹ Neuroblastoma accounts for 8–10% of all childhood cancers, and approximately 40% of patients have metastatic disease refractory to treatment.² While many other solid tumors respond to chemotherapeutic agents by undergoing a caspase-dependent death,³ neuroblastoma tumors frequently overexpress the antiapoptotic protein Bcl-2 and/or have defects in the caspase-8 mediated apoptotic pathway, resulting in chemoresistant disease.⁴ In our laboratory, we have focused on alternative methods for apoptotic induction in human neuroblastoma cells, including high-mannitol or -glucose exposure, in attempts to develop novel ways to augment the treatment of aggressive neuroblastoma.^{5,6}

Increased expression of the insulin-like growth factor (IGF) ligands, IGF-I, IGF-II, and the type I IGF receptor (IGF-IR) is present in a wide range of human cancers,⁷ including lung, breast, thyroid, prostate, glioblastomas, rhabdomyosarcomas, leukemias and neuroblastoma (reviewed in Nechushtan *et al.*⁸). IGF-IR overexpression leads to cellular transformation,^{7,9} tumor proliferation^{10,11} and growth¹² while disruption of IGF-IR expression reverses the transformed phenotype.^{7,9} In neuroblastoma, IGF expression is present in all stages of primary tumors.¹³ IGF-I or IGF-II coupled to IGF-IR promotes survival of human neuroblastoma cell lines,^{14,15} and inhibition of IGF-IR expression using antisense strategies inhibits tumor growth and induces regression of NBL tumors in mice.¹⁶ IGF-I and IGF-IR expression also protects neuroblastoma cells from apoptosis, primarily through downstream PI3K pathway signaling.^{5,6,10,17–19} Therefore, targeting the IGF-IR may provide a new therapeutic approach to cancer treatment.^{12,20,21} Antisense IGF-IR strategies also enhance the susceptibility of Ewing's sarcoma to doxorubicin-induced apoptosis,²² implying a use for IGF-IR disruption in combinatorial drug therapy.²³ Based on the above evidence, our laboratory seeks to understand how the IGF-IR protects neuroblastoma cells from apoptosis induced through different mechanisms.

One potential mechanism by which IGF-I blocks apoptosis is by preventing depolarization of the inner mitochondrial membrane potential ($\Delta\Psi_M$). Depolarization of mitochondria (loss of $\Delta\Psi_M$) is now known to initiate the apoptotic cascade in many cells, including cells of neuronal origin.^{24–26} $\Delta\Psi_M$ is regulated by the permeability transition pore (PTP), a complex

of proteins that includes the adenine nucleotide transporter (ANT) in the inner mitochondrial membrane and the voltage-dependent anion channel (VDAC)/porin in the outer mitochondrial membrane.^{24,27,28} These changes in mitochondrial function are associated with release of cytochrome *c* and other proteins from the inner membrane space to the cytoplasm.^{24,29}

The release of mitochondrial proteins in response to toxic stimuli activates the caspase cell death proteases, essential end effectors of cell death.^{30–32} Upon release from the mitochondria, cytochrome *c* binds to Apaf-1 and caspase-9, forming a complex known as the apoptosome.^{33–36} Apoptosome formation results in activation of caspase-9, initiating a caspase cascade.^{33–36} Typically, the caspase cascade ends with caspase-3 activation, the caspase most critical for apoptosis induction.^{24,25} In contrast to this pathway, apoptosis may also occur via a caspase-8-dependent pathway.³³ Caspase-8 is activated by death receptors; this leads to downstream mitochondrial alterations, once again producing caspase-9 cleavage.³³ However, as previously stated, the caspase-8 pathway is often compromised in neuroblastoma, leading to resistant disease.⁴

In neuroblastoma, the relationship between high-glucose, mitochondrial membrane depolarization (MMD), caspase activation, apoptosis and IGF-I rescue is unknown. In the current studies, we utilized SH-SY5Y human neuroblastoma cells to examine the role of mitochondrial changes and caspase cleavage in glucose-induced apoptosis and to determine the relevance of these changes to the survival activity of IGF-I. We show that glucose induces MMD, mitochondrial swelling and caspase-3 activation in SH-SY5Y cells. These events are dependent on PTP. We present new evidence that IGF-I-mediated signaling prevents apoptosis due to stabilization of $\Delta\Psi_M$ leading to inhibition of MMD and mitochondrial swelling. We further show that the effects of glucose on mitochondria are associated with a decrease in Bcl-2 protein levels and with downregulation of uncoupling proteins (UCPs), events that will promote induction of apoptosis. Finally, glucose induces a rapid induction of caspase-9, and caspase-9 inhibition prevents glucose-mediated apoptosis. Caspase-3 activation occurs independent of caspase-9 induction, an event preventable by IGF-I. These studies advance our understanding of IGF-I-mediated rescue in neuroblastoma cells, which may ultimately lead to intervention in resistant disease.

Results

High glucose induces MMD and mitochondrial enlargement

Although there is excellent evidence for a role of changes in $\Delta\Psi_M$ and swelling in non-neuronal apoptosis, the role of these mitochondrial changes in neuroblastoma is unknown. We were therefore interested in the contribution of these mitochondrial events to glucose-induced neuroblastoma apoptosis. To facilitate these investigations, we utilized SH-SY5Y human neuroblastoma cells, an N-type neuroblastoma cell line, which is tumorigenic *in vivo* and *in vitro*, expresses N-Myc and expresses Bcl antiapoptotic proteins.^{5,37,38} In the first

set of experiments, we examined the effect of glucose on $\Delta\Psi_M$ in SH-SY5Y cells using rhodamine 123 (Rh123). Rh123 is a vital fluorescent dye preferentially taken up by mitochondria in a voltage-dependent manner such that Rh123 fluorescence is proportional to $\Delta\Psi_M$.³⁹ In these experiments, cells were co-stained with propidium iodide (PI), a DNA intercalating dye commonly used to measure the extent of cell death.⁴⁰ This co-staining with PI coupled with fluorescent activated cell sorting (FACS) allowed us to gate only on live cells, thereby ignoring any Rh123 fluorescence due to dead cells.

