Invited Symposium: Reactive Oxygen Species and Neurodegenerative Diseases *(7 Presentations in this Symposium)*

Reactive Oxygen Species, Apoptosis and Altered NGF-Signaling in PC12 Pheochromocytoma Cells Cultured in Elevated Glucose: An *in vitro* Cellular Model for Diabetic Neuropathy

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ABSTRACT

Diabetic neuropathies, affecting the autonomic, sensory, and motor peripheral nervous system, are among the most frequent complications of diabetes. The symptoms of diabetic polyneuropathies are multifaceted; the etiology and the underlying mechanisms are as yet unclear. Clinical studies established a significant correlation between the control of the patients´ blood glucose level and the severity of the damage to the peripheral nervous system. Recent in vitro studies suggest that elevated glucose levels induced dysfunction and apoptosis in cultured cells of neuronal origin, possibly through the formation of reactive oxygen species (ROS). Based on these results, we hypothesized that elevated glucose levels impair neuronal survival and function via ROS dependent intracellular signaling pathways. In order to test this hypothesis, we cultured neural crest-derived PC12 pheochromocytoma cells under euglycemic (5 mM) and hyperglycemic (25 mM) conditions. Continuous exposure of undifferentiated PC12 cells for up to 72 h to elevated glucose induced the enhanced generation of ROS, as assessed from the increase in the cell-associated fluorescence of the ROS-sensitive fluorogenic indicator, 2,7-dichlorodihydrofluorescein diacetate. In cells cultured in high glucose, both basal and secretagogue-stimulated catecholamine release were enhanced. Furthermore, high glucose, reduced (by ca. 30%) the rate of cell proliferation and enhanced the occurrence of apoptosis, as assessed by DNA fragmentation, TUNEL assay and the activation of an apoptosis-specific protease, caspase CCP32. Elevated glucose levels significantly attenuated nerve growth factor (NGF)-induced neurite extension, as quantitated by computer-aided image analysis. Culturing PC12 cells in high glucose resulted in alterations in basal and NGF-stimulated mitogen-activated protein kinase (MAPK) signaling pathways, specifically in a switch from the neuronal survival/differentiation-associated MAPK ERK to that of apoptosis/stress-associated MAPK p38 and JNK. Based on our results we present a model in which the prolonged, excess formation of ROS represents a common mechanisms for hyperglycemia-induced damage to neuronal cells. We propose that this simple in vitro system might serve as an appropriate model for evaluating some of the effects of elevated glucose on cultured cells of neuronal origin.

Keywords: PC12 pheochromocytoma - glucose - MAP Kinase - ROS - diabetic neuropathy -

Discussion Board

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INTRODUCTION

Diabetes mellitus is an endocrine disease characterized by the inability of the pancreas to secrete enough insulin to maintain physiological levels of blood glucose. Diabetic neuropathies, a complex array of nerve disorders which affect the autonomic, sensory, and motor peripheral nervous system, are among the most frequent late complications of diabetes, affecting some 60-70 % of all diabetic patients. The symptoms of diabetic neuropathies are multifaceted and involve both destruction of peripheral nerves and neuronal hyperactivity [13,18].

In support of the clinical observation that the on-set and progression of diabetic neuropathies can be mitigated by proper control of the blood sugar levels, recent studies suggest that elevated blood glucose (hyperglycemia) is a major cause for damage to the nervous system [23]. Hyperglycemia may act during embryogenesis by inducing defects in the development of the neural tube [38,43,44]. Also, in experimental rat models of diabetes hyperglycemia was found to affect nerve conduction velocity in patients [31] and to alter the pattern of neurotransmitter release in the adult cerebral cortex [15].

The mechanisms underlying these pathological changes are as yet obscure, but hyperglycemia-induced neuronal damage may result from the induction of programmed cell death, or apoptosis [44]. High among the possible damaging mechanisms ranks the hyperglycemia-induced non-enzymatic modification of sugar moieties on proteins and lipids, which leads the formation of advanced glycosylation end-products (AGEs). AGEs are involved in the possible disturbances of carbohydrate-, protein-, and lipid-metabolism. Importantly, AGEs generate Reactive Oxygen Species (ROS), suggesting that hyperglycemia causes oxidative damage to the cells through NF-kB dependent pathways [11,34]. AGEs and ROS-induced cellular dysfunctions can interfere with gene expression of peptides and cytokines involved in the regulation of cell proliferation [7]. Extensive production of ROS may mediate a signal for apoptotic cell death [16].

Apoptosis is a complex, highly controlled process which results in programmed cell death [32,33]. Along the way, there are several characteristic stepping stones/indicators, which will distinguish apoptosis from necrosis (chaotic cell death due to overt injury). One of the early markers for apoptosis is the activation of specific the caspases ,such as caspase 3 (CPP32), which are members of the Ced/ICE family of cystein proteases [22]. Progressive DNA fragmentation is a marker for the later stages of apoptosis. There is increasing evidence for the enhanced induction of apoptosis of numerous cell types in diabetes including neuronal cells [3,40,59].