Mitochondrial dysfunction has been associated with other models of cell death, but not with glucose-induced injury.^{24,30,31} When $\Delta\Psi_M$ was measured over 24 h, there was an initial increase in the mean Rh123 level to 150% of control (maximum at 3 h) followed by a decline to sub-basal levels by 8 h. This initial increase in Rh123 levels indicates either mitochondrial swelling or hyperpolarization²⁷ (Figure 1a). Rh123 measurements were standardized during the time course experiments, and between experiments, by measuring values as a percent of control at the corresponding time period, and represent combined data from seven separate experiments. In the presence of increased glucose (45 mM), there was a rapid two-fold rise in the percent of dead cells at 6 h, followed by a progressive increase to three-fold above the

Figure 1 High glucose results in initial hyperpolarization followed by depolarization of $\Delta\Psi_M$. (a) Using FACS analysis, there is an initial increase in Rh123 levels measured as peak Rh123 levels in relative fluorescent units and expressed as percent control. Rh123 levels peak at 3 h, corresponding to enlargement of isolated mitochondria measured by FLS. This is followed by a decrease in $\Delta\Psi_M$ corresponding to MMD over the next 24 h. (b) Increasing glucose levels are associated with an increase in $\Delta\Psi$ measured as the % Rh123-positive cells at 3 h after addition of high glucose. The % Rh123-positive cells is significantly increased with the addition of 45 or 175 mM glucose compared to control media (* $P < 0.01$). (c) Increased concentrations of added glucose increase the size of isolated mitochondria from SH-SY5Y cells at 3 h, measured by FACS analysis of FLS. Results indicate the % change (increase) compared to control (% Δ Control). The mitochondrial size is significantly increased with the addition of 45 or 175 mM glucose compared to control media (* $P < 0.05$)

corresponding controls at 24 h ($P < 0.01$). There was no significant change in control apoptosis during the experiment, although cell death was mildly increased at 24 h.

To determine if higher glucose levels affected $\Delta\Psi_M$ and mitochondrial size, increasing concentrations of glucose (45–175 mM) were added to cultured SH-SY5Y cells; the inner mitochondrial membrane polarity was measured using Rh123 in whole cells, and mitochondrial size was measured in isolated mitochondria. Mitochondrial hyperpolarization increased at 3 h with higher concentrations of glucose as measured by the % Rh123-positive cells (Figure 1b), and was significantly greater with 45 mM glucose compared to control ($P < 0.01$). In contrast, by 6 h there was a dose-dependent decrease in $\Delta\Psi_M$ to 30% less than control with 45 mM glucose and 60% less than control with 175 mM glucose at 6 h ($P < 0.001$). High glucose also induced mitochondrial enlargement in isolated mitochondria, to 10% greater than control with 45 mM glucose ($P < 0.05$) and 14% greater than control with 175 mM glucose (Figure 1c) at 3 h. The maximal mitochondrial size using forward light scatter (FLS) was most easily measured at 3 h.

High glucose induces caspase-3 cleavage

Activation of the caspase family of cell death proteases is essential for most types of apoptosis.^{24,25} The caspases normally exist as inactive proenzymes until they are either cleaved by other proteases or are activated by factors released from mitochondria. We and others have shown that activation of caspase-3 by a variety of toxic insults including high-dose mannitol in hyperosmolar concentrations plays an important role in apoptosis in SH-SY5Y cells.^{5,37,41} However, it has not been previously established whether high glucose, in non-hyperosmolar concentrations, induces caspase-3 activation in SH-SY5Y cells. As shown in Figure 2a, addition of glucose causes a dose-dependent increase in cleavage to the 17 kDa cleavage band that is initially detected at 3 h, corresponding to hyperpolarization of $\Delta\Psi_M$ and increases to maximal levels at approximately 12 h (Figure 2b). Cleavage of the 32 kDa procaspase protein can be observed with as little as 45 mM glucose, but is greater with 175 mM glucose, except at 24 h where caspase levels begin to fall as increasing numbers of cells die.

To further illustrate that caspase-3 is activated during glucose-induced apoptosis in neuroblastoma cells, we plotted the ratio of cleaved caspase-3:GAPDH for each dose of glucose as a function of time. The percent cells showing cleaved caspase-3 was $175 \pm 25\%$ of control with 45 mM glucose ($P < 0.01$), and $267 \pm 53\%$ of control with 175 mM glucose ($P < 0.001$) at the 12 h time point. These results show that glucose causes a dose-dependent activation of caspase-3 in SH-SY5Y cells.

Bongkreik acid inhibits MMD, downregulation of UCPs and caspase-3 cleavage

The next series of experiments determined the effect of adenine nucleotide translocase/voltage-dependent anion channel (ANT/VDAC) complex inhibition on the $\Delta\Psi_M$. Bongkreik

Figure 2 High glucose induces a dose-dependent cleavage of caspase-3 in SH-SY5Y cells that is blocked by IGF-I. (a) High glucose (45–175 mM) increases cleavage of caspase-3 in a dose-dependent manner. Immunoblotting of whole SH-SY5Y lysates with anti-caspase-3 antibody showed cleavage of procaspase-3 to the active p17 subunit. At each time point 3–24 h, there is an increase in caspase-3 cleavage compared to control. Results were plotted as a ratio to GAPDH used as a loading control. Increased cleavage is observed even with 45 mM total glucose but is maximal at earlier time points with 175 mM total glucose. In each case, caspase-3 cleavage was blocked by 10 nM IGF-I. (b) Results for the ratio of cleaved caspase-3:GAPDH are plotted over 24 h. At 0 h, results were identical between groups. With both 45 and 175 mM glucose, increased caspase-3 cleavage is seen at 3 h and there is a gradual increase in caspase-3 levels peaking at approximately 12 h. The asterisk (*) represents statistically significant increases in cleaved caspase-3 with increased glucose concentration at the 12 h time point ($P < 0.01$)

reik acid (BKA) is an inhibitory ligand for the mitochondrial ANT.^{42,43} To determine if stabilization of $\Delta\Psi_M$ prevented cleavage of caspase-3 and apoptosis, SH-SY5Y cells were treated with 100 μ M BKA. In the presence of 45 mM glucose, there was a decrease in $\Delta\Psi_M$ ($P < 0.001$) (Figure 3a), and an increase in the percent depolarized cells. Although the graph indicates data at 24 h, similar results were obtained at 6 and 12 h. Glucose induced a two-fold increase in the mean caspase level ($P < 0.001$) and a nine-fold increase in the percent caspase-positive cells at 6 h (Figure 3b). With 100 μ M BKA, glucose-induced MMD (Figure 3a) and caspase-3

Figure 3 Inhibition of the ANT/VDAC complex by BKA inhibits MMD and caspase activation. (a) SH-SY5Y cells were treated with DMEM $\pm$ high glucose for a period of 24 h. The graph indicates the mean Rh123 level compared to control (control = 100%). With 45 mM total glucose, there was an 84% decrease in $\Delta\Psi_M$ using Rh123, compared to control ($P < 0.001$). Depolarization of the inner mitochondrial membrane was blocked by 100 μ M BKA. There was no statistically significant difference between control and control + BKA at 24 h. (b) BKA blocks glucose-induced cleavage of caspase-3 in SH-SY5Y cells measured using a DEVD-AMC fluorogenic substrate. The graph indicates cleavage of caspase-3 compared to control (control = 100%). Neuroblastoma cells were treated with high glucose (45 mM total glucose) $\pm$ 100 μ M BKA for a period of 6 h. BKA acts by blocking the ANT/VDAC complex of the mitochondria and prevents loss of $\Delta\Psi_M$. In the presence of BKA, there is reduced caspase-3 cleavage even in the presence of 45 mM total glucose, indicating that inhibition of mitochondrial depolarization is critical in preventing apoptosis. *** $P < 0.001$ compared to control

cleavage (Figure 3b) is blocked. With 100 μ M BKA, there was no statistical difference between control and glucose + BKA for MMD or for caspase-3 activation. BKA alone had no significant effect on MMD or caspase cleavage in control cells. Therefore, our results demonstrate that in SH-SY5Y cells, PTP opening results in caspase-3 activation.

We then determined if stabilization of $\Delta\Psi_M$ by BKA would affect UCPs known to act as proton pump regulators. In the presence of 45 mM glucose, there was a decrease in uncoupling protein 3 (UCP3) protein levels that was inhibited by optimal levels of BKA consistent with BKA preventing ultimate MMD (Figure 4). BKA under control conditions slightly decreased the ratio of UCP3:GAPDH, possibly because BKA in the absence of high glucose would cause sustained mitochondrial membrane hyperpolarization that in

Figure 4 High-glucose-induced reduction in UCP3 levels is blocked by BKA. The blot indicates UCP3 protein levels in SH-SY5Y cells at 6 h. SH-SY5Y cells were cultured in control medium or high (45 mM) glucose + 100 μ M BKA for a period of 6 h. Blots were developed with UCP3 antibody (32–35 kDa) on a 12% gel, and for GAPDH as a loading control. The graph indicates the ratio of UCP3:GAPDH in each lane. UCP3 levels decrease in the presence of high glucose. This is blocked by 100 μ M BKA

Figure 5 IGF-I prevents a decrease in Bcl-2 protein levels induced by high glucose. Immunoblots were performed on whole SH-SY5Y lysates using a monoclonal antibody against Bcl-2. The graph indicates changes in the ratio of total Bcl-2:GAPDH on immunoblots performed over 12 h. Bcl-2 protein levels were unchanged in high glucose compared to control conditions at 1–6 h, but were measurably smaller in high glucose at 12 h

itself would induce mitochondrial damage.⁴⁴ Uncoupling protein 2 (UCP2) levels in the presence of high glucose were reduced similar to UCP3, although levels of protein expression of UCP2 are less in SH-SY5Y cells (data not shown).

Effects of glucose on Bcl family members

The Bcl-2 family is a group of related proteins that either promote or counteract apoptosis and are thought to regulate mitochondrial function.^{24,25} Bcl-2 prevents apoptosis in response to a wide variety of stimuli, apparently by inhibiting MMD.^{27,45} In contrast, Bax promotes apoptosis by enhancing MMD.⁴⁶ A reduction in Bcl-2 levels was not visible at 6 h but could be observed by 12 h (Figure 5). However, glucose did not affect Bax levels in SH-SY5Y cells (data not shown).

Effects of IGF-I on $\Delta\Psi_M$ and mitochondrial enlargement

IGF-I and IGF-IR expression protects neuroblastoma cells from apoptosis and inhibition of IGF-IR expression induces neuroblastoma tumor regression in mice, likely through an apoptotic death of tumor cells.¹⁶ Moreover, we have shown that IGF-I prevents glucose-induced apoptosis in untransformed cells of neuronal origin,^{47,48} although the intermediate pathways leading to apoptosis are unknown. We therefore wanted to determine whether IGF-I affects mitochondrial function in neuroblastoma cells. Figure 6a shows the effects of 45 mM glucose and 10 nM IGF-I alone and in combination on mitochondrial size. Similar to the results in Figure 1, a 3 h treatment with 45 mM glucose caused a 10% increase in mitochondrial size compared to control. IGF-I alone at 10 nM caused a very slight change in mitochondrial size (Figure 6a). Most importantly, IGF-I prevented glucose-induced mitochondrial swelling (1% decrease *versus* control). The PI3K inhibitor LY294002 blocked IGF-I regulation of mitochondrial size, allowing mitochondrial enlargement in the presence of high glucose (Figure 6a). In contrast, the mitogen-activated protein kinase extracellular signal-regulated kinase (MEK1/2) inhibitor PD98059 did not block IGF-I regulation of mitochondrial size. LY294002 and PD98059 alone had no significant effect on mitochondrial size compared to control (data not shown).