Recent studies established an in vivo link between diabetes and apoptosis in the nervous system [3,44,49]. Some of these studies suggest that hyperglycemia- induced apoptosis in neurons may be driven by the formation of ROS [7]. In the past, the cellular mechanisms involved in the pathophysiology of diabetes have been tested in several *in vitro* models, most notably in cultured vascular endothelial cells [4,37,54], fibroblasts [2] and kidney cells [41]. To date, there are only very few models describing the effects of hyperglycemia on neuronal cell function [6,27]. Most recently, Feldman and coworkers, in corroborating in part some of our previously presented results (E. Lelkes et al., American Academy of Neurology 49th Annual Meeting, 1997 Boston, MA), described the elevated glucose inhibits neurite outgrowth and induces apoptosis in cultured rat dorsal root and that these changes can be prevented by addition of exogenous insulin-like growth factor I [48,49].

In this study, neural-crest derived PC12 pheochromocytoma cells [56], a well established model for neuronal differentiation and neurotransmitter secretion, were used to examine the role of hyperglycemia on several aspects of diabetic neuropathy, such as enhanced apoptosis, generation of free radicals and altered neurotransmitter release. Undifferentiated PC12 cells proliferate, just like embryonic neuronal cells. However, in the presence of neurotrophic growth factors, such as Nerve Growth Factor (NGF), these cells cease to proliferate and differentiate into sympathetic neurons [17]. The cellular signaling mechanism by which NGF binds to specific receptors (trk and p75) and promotes neuronal differentiation and survival (through trk), but also can initiate apoptosis (through p75), is under intensive investigation [26].

These two aspects of neurotrophin signaling involve the activation of different branches of the MAP - Kinase signaling pathway. MAP-Kinases represent a central "switchboard" of intracellular signal transduction pathways [8]. In addition to the "classical" MAPK-pathway involving the growth-factor stimulated activation (phosphorylation) of the extracellular signal regulated kinase (ERK), several other MAP kinase subclasses, such as c-Jun-N-terminal kinase/stress activated protein kinase (JNK/SAPK) and p38, have recently been identified. These kinases are activated by stress and may lead, by as yet unknown mechanisms, to apoptosis [28]. NGF- signaling involves activation of these three different MAP-Kinase pathways which can either result in differentiation and cell survival, mediated through the ERK pathway, or lead to apoptosis, through the p38 and the JNK/SAPK pathways [26]. Conversely, induction of apoptosis in differentiated PC12 cells results in altered MAP-kinase activation [10].

Catecholaminergic in nature, PC12 cell serve also as an establish model system for studying mechanisms of exocytotic neurotransmitter secretion [1]. In this process, membrane depolarizing agents, such as potassium, or specific receptor agonists, such as nicotine, stimulate the regulated secretion of neurotransmitters, in which storage vesicles containing catecholamines fuse with the plasma membrane and release their contents into the outer environment [21]. Several of the proteins involved in this tightly regulated process require activation by phosphorylation [50,52]. Previous studies suggest that neurotransmitter release may be compromised in diabetes [58].

Our working hypothesis is that hyperglycemia causes diabetic polyneuropathies by a mechanism which involves generation of ROS and alterations in the MAPK- dependent cellular signal transduction pathways leading to apoptosis. We further hypothesized that some of the symptoms of diabetes-related neuronal dysfunction, such as axonal/neurite degeneration and apoptosis can be reproduced by culturing neural crest-derived PC12 pheochromocytoma cells in the presence of elevated levels of glucose.

MATERIALS AND METHODS

1. Cell Culture: PC12 rat pheochromocytoma cells, were maintained in Dulbecco´s Modified Eagle´s Medium DMEM-LG) containing 0.1 % (5 mM) glucose and supplemented with 7.5% horse serum, 7.5% fetal calf serum, 2.0 mM L-glutamine, 100 U/ml penicillin and 100µg/ml streptomycin, as previously described [29]. For some of the experiments, the cells were adapted to grow in DMEM containing (0.5% , viz. 25 mM) glucose (DMEM-HG). Intermediate glucose levels were obtained by adding aliquots from a 10 % glucose stock solution to DMEM-LG to attain glucose levels of 0.2 - 0.5 % . Neuronal differentiation was induced by addition of 50 ng/ml nerve growth factor (NGF, gift from Dr. Philip Lazarovici, Hebrew University, Jerusalem).

2. Cell Proliferation Assays: For each experimental point, 50,000 PC12 cells were plated in the appropriate medium into one well of a 24 well tissue culture plate. After three days, cell proliferation was determined using two independent techniques based on fluorescent quantitation of, respectively, cellular metabolic activity (Alamar Blue assay) [39] and DNA content (Bisbenzimide assay) [42].

3. Morphometric Assessment of PC12 Cell Differentiation: To assess the effects of elevated glucose on NGF-induced neurite extension, 10,000 PC12 cells were plated in 6 well plates in control medium (0.1 % glucose) . After 24 h the control medium was exchange to medium containing 0.1 -0.5% glucose and 50 ng/ml NGF . The cultures were re-fed 2 days later with the diverse, NGF-containing media. Neurite length was determined from micrographs taken on day 5 from three random fields/well. To measure the complexity of established neuronal networks, the cells were plated as above and maintained in control medium (0.1% glucose) for 10 days in the presence of 50 ng/ml NGF until a complex neuronal network was established, At that point, the medium was replaced with experimental media containing NGF and the various glucose levels. After another 4 days, the complexity of the neuronal networks was determined from micrographs taken form 3 random fields from each well. All micrographs were analyzed by computer-aided video microscopic image analysis using a PC-based software program (Image Pro), evaluating the average length, total length, average width of each neurite, as well as the total area covered with neurites in a given field.