Figure 6b shows a similar protective effect of IGF-I on glucose-induced MMD. Treatment of cells for 6 h with 45 mM glucose alone caused an approximately 40% decrease in $\Delta\Psi_M$. When 10 nM IGF-I was added along with 45 mM glucose, there was a 5% increase in $\Delta\Psi_M$ compared to control. Interestingly, 10 nM IGF-I alone caused an increase in Rh123 fluorescence, but Figure 5a shows that this is not due to mitochondrial swelling. Rather, this effect of IGF-I likely reflects hyperpolarization of $\Delta\Psi_M$. Inhibition of PI3K by LY294002 blocked IGF-I stabilization of $\Delta\Psi_M$, whereas the MEK inhibitors PD98059 and U0126 only partially reversed the effect of IGF-I on $\Delta\Psi_M$.

IGF-I prevents caspase activation

Next, a protective effect of IGF-I was also observed at the level of caspase-3 activation. We examined caspase-3 activity at 6 h after adding glucose using DEVD-AMC, a fluorogenic and cell-permeable substrate for caspase-2, -3 and -7.⁴⁹ As in Figure 2, similar results were found using the cell-permeable caspase-3 substrate DEVD-AMC (Figure 7). The addition of 45 mM glucose increased the mean caspase-3 level (Figure 7) on FACS ($P < 0.001$) as previously observed using Western immunoblotting (Figure 2). IGF-I decreased the mean caspase-3 level 20% below control in the presence of glucose, and was statistically different from both high-glucose conditions ($P < 0.001$) and from control ($P < 0.001$). Similarly, IGF-I alone decreased the mean caspase-3 level 23% below control ($P < 0.001$). Cleavage of DEVD-AMC was blocked by Ac-DEVD-CHO (data not shown), indicating that this activity is primarily due to activated caspase-3.⁴⁹

Similar to the results with MMD, inhibition of both PI3K and MAPK/MEK signaling reduced the antiapoptotic effect of IGF-I in cells treated with high glucose for 6 h (Figure 7). LY294002

Figure 6 IGF-I inhibits changes in mitochondrial function induced by high glucose. **(a)** The graph indicates the percent change from control in mitochondrial size (% Δ Control) measured using FLS of Rh123 from FACS analysis. With 45 mM total glucose, there was a 10% increase in the mean size of isolated mitochondria from SH-SY5Y cells, compared to control. IGF-I (10 nM) prevented enlargement in mitochondria even in the presence of 150 mM glucose. The PI3K inhibitor LY294002 (10 μM) blocked IGF-I inhibition of mitochondrial swelling. In contrast, the MEK inhibitor PD98059 (10 μM) and IGF-I alone had no effect on mitochondrial size compared to control mitochondria. **(b)** The graph indicates the % change in $\Delta\Psi_M$ compared to control ($\Delta\Psi_M$) using the mean Rh123 level from FACS analysis. Values are expressed as percent change from control (% Δ Control). IGF-I inhibits the high-glucose-induced decrease in $\Delta\Psi_M$. IGF-I alone increases the mean Rh123 levels above control, consistent with an elevated $\Delta\Psi_M$. In contrast, LY294002 (10 μM) blocks IGF-I inhibition of MMD. The MEK inhibitors PD98059 and U0126 (both 10 μM) partially inhibit IGF-I regulation of $\Delta\Psi_M$. ** $P < 0.01$; *** $P < 0.001$ compared to control

almost completely blocked IGF-I protection, whereas PD98059 and U0126 reduced the neuroprotective effect of IGF-I by 50% in the presence of high glucose. PD98059 and U0126 had no significant effect on apoptosis in control cells. However, LY294002 alone increased apoptosis by 20% in

Figure 7 IGF-I blocks activation of caspase-3 in SH-SY5Y cells through a PI3K-dependent mechanism. SH-SY5Y cells were cultured in control or 45 mM total glucose for a period of 6 h, and then caspase-3 activation was measured using a DEVD-AMC fluorogenic substrate in the presence of PI exclusion. The graph indicates % control (where control = 100%). IGF-I (10 nM) inhibits apoptosis in the presence of added glucose, an effect that is blocked by LY294002 and to a lesser extent by PD98059 and U0126. IGF-I alone marginally decreased caspase-3 activation below control levels. ** $P < 0.01$; *** $P < 0.001$ compared to control

control cells, although it does not further increase glucose-induced apoptosis (data not shown).

Glucose activates initiator caspases

MMD often leads to the release of cytochrome *c* from the mitochondria, followed by formation of the apoptosome, a complex formed by cytochrome *c*, Apaf-1 and caspase-9 binding.^{33–36} This in turn promotes caspase-9 cleavage and activation of the downstream caspase cascade.^{33–36} To test whether caspase-9 is involved in glucose-mediated apoptosis, SH-SY5Y cells were stably transfected with caspase-9 dominant-negative (C9DN) constructs. As a control, SH-SY5Y cells were also transfected with caspase-8 dominant-negative constructs (C8DN), as caspase-8 activation is normally activated by death receptor induction rather than mitochondrial events. Transfected cells were then exposed to increasing concentrations of glucose and apoptosis measured using PI staining. As previously discussed, 175 mM glucose increases apoptosis in control, vector-transfected cells; however, this increases three-fold in 325 mM glucose at 24 h (Figure 8a). Interestingly, when cells are transfected with C8DN, apoptosis levels are approximately 60–70% of vector control cells (Figure 8a). C9DN expressing cells show apoptosis levels of approximately 20% of vector control cells (Figure 8b). These data suggest that although caspase-8 is responsible for a portion of the apoptosis seen in glucose-treated SH-SY5Y cells, caspase-9 plays a larger role.

We next investigated the time course over which caspase-8, -9 and -3 are activated in response to 325 mM glucose, the dose that produces the greatest amount of apoptosis in SH-SY5Y cells. Caspase-8, a 55/53 kDa protein, is cleaved into several products, including 43/41, 26/24 and 18 kDa products.⁵⁰ The 55 kDa proform is found in every condition,

Figure 8 Caspase-9 mediates apoptosis induced by high glucose in SH-SY5Y neuroblastoma cells. (a) SH-SY5Y cells stably transfected with vector, dominant-negative caspase-8 (C8DN) or dominant-negative caspase-9 (C9DN) constructs were treated with 25, 175 or 325 mM glucose $\pm$ 10 nM IGF-I for 24 h. Cells were then analyzed for apoptosis via flow cytometry. C9DN cells treated with 325 mM glucose exhibit significantly less apoptosis than vector cells ($P < 0.001$). Addition of IGF-I to 325 mM glucose inhibits apoptosis significantly in vector, C8DN and C9DN cells ($P < 0.001$). (b) SH-SY5Y cells were treated with control DMEM media for 60 min (c) or 325 mM glucose for 5–60 min as indicated. Cells were analyzed for caspase cleavage via Western immunoblotting. Levels of the caspase-8 proform (55 kDa) do not change upon glucose exposure. In contrast, caspase-9 and caspase-3 show increased cleavage after 30 min of high-glucose treatment (37 and 17 kDa, respectively). Equal protein loading between samples is shown with the GAPDH blot presented. (c) SH-SY5Y cells stably transfected with vector, C8DN or C9DN constructs were treated with control DMEM media for 6 h or with 325 mM glucose $\pm$ 10 nM IGF-I for 2, 4 or 6 h as indicated. Cells were then analyzed for caspase-3 cleavage via Western immunoblotting. A caspase-3 cleavage band (17 kDa) is detected in all three cell lines in 325 mM glucose. IGF-I addition prevents the appearance of the caspase-3 cleavage band in all three cell lines

unregulated by glucose exposure (Figure 8b). Interestingly, the p18 cleavage product, which is the active subunit capable of cleaving downstream caspases, is undetectable in glucose-treated SH-SY5Y cells. In contrast, the 35 kDa caspase-9 cleavage product, absent in untreated samples, is detected within 30 min of exposure to high glucose and remains steady through 60 min. (Figure 8b). The caspase-9 cleavage corresponds to the time course of caspase-3 cleavage at this high glucose dose (Figure 8b). This indicates that glucose-mediated apoptosis in SH-SY5Y cells may occur through

regulation of the caspase-9-dependent pathway. Furthermore, these caspase-mediated events begin prior to mitochondrial disruption.

Finally, we examined the effect of IGF-I on glucose-mediated apoptosis in the C8DN and C9DN SH-SY5Y cells. IGF-I protects 60–75% of SH-SY5Y cells from glucose-mediated apoptosis in vector-transfected control cells (Figure 8a). Interestingly, the IGF-I and C8DN or C9DN constructs have an additive effect on protection, with the combination of both IGF-I and caspase inhibition protecting more than 96% of cells (Figure 8a). Therefore, IGF-I likely exerts protective effects on SH-SY5Y cells independent of initiator caspase regulation. To confirm these observations at the caspase-3 level, vector-transfected control, C8DN and C9DN cells were exposed to high glucose (325 mM) for 2, 4 and 6 h, time points prior to mitochondrial dysfunction. At each time point, caspase-3 is cleaved in all three cell lines with glucose exposure (Figure 8c). This observation suggests that caspase-3 is cleaved independent of caspase-8 or caspase-9 in SH-SY5Y cells. IGF-I prevents caspase-3 cleavage at every time point (Figure 8c), consistent with our previous results. Therefore, our studies suggest that caspase-3 is activated very early after glucose exposure, independent of caspase-8 or caspase-9 cleavage. An additional activation of caspase-3 occurs after MMD, which is dependent on PTP opening. Therefore, caspase-3 is activated in multiple, likely parallel, places along the apoptotic cascade in response to glucose exposure. However, IGF-I prevents caspase-3 activation in each case, providing the greatest amount of protection against glucose-induced apoptosis.

Discussion

Proteins involved in apoptosis are often altered in aggressive neuroblastoma tumors.^{4,51} In particular, the proapoptotic protein caspase-8 is frequently absent or inactive, and the antiapoptotic protein Bcl-2 is often upregulated.^{4,52} Therefore, understanding alternative methods of apoptosis induction in neuroblastoma cells is critical for future treatment development.

Recent studies indicate that mitochondria play a central role in most forms of apoptosis.^{24,25} For example, cytochrome *c* is lost by mitochondria to the cytosol where it acts as a cofactor for caspase activation. Also, Bcl-2 family proteins appear to carry out their functions, at least in part, by regulating $\Delta\Psi_M$ and mitochondrial volume.^{24,27,53} Moreover, kinetic data show that mitochondria undergo major changes in membrane permeability, polarity and volume prior to other well-recognized signs of apoptosis such as caspase activation and chromatin condensation.²⁵ In neuroblastoma, doxorubicin induces apoptosis in caspase-deficient cells through direct effects on the mitochondria.⁵⁴ Therefore, the current studies were carried out to determine the contribution of mitochondrial changes to apoptosis in neuroblastoma, with particular focus on identifying the cellular biochemical pathways that lead to apoptosis after high-glucose exposure.