4.DNA Fragmentation Analysis: To assess the effects of various glucose levels on DNA fragmentation, PC12 cells were grown for 10 days in either DMEM-LG or DMEM-HG. At the end of the experiments, DNA was isolated using a commercial kit (Puregene, by Gentra, Minneapolis) according to the manufacturer´s instructions. The degree of DNA fragmentation was evaluated by agarose gel-electrophoresis according to a published procedure [35].

5. TUNEL Assay: To ascertain that the observed glucose-induced fragmentation of DNA was due to the induction of apoptosis, 10,000 PC12 cells were plated into poly-ornithine-coated tissue culture/ chamber slides (from Nunc) and grown for 7 days in either DMEM-LG or DMEM-HG in the presence or absence of 50 ng/ml NGF. Apoptosis was assessed using a commercially available kit (Apoptag *in situ* apoptosis kit, from Oncor) according to the manufacturer´s instructions.

6. Activation of CPP32: To assess the involvement of apoptosis -specific proteases in the high-glucose mediated apoptosis of PC12 cells, we determined the level of caspase CCP32 activity in cells grown for 7 days in either high or low-glucose containing medium in the presence or absence of NGF. Caspase activity was determined by using the ApoAlert™ assay kit form Clontech, according to the manufacturer´s protocol. Fluorescence readings were taken in a miniplate reader (Cytofluor from Millipor) with excitation/emission filters set at 360 and 530 nm, respectively.

7. Catecholamine Release: PC12 cells were plated (500,000 cells/well) into poly-ornithine(0.1 %) coated 24 well tissue culture plates and grown for 48 h in either DMEM-LG or DMEM-HG. Prior to the experiments, the cells were loaded overnight with 0.5 µCi [^{3} H]/well tyrosine, the precursor molecule for catecholamines [30]. Catecholamine release, basal and following stimulation for 15 min with either 50 mM potassium (K^+), a general depolarizing agent, or 50 µM nicotine, a nicotinic receptor agonist, was determined as previously described [1]. To facilitate comparison between individual experiments, the amounts of catecholamine released is given as a percent of total cellular content.

8. Cell Signaling: In order to test the hypothesis that elevated glucose levels might affect intracellular signaling, PC12 cells were grown for 3 days in either DMEM-LG or DMEM-HG and stimulated for 30 min with 50 ng/ml NGF. Subsequently, the activity, viz. the level of tyrosine phosphorylation, of the extracellular-receptor-regulated MAP kinase (ERK-1) as well as of two other, recently identified subgroups of MAP kinases involved in cellular stress/apoptosis responses (p38 and JNK), were analyzed by immunoprecipitation and western blotting as previously described [47].

9. Generation of Reactive Oxygen Species: The formation of reactive oxygen species (ROS) was measured by using the ROS sensitive dye, 2,7-dichlorodihydrofluorescein diacetate (DCF), from Molecular Probes) as an indicator [60]. For visualization purposes, PC12 cells were plated (50/000/well) in 35 mm cell culture dishes. For quantitative analysis the cells were plated at higher concentrations (250,00/well) in poly-ornithine-coated 24 well plates. The cells were grown for 3 days in DMEM-LG and DMEM-HG, respectively. Prior to the experiments, the medium was replaced with phenol-free M199 medium, containing either 0.1% or 0.5% glucose, respectively. Control cells were pre-incubated for 10 min with the ROS scavenger pyrollidine dithiocarbamate (PDTC, 100 µM) to obtain the "background" fluorescence. Subsequently all cells were incubated for 15 min with 20 µM DCF. At the end of the experiments all cells were washed with M199 containing 100 µM PDTC to stop the further formation of ROS. Cell associated fluorescence was visualized in a Nikon Microphot fluorescence microscope, or measured in a fluorescent microplate reader (Cytofluor from Millipore) using the appropriate fluorescein excitation/emission filter combinations. For normalization purposes, the cells were incubated at the end of the experiments in the latter study with Alamar Blue and the cell numbers determined, as described above.

10. Statistical Evaluation of the Data: Unless indicated otherwise, all experiments were repeated at least 3 times. For each experimental point, at least 3 wells (6 wells for the proliferation assays) from each experiment were analyzed. In plotting the results, the date were, in most cases, normalized to the control values (0.1% glucose).Unless stated otherwise, all data are given as means + standard deviation. The statistical significance between the data was evaluated by ANOVA (analysis of variance) using the Instat software program (Graphpad Inc.). A P value of <0.5 was accepted as statistically significant.