In previous studies, we have shown that mannitol induces apoptosis in SH-SY5Y cells.^{5,18,41} However, high concentrations of mannitol are needed to induce apoptosis in these

cells.⁵ We and others have shown that even a mild increase in extracellular glucose (20 mM) enhances apoptosis in primary neuronal cells.^{44,47,48,55,56} Even at higher concentrations (> 150 mM), cell death is considerably higher with equiosmolar glucose than with mannitol, indicating that glucose induces cell death independent of an osmolar effect and can be used at lower concentrations to initiate apoptosis.^{44,47} Therefore, we used glucose as a cell death inducer in the current study, as we could use milder conditions to investigate more fully the apoptotic mechanisms activated. In this study, glucose clearly induces a dose-dependent increase in caspase-3 cleavage. With 45 mM total glucose used, in most of the experiments there is only a 5% increase in total osmolarity of the medium, insufficient to account for the observed apoptosis.⁴⁷

In this study, we investigated the effect of increased glucose on the mitochondria. Hyperpolarization of $\Delta\Psi_M$, an event associated with induction of apoptosis,^{44,57–59} is observed with addition of extracellular glucose and is maximal in this study at 3 h, corresponding to early cleavage of caspase-3 at the same time point. One of the key events preceding apoptosis is a change in PTP. Mitochondrial permeability transition is associated with opening of the ANT/VDAC channel spanning the inner and outer mitochondrial membranes. This results in osmotic swelling that in turn disrupts the integrity of the outer mitochondrial membrane,⁶⁰ and is associated with release of proapoptotic factors into the cytoplasm that activate the caspase cascade.²⁴ In contrast, inhibition of the ANT/VDAC channel stabilizes $\Delta\Psi_M$.⁴³ In this study, MMD induced by high glucose is blocked by BKA, a ligand for the ANT that inhibits opening of the ANT and stabilizes the inner mitochondrial membrane.⁴² Furthermore, BKA inhibits downstream cleavage of caspase-3 indicating that stabilization of $\Delta\Psi_M$ in the presence of high glucose is important in preventing apoptosis. One important association of increased stability of $\Delta\Psi_M$ is to prevent the loss of UCPs.

Glucose oxidation in the mitochondria leads to the supply of electrons to the electron transfer chain, leading to increased proton pumping from the mitochondria. Overproduction of electron donors by the tricarboxylic acid (TCA) cycle increases the proton gradient and results in an increased $\Delta\Psi_M$ that would predispose the cell to the induction of apoptosis. The UCPs are inner mitochondrial membrane proteins that dissipate the proton electrochemical gradient as heat, that is, they uncouple mitochondrial electron transfer from oxidative phosphorylation and regulate mitochondrial proton conductance.^{61,62} Furthermore, overexpression of UCPs prevents apoptosis induction.⁵⁷ UCP3 is expressed in skeletal muscle and neurons, and UCP2 in neurons.^{61,63,64} The UCPs also have sequence homology to mitochondrial transporters including the Bcl proteins, suggesting that they may be mitochondrial carriers.^{65–67} When UCP levels are reduced, $\Delta\Psi_M$ is abnormally high, increasing back pressure on the inner mitochondrial membrane proton pumps, events that may further promote induction of apoptosis. In this study, UCP3 protein levels are decreased in the presence of high glucose and maintained by the presence of BKA consistent with regulation of UCPs by $\Delta\Psi_M$. Interestingly in neurons, overexpression of UCPs prevents glucose-induced apoptosis,⁵⁷ suggesting that similar to the Bcl proteins, UCPs may act as important regulators of apoptosis.

Changes in PTP leading to mitochondrial swelling, MMD, cytochrome *c* release and eventual caspase activation may also be due to regulation of Bcl-2-related proteins. For example, Bcl-2 possesses BH1 and BH2 domains that may have a pore-forming function in the mitochondrial membrane. This would counteract apoptosis by facilitating the activity of PTP, whereas Bax promotes apoptosis by inhibiting PTP activity.^{27,28} This concept was supported by our findings that elevated glucose causes a reduction in the levels of Bcl-2, similar to our previous findings with mannitol exposure.¹⁷ However, Bcl-2 is also expressed at extramitochondrial sites and may separately regulate apoptosis through Apaf-1 and binding to the apoptosome.⁶⁸

Normally, Apaf-1 binds to cytochrome *c*, released from the mitochondria, and caspase-9, which leads to caspase-9 cleavage and activation of the downstream caspase cascade.^{33–36} Many neuroblastoma tumors and cell lines show N-Myc-independent defects in the caspase-8 signaling pathway, whereas the caspase-9 pathway remains intact,^{69,70} suggesting that targeting this pathway is important for chemotherapeutic treatment. In the current study, glucose treatment induces caspase-9 cleavage in neuroblastoma cells, and inhibition of this caspase prevents apoptosis. Caspase-8 inhibition also prevents apoptosis in a small percentage of cells, although no cleavage of this protein is detected. These results are similar to those seen in glucose-induced apoptosis in proximal tubular epithelial cells.⁷¹ Interestingly, C8DN cells show apoptosis inhibition even greater than C9DN in serum-containing conditions. Therefore, C8DN may prevent basal levels of apoptosis in SH-SY5Y cells.

In contrast to caspase-8, in our study, caspase-9 cleavage is regulated by glucose exposure, followed closely by caspase-3 activation at 30 min. Furthermore, inhibition of caspase-9 with the dominant-negative construct prevents over 80% of apoptosis in neuroblastoma cells. However, C9DN cells still show caspase-3 cleavage after glucose exposure, suggesting that caspase-3 may be cleaved independently of caspase-9 activation. Previous reports indicate that caspase-9 and -3 operate in a feedback amplification loop, such that initial low levels of activated caspase-9 lead to caspase-3 activation, but full caspase-9 processing is only achieved after caspase-3 activation; this in turn leads to additional caspase-3 activation.⁷² Therefore, we hypothesize that glucose induces caspase-3 activation through several parallel pathways, only one of which is caspase-9 dependent. Furthermore, we believe that caspase-3 and caspase-9 act in an amplification loop, ultimately ensuring the apoptotic death of glucose-exposed neuroblastoma cells. In contrast, Bcl protein alterations, which would destabilize the mitochondrial membrane allowing cytochrome *c* release, occur late in the apoptotic process. This suggests that glucose-mediated caspase-9 activation occurs independent of mitochondrial involvement or Bcl protein expression changes. Early caspase activation could be mediated through Bax translocation. Bax translocation from the cytosol to the mitochondria occurs within 30 min of staurosporine treatment in SH-SY5Y cells.⁷³ Bax translocation is also induced by hypoxia and ATP depletion in kidney cells⁷⁴ and by reactive oxygen species in cardiomyocytes.⁷⁵ Since reactive oxygen species are produced by hyperglycemia,⁷⁶ Bax translocation

is a good candidate for an early event in glucose-mediated apoptosis. Upon insertion into the mitochondrial membrane, Bax may mediate cytochrome *c* release, an event that can occur without permeability transition.^{43,73,77} We are currently investigating Bax translocation and cytochrome *c* release in glucose-mediated apoptosis in neuroblastoma cells.

IGF-I signaling through the IGF-IR is important in cellular transformation and the proliferation of tumor cells, while disruption of the IGF-IR reverses the transformed phenotype.^{7,9–11} Therefore, targeting the IGF-IR provides a novel therapeutic approach for cancer treatment.^{12,20,21} In neuroblastoma, IGF-I leads to increased cell growth and survival.^{6,10,13,14,16,17} Our current studies indicate that IGF-I is neuroprotective by blocking mitochondrial swelling, MMD and caspase-3 activation. IGF-I prevents mitochondrial events induced by several stressors in SH-SY5Y neuroblastoma cells. Mannitol-mediated MMD and subsequent apoptosis are inhibited by IGF-I.⁶ IGF-I also protects SY5Y cells from peroxynitrite-induced apoptosis by preventing cytochrome *c* release and caspase-3 activation.⁷⁸ Finally, IGF-I induces UCP3 expression,⁷⁹ which could counteract the effect of glucose on this protein.

Two important IGF-I signaling pathways, PI3K and MAPK, are implicated in the regulation of apoptosis.^{80,81} While there is previous evidence that IGF-I regulates $\Delta\Psi_M$,⁶ this study shows regulation of $\Delta\Psi_M$ and mitochondrial enlargement by discrete signaling pathways. Overall, the data in this study indicate that the PI3K pathway is responsible for the inhibition of glucose-mediated MMD and apoptosis. There is further support for PI3K and downstream Akt regulation of Bcl-2 in neuronal cells; LY294002 blocks the ability of IGF-I to maintain Bcl-2 expression during apoptosis,⁸² while a dominant-negative form of Akt eliminates the protective effects of IGF-I.⁸² Akt also acts downstream of IGF-I to prevent cytochrome *c*-induced caspase-3 activation in peroxynitrite-treated SH-SY5Y cells.⁷⁸ In mesangial cells, IGF-I prevents glucose-mediated apoptosis and mitochondrial changes via both the PI3K and MAPK pathways.⁸³ However, in our study, inhibition of the MAPK/MEK pathway independently and partially blocked IGF-I inhibition of MMD, but did not significantly affect IGF-I mitochondrial enlargement, indicating that IGF-I regulates different components of mitochondrial function through discrete signaling pathways.

Collectively, our studies indicate that changes in $\Delta\Psi_M$ are central to apoptosis and induced by increases in extracellular glucose. This change in the function of PTP is linked to a reduction in UCP2 and 3, altered Bcl-2 activity and activation of caspases. In contrast, IGF-I regulates $\Delta\Psi_M$ and glucose-induced apoptosis via discrete signaling pathways. Together, our results suggest that the following pathway contributes to glucose-induced apoptosis in neuroblastoma: (i) caspase-9 is activated; (ii) PTP function is reduced; (iii) mitochondrial membranes are disrupted, releasing proapoptotic proteins and activated caspases, initiating the execution phase of apoptosis; and (iv) Bcl-2 protein levels are altered, ensuring an apoptotic death. Therefore, initiation of this cascade of events could promote apoptosis in aggressive neuroblastoma tumors resistant to other treatment strategies.

Materials and Methods

Materials

Tissue culture plastic was purchased from Corning (Corning, NY, USA). Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), trypsin-EDTA and calf serum (CS) were purchased from Gibco BRL (Gaithersburg, MD, USA). IGF-I was provided by Cephalon, Inc. (Westchester, PA, USA). BKA, LY294002, PD98059 and U0126 were from Biomol (Plymouth Meeting, PA, USA). The caspase-3 fluorometric assay kit was from PharMingen (San Diego, CA, USA). Bcl-2 antibodies were obtained from Santa Cruz (SC-7382, Santa Cruz, CA, USA), caspase-3 antiactive rabbit polyclonal antibodies (Asp 175) and caspase-9 antibodies were from Cell Signaling (Beverly, MA, USA), caspase-8 antibody was from Calbiochem (Cat #218778; San Diego, CA, USA), UCP3 antibodies were from Research Diagnostics Inc. (Flanders, NJ, USA) and GAPDH monoclonal antibody was from Chemicon (Temecula, CA, USA). Enhanced chemiluminescence (ECL) reagents and molecular weight standards were from Amersham (Arlington Heights, IL, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

SH-SY5Y human neuroblastoma cells were grown in DMEM with 10% calf serum at 37°C in a humidified atmosphere containing 10% CO₂ as described previously.⁵ In experiments, cells were subcultured in DMEM alone with the experimental condition. Control DMEM contains 25 mM basal glucose; all additional glucose concentrations are expressed as total glucose levels (25 mM control glucose concentration + exogenous glucose concentration). SH-SY5Y cells were stably transfected with pCDNA3 vector control, dominant-negative caspase-8 (C8DN) or dominant-negative caspase-9 (C9DN) constructs, kindly provided by Dr. Vishva Dixit, Genentech (CA), formerly of the University of Michigan. These dominant-negative constructs contain point mutations at the active cysteine site to an alanine residue, resulting in caspases that can be cleaved, but are not capable of cleaving downstream targets.^{84,85} Cells were selected for at least 1 month in DMEM + 500 μM G418 prior to use.

Isolation of mitochondrial and cytosolic fractions

Differential centrifugation of SH-SY5Y cells was used to isolate mitochondrial and cytosolic fractions as described previously.²⁷ For FACS analysis, the following isolation method was used: cells were lysed with a tissue tearator while suspended in ice-cold buffer A containing 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 μg/ml phenylmethylsulfonyl fluoride, 8 μg/ml aprotinin and 2 μg/ml leupeptin at pH 7.4. Unlysed cells and nuclei were pelleted at 750 × *g* for 10 min, then the supernatant was spun at 10 000 × *g* for 25 min and the pellet was resuspended in buffer A. This represents the mitochondrial fraction. The supernatant is then spun at 100 000 × *g* for 1 h, and the supernatant from this spin represents the cytosolic fraction.