RESULTS

Effect of Glucose on PC12 Cell Proliferation: The proliferation of undifferentiated PC12 cells decreased by approximately 35% when the glucose concentration was increased from 0.1% to 0.3% (Figure 1)

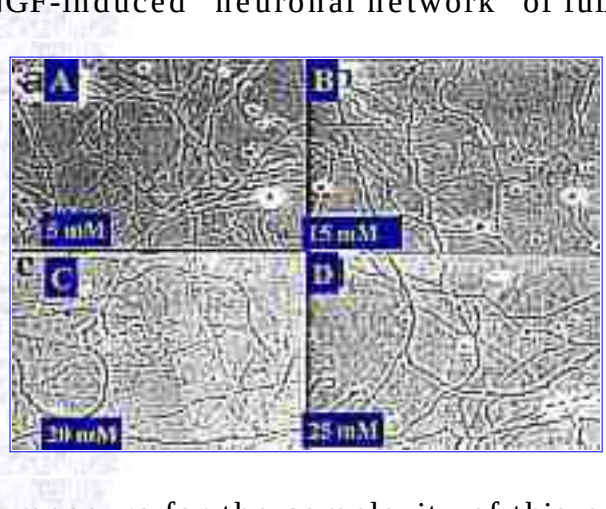

Figure 1: Effects of elevated glucose levels on PC12 cells proliferation. Undifferentiated PC12 cells were cultured for 3 days in media containing various glucose levels. After 3 days, the number of cells in each well was determined using the Alamar Blue assay. The data, obtained from three independent experiments, carried out in triplicates, were averaged and normalized to the controls (0.1% glucose). * P<0.05

To examine the possible effects of osmotic pressure on the cell proliferation, the culture media were supplemented with, respectively, 25 mM NaCl and 25 manitol, rather than with 0.5% (or 25 mM) glucose. The rate of PC12 cell proliferation in these controls was identical to that of untreated controls grown in DMEM-LG, thus ruling out a possible artifact due to changes in the osmotic pressure (data not shown).

Effects of Elevated Glucose Levels on Neuronal Differentiation: After 4 days exposure to 50 ng/ml NGF in media containing various glucose concentrations, the length of the ensuing neurites was decreased with increasing glucose concentration (Figure 2).

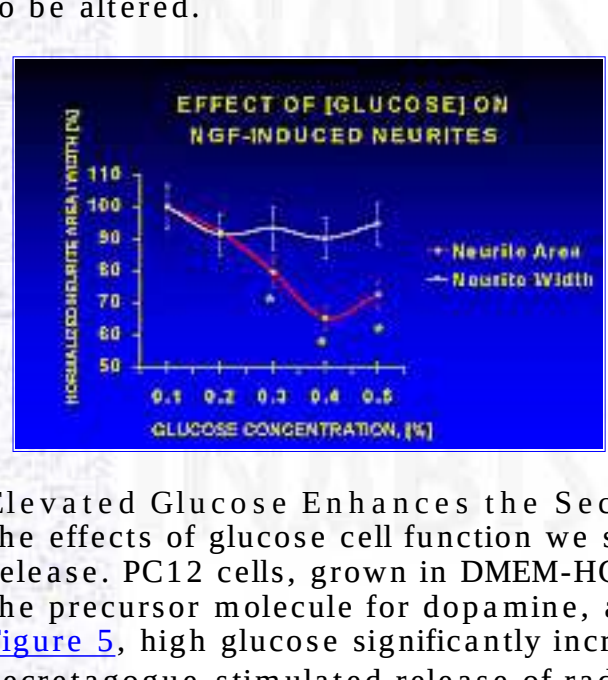

Figure 2: Effects of elevated glucose on neurite extension from PC12 cells. PC12 cells w ere cultured in the presence of 50 ng/ml NGF in media containing various glucose levels, described in Materials and Methods. 4 days after addition of NGF, neurite extension was analyzed morphometrically from phase contrast photo micrographs of 3 independent experiments, performed in triplicate. The data (Mean ± SEM) is normalized to neurite length in 01.% glucose. . * p<0.05

Furthermore, increasing glucose levels diminished the complexity (density) of a NGF-induced "neuronal network" of fully differentiated PC12 cells (Figure 3).

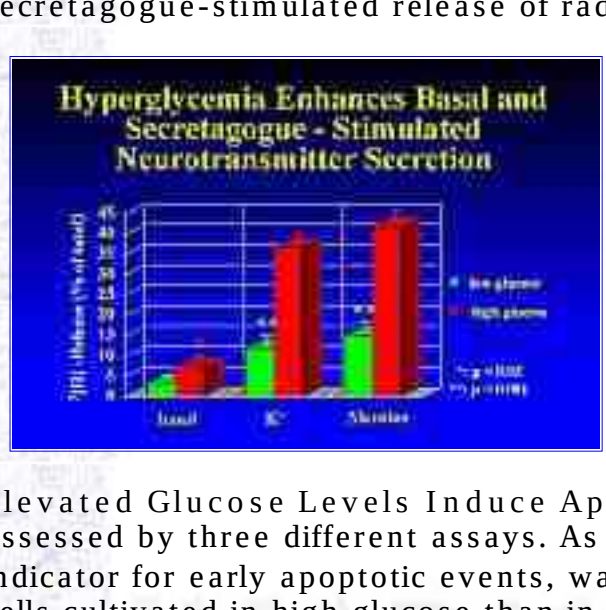

Figure 3: Effects of Glucose on Neuronal Networks. PC12 cells were grown in control medium (0.1% glucose) and differentiated for 10 days with 50 ng/ml NGF. Thereafter, the glucose level s were changed and the cells, in the continuous presence of NGF, maintained for 4 more days at 5 mM (0.1%), Panel A ; 15 mM (0.3%), Panel B ; 20 mM (0.4%), Panel C ; and 25 mM (0.5%), Panel D . Original magnification of these computer enhanced micrographs: 125 x.

A measure for the complexity of this network is the fractional (%) area, which is occupied by neurites in each field. As seen in Figure 4, the total area occupied by neurons was reduced by approximately 35% when glucose levels were increased from 0.1% to 0.4%.By contrast, the width of the "surviving" individual neurites seemed not to be altered.

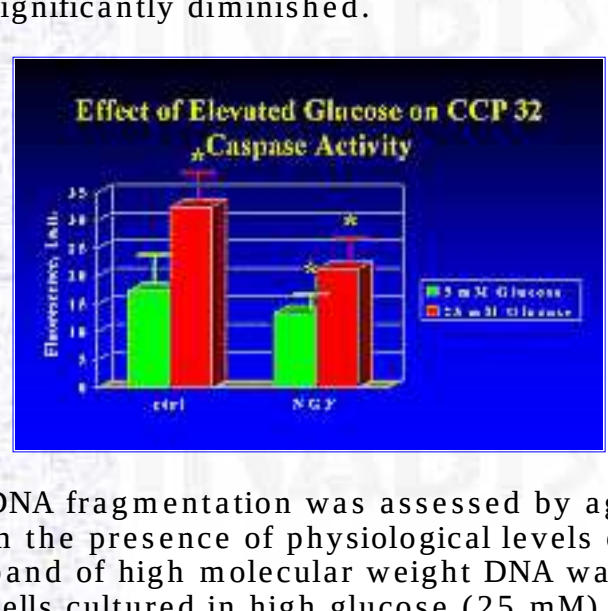

Figure4: Effects of Various Glucose Levels on Neuronal Networks: Quantitative Evaluation by Computer-Aided Image Analysis. Elevated glucose levels decrease the total area occupied by neurites in a dose-dependent fashion, but do not affect their width. For details see methods. Data mean ±SEM, nomalized to values at 0.1 % glucose * p < 0.05, n =5

Elevated Glucose Enhances the Secretory Competence of PC12 Cell: To test for the effects of glucose cell function we studied basal and stimulated neurotransmitter release. PC12 cells, grown in DMEM-HG or DMEM-LG, were loaded with [3H]-tyrosine, the precursor molecule for dopamine, as detailed in Materials and Methods. As seen in Figure 5, high glucose significantly increased both the basal and secretagogue-stimulated release of radioactivity.

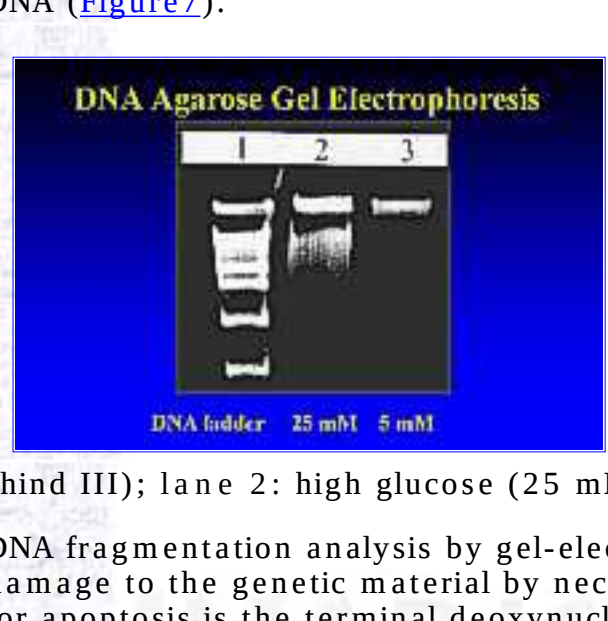

Figure 5: Effects of Glucose on Catecholamine Secretion. PC12 cells, loaded with [3H] - tyrosine, were stimulated for 15 minutes with either nicotine (50 µM) or K+ (50 mM), or neither (basal). Catecholamine release was normalized to total catecholamine contents. The data indicate means ± standard deviation of duplicate experiments, with 8 wells per data point.

Elevated Glucose Levels Induce Apoptosis: The occurrence of apoptosis was assessed by three different assays. As seen in Figure 6, the activity of CPP32, an indicator for early apoptotic events, was about 2 fold higher in undifferentiated PC12 cells cultivated in high glucose than in low glucose (p < 0.05). In the presence of NGF, caspase activity in both low glucose and, more significantly in high glucose was significantly diminished.