Analysis of mitochondrial membrane polarity in whole cells

SH-SY5Y cells were plated at 1 × 10⁵ cells/cm² in six-well plates. After reaching approximately 90% confluence, cells were placed in serum-free DMEM and the experimental condition. Rh123 is a potentiometric dye that

is preferentially taken up by mitochondria. A decrease in Rh123 levels is consistent with MMD. Cells were then incubated with 5 μg/ml Rh123 for 30 min at 37°C. Next, cells were removed from plates with trypsin-EDTA and combined with medium to collect all cells. Following centrifugation at 500 × *g*, the trypsinized cells were washed with HBSS (without Ca²⁺, Mg²⁺ and phenol red). The cells were then stained for 15 min with 18 μg/ml PI in the presence of 40 μg/ml RNase A, followed by washing in HBSS. Live cells were then analyzed using an Epics Elite FACS system (Coulter Cytometry, Hialeah, FL, USA) reading Rh123 (absorbance: ± 480 nm, emission: ± 530 nm), and using gating against PI to indicate PI exclusion (live cells).

Determination of mitochondrial size using FLS

Mitochondria were isolated as described above, stained with 2.5 μg/ml Rh123 in buffer A for 30 min, and then washed with cold MSH buffer (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 0.2 mM EGTA, 5 mM succinate, 0.15% BSA, 5 μM rotenone, 0.01% saponin) to stabilize the mitochondria prior to FACS analysis. The mitochondria were repelleted at 10 000 × *g*, resuspended in MSH buffer, and FACS analysis with FLS was performed as described previously.²⁷ Measurements from experimental mitochondria were compared against control mitochondria, and 1–10 μm measurement beads. Mitochondria were examined histologically to confirm pure isolates and to confirm morphological integrity of the inner and outer membranes.

Immunoblotting for caspases, Bcl and UCP proteins

SH-SY5Y cells were plated at an initial density of 5 × 10⁶ cells/cm² and grown to approximately 90% confluence. Cells were washed twice with DMEM before experimental treatments. Western blots were performed as described previously⁸⁶ using 30–75 μg of total protein for each sample. The same quantity of protein was loaded in each well. Anti-Bcl-2 immunoblotting was performed using 0.1 μg/ml rabbit polyclonal antibody. Anti-caspase-3 polyclonal antibody was used at a 1 : 1000 dilution for 2 h and probed with secondary goat anti-rabbit at a dilution of 1 : 2500 for 1 h. UCP3 polyclonal antibody was used at a 1 : 1000 dilution, and secondary goat anti-rabbit at 1 : 2500. GAPDH monoclonal antibody was used at a dilution of 1 : 300. Immunoblots were exposed following ECL reaction (Amersham, Arlington Heights, IL, USA) to autoradiography film (Hyperfilm-MP, Amersham). Immunoblots shown are one of at least three independent experiments. GAPDH antibody (36 kDa) was used as a loading control, and was applied to the same blot for antiactive caspase-3. Blots for Bcl-2 and UCP3 were stripped and reprobed with anti-GAPDH.

Caspase-3 activation assay

A caspase-3 fluorometric assay kit from Pharmingen (San Diego, CA, USA) was used for the determination of caspase-3 activation within cells. The assay was conducted according to the manufacturer's instructions⁸⁷ and in conjunction with an Epics Elite flow cytometry system using excitation at 380 nm and emission at 440 nm. All experiments were conducted with the following controls: cell lysate alone, Ac-DEVD-AMC alone, non-apoptotic cells and positive apoptotic controls. Caspase cleavage was gated against PI (to determine cell viability) using FACS, and the mean of the peak caspase-3 level and percent cells expressing cleaved caspase-3 were measured.

Flow cytometry

Analysis of DNA content was performed using flow cytometry. Cells were plated in six-well plates at 1×10^5 cells/cm². After reaching near confluency, cells were serum deprived for 4 h and exposed to experimental conditions for the indicated times. Cells were removed from the plates with trypsin-EDTA, rinsed in HBSS, fixed in ice-cold 70% ethanol and stored at 4°C. Cells were stained for 2–12 h with 18 µg/ml PI and 40 µg/ml RNase A at 4°C. DNA content of PI-stained cells was measured and separated into phases of the cell cycle based on the PI fluorescence. Percent apoptotic cells in all cases was taken as percent sub-g₀ DNA as measured on an Epics Elite flow cytometry system (Coulter Cytometry, Hialeah, FL, USA). All results are expressed as the mean percent apoptotic cells of three experiments $\pm$ standard error of the mean (S.E.M.).

Statistical analysis

Assumptions about the Gaussian distribution of data and rules for transformation of non-normative data were made as described previously.⁴⁷ Comparison of dependent variables was performed using factorial analysis of variance (ANOVA). Unless otherwise indicated, experiments were replicated at least three times, and data pooled after standardization against matching control values. An observer blinded to the experimental condition made measurements. For graphic and descriptive purposes, data are expressed as value $\pm$ S.E.M.

Acknowledgements

This work was supported in part by NIH NS42056, The Juvenile Diabetes Research Foundation Center for the Study of Complications in Diabetes (JDRF), Office of Research Development (Medical Research Service), Department of Veterans Affairs (JWR); NIH NS36778, NIH NS38849, and grants from the JDRF and Program for Understanding Neurological Diseases (ELF); NIDDK #P60DK-20572 – Michigan Diabetes Research and Training Center; University of Michigan Core Flow Cytometry facility (supported in part by the UM-Comprehensive Cancer Center NIH P30 CA46592 and the UM-Multipurpose Arthritic Center NIH AR20557). We extend our appreciation to Dr. T Schwab, Ms. K Cherian and Mr. A. Parekh for assistance with experiments and to Dr. B Kim for aid in manuscript preparation.

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