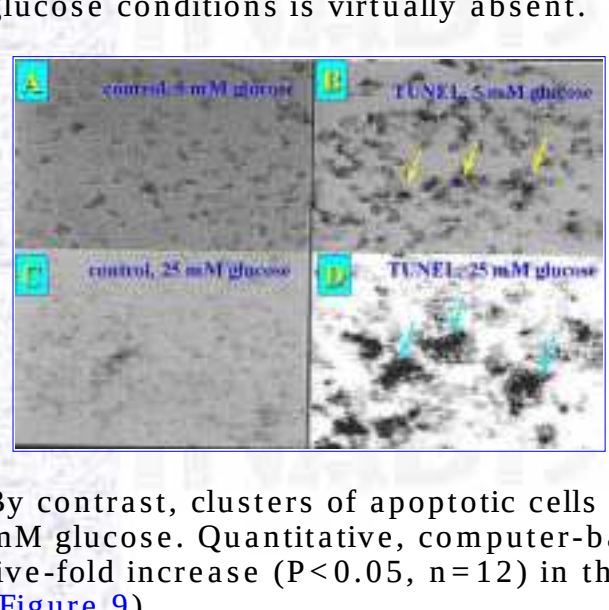

Figure 6: Analysis of Apoptosis by CPP32 Protease. PC12 cells were cultured for 7 days in high and low glucose in the presence or absence of 50 ng/ml NGF. CPP32 assay was used based on the ApoAlert kit from Clontech.. The data, normalized to CPP32 activity in control low glucose cells, represent means ± SEM from two independent experiments carried out in triplicate. *: P<0.05;

DNA fragmentation was assessed by agarose gel electrophoresis. In PC12 cells cultured in the presence of physiological levels of glucose (5 mM), only a single, well defined band of high molecular weight DNA was visible. In contrast, DNA isolated from PC12 cells cultured in high glucose (25 mM) contained not only intact but also fragmented DNA (Figure7).

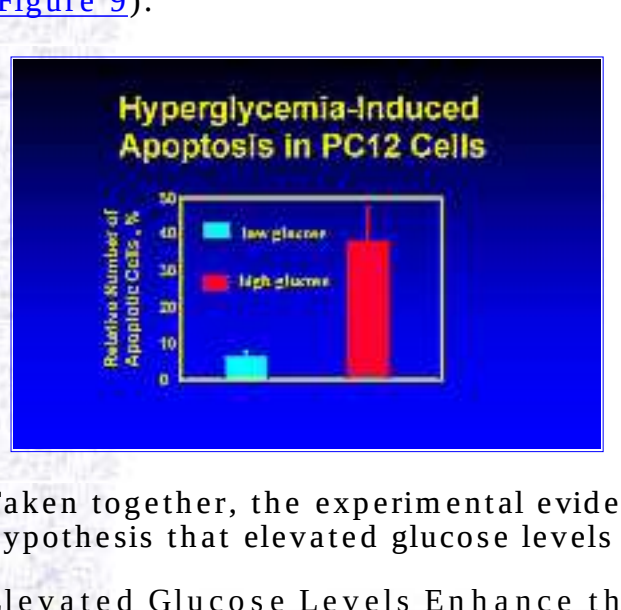

Figure7: Glucose-Induced DNA Fragmentation Total genomic DNA was isolated from several independent experiments, in which PC12 cells were grown for 10 days in either low or high glucose. Total DNA was analyzed by agarose gel electrophoresis and, after ethidium bromide staining, visualized under UV illumination. The figure is representative of 6 experiments, all of which had similar results. lane 1: standard whale sperm DNA, digested with an endonuclease (hind III); lane 2: high glucose (25 mM); lane 3: low glucose (5 mM).

DNA fragmentation analysis by gel-electrophoresis does not discriminate between damage to the genetic material by necrosis or by apoptosis. A more suitable indicator for apoptosis is the terminal deoxynucleotidyl (TdT) dUTP nick-end labeling method (TUNEL assay). As seen in Figure 8, apoptosis in PC12 cells grown under physiological glucose conditions is virtually absent.

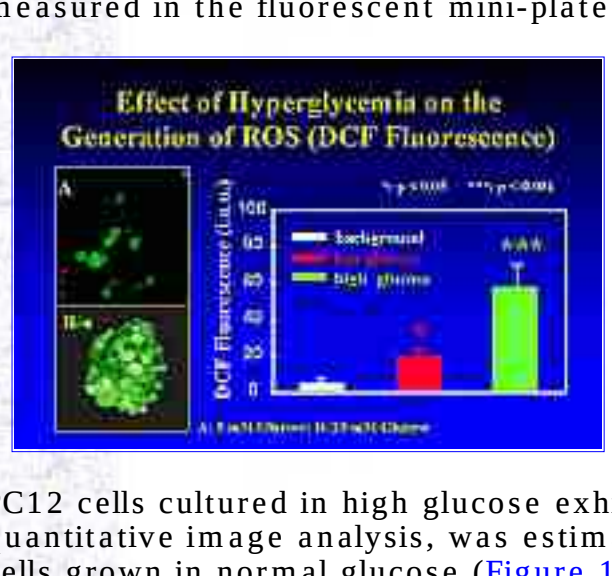

Figure 8: Hyperglycemia-Induced Apoptosis. The occurrence of apoptosis in PC12 cells cultured for 7 days in, respectively, high and low glucose containing media, was analyzed by in situ TUNEL labeling (Apoptag assay). Panel A: low glucose control (background, no TdT); Panel B: low glucose Panel C: high glucose control (background, no TdT); Panel D: high glucose. Original magnification: 250x

By contrast, clusters of apoptotic cells are clearly discernable in PC12 cells grown in 25 mM glucose. Quantitative, computer-based image analysis suggests an approximately five-fold increase (P<0.05, n=12) in the number of apoptotic cells in high glucose (Figure 9).

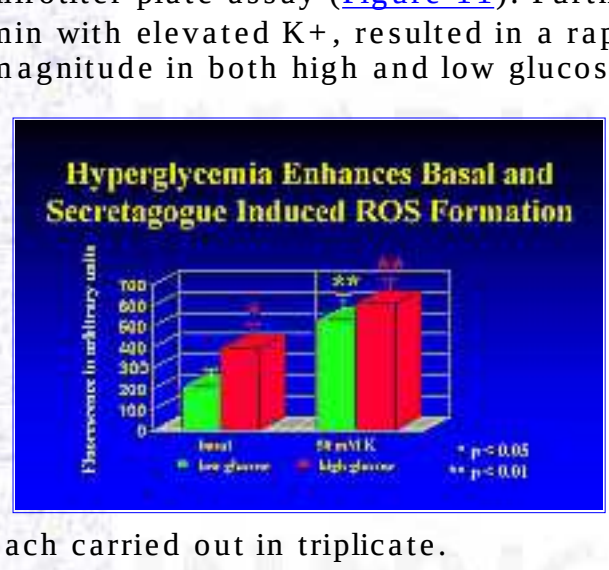

Figure 9: Quantitative Analysis of Apoptosis in PC12 Cells. The relative number of TUNEL -labeled apoptotic cells was determined by quantitative image analysis of images such as shown in Figure 8, evaluating 3 random fields from 4 independent assays. Data represent means ± SEM, p <0.01

Taken together, the experimental evidence presented here is in agreement with the hypothesis that elevated glucose levels induces apoptosis in undifferentiated PC 12 cells.

Elevated Glucose Levels Enhance the Generation of Reactive Oxygen Species: PC12 cells, grown for 3 days in low and high glucose, were loaded with the ROS-sensitive fluorogenic, membrane permeant dye 2,7 dichlorofluorescin diacetate (DCF) and observed under a fluorescent microscope. To quantitate ROS production in a large cell population, PC12 cells were plated in 24-well and ROS production was measured in the fluorescent mini-plate reader.

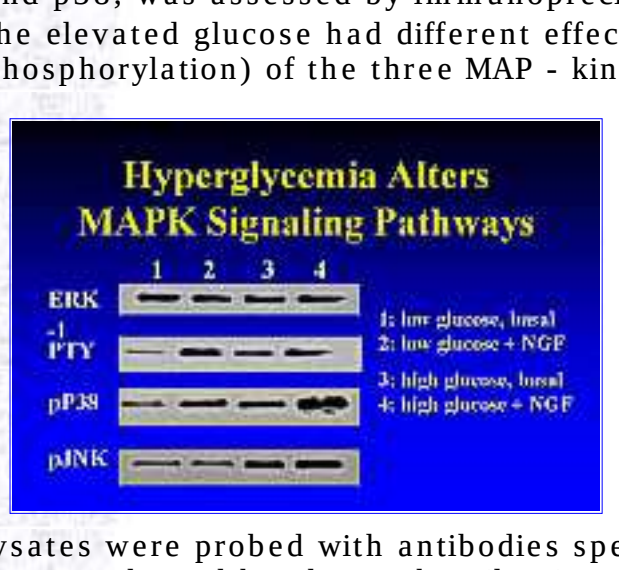

Figure 10: Elevated Glucose induced ROS Formation. PC12 cells cultured for 3 days in 35 mm culture dishes were loaded with the ROS sensitive flourescent dye 2,7dichloroflourescin (DCF) as described in Materials and Methods. The fluorescence in individual cells/cell clusters was visualized in a fluorescence microscope. Images were captured digitally and cell-associated fluorescence evaluated by computer aided image analysis.

PC12 cells cultured in high glucose exhibited higher levels of DCF fluorescence which, by quantitative image analysis, was estimated to be approx. 2-3 fold higher than that in cells grown in normal glucose (Figure 10). This finding was also confirmed using the mirotiter plate assay (Figure 11). Furthermore, stimulation of the DCF loaded cells for 5 min with elevated K+, resulted in a rapid oxidative burst, which was similar in magnitude in both high and low glucose.

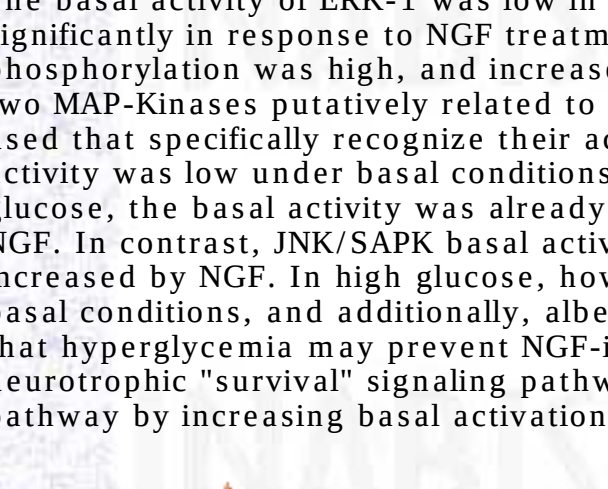

Figure 11: Effects of Glucose on the Formation of Reactive Oxygen Species. The ROS sensitive flourescent dye 2,7dichloroflourescin (DCF) was used to measure the level of ROS production in PC12 cells cultured for 3 days in high and low glucose. Some of the cells were stimulated with 50 mM K+, while others were left untreated. After 5 minutes, DCF was read in a flourescent microplate reader. The data represent means ± SEM for duplicate independent experiments, each carried out in triplicate.

Elevated Glucose Levels Affect Mitogen-Activated Protein Kinase (MAP-Kinase)-Dependent Cellular Signaling Pathways: PC12 cells, cultured for 3 days in low and high glucose, respectively, were stimulated for 30min with NGF. The level of tyrosine phosphorylation of 3 members of the MAP - Kinase family, ERK-1, JNK/SAPK and p38, was assessed by immunoprecipitation/western blotting. As seen in Figure 12, the elevated glucose had different effects on the activities (as assessed by the levels of phosphorylation) of the three MAP - kinases studied.

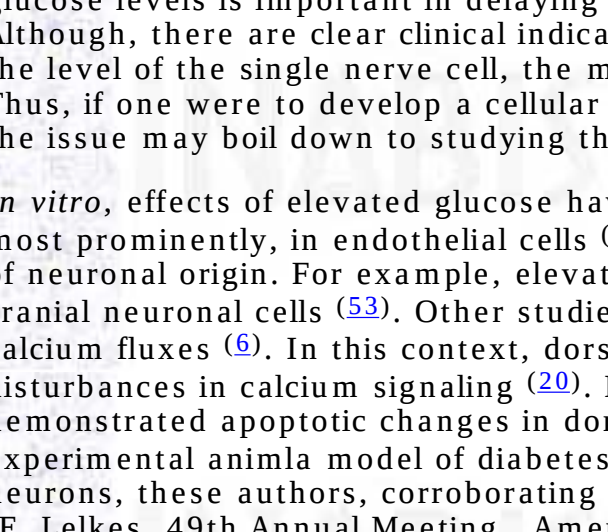

Figure 12: Analysis of MAP - Kinase Activity: PC12 cells were cultured for 3 days in high and low glucose, and stimulated for 30 minutes with NGF. MAP-Kinase activity was evaluated by immunoprecipitation / western blotting. For ERK-1, cell lysates were immunoprecipitated with anti-ERK-1. Identical samples were probed in parallel with anti-ERK-1 for quantitation and with PTY (anti-phosphotyrosine antibody) for activity. For p38 and JNK/ SAPK, western blots of whole cell lysates were probed with antibodies specific for their phosphorylated forms. The blots were evaluated by electrochemiluminescence. Lane 1:Low glucose basal activity. Lane 2: Low Glucose + 50 ng/ml NGF. Lane 3: High glucose basal activity.Lane 4: High Glucose + 50 ng/ml NGF.

The basal activity of ERK-1 was low in physiological (5 mM) glucose and increased significantly in response to NGF treatment. In contrast, in high glucose, ERK-1 base line phosphorylation was high, and increased only slightly upon activation by NGF.For the two MAP-Kinases putatively related to apoptosis, p38 and JNK/SAPK, antibodies were used that specifically recognize their active/ phosphorylated forms. In low glucose,p38 activity was low under basal conditions and was significantly enhanced by NGF. In high glucose, the basal activity was already high, but p38 was further strongly enhanced by NGF. In contrast, JNK/SAPK basal activity appeared low in low glucose, and was not increased by NGF. In high glucose, however, JNK/SAPK phosphorylation was high under basal conditions, and additionally, albeit slightly, elevated by NGF. These results indicate that hyperglycemia may prevent NGF-induced neuronal differentiation by decreasing the neurotrophic "survival" signaling pathway (via ERK-1) while enhancing the apoptotic pathway by increasing basal activation of JNK/SAPK and p38.

DISCUSSION

Diabetic neuropathy is a complex disease that affects all three nerve types (motor, sensory and autonomic) in the peripheral nervous system. While in some forms of diabetic neuropathy, nerve fibers are lost, other manifestations indicate a neuronal hyper-reactivity [5,13,18]. Clinical studies indicate that proper management of the blood glucose levels is important in delaying and/or ameliorating the syndromes [13,18,51]. Although, there are clear clinical indications that high blood glucose leads to damage at the level of the single nerve cell, the mechanisms by which this occurs is as yet unclear. Thus, if one were to develop a cellular model for diabetic neuropathy, experimentally, the issue may boil down to studying the effects of glucose on cultured neuronal cells.

In vitro, effects of elevated glucose have been studied in numerous cultured cells types, most prominently, in endothelial cells [4,12,36], but, until recently, not with cultured cells of neuronal origin. For example, elevated glucose levels inhibit the migration of cultured cranial neuronal cells [53]. Other studies suggested effects of hyperglcemia on neuronal calcium fluxes [6]. In this context, dorsal root ganglion neurons from diabetic rats exhibit disturbances in calcium signaling [20]. Most recently, Feldman and coworkers, demonstrated apoptotic changes in dorsaloot ganglian neurons and Schwann cells in an experimental animla model of diabetes [49]. Furthermore using rat dorsal root ganglion neurons, these authors, corroborating in part some of our previously presented results (E. Lelkes, 49th Annual Meeting , American Academy of Neurology, Boston, MA, 1997), also showed that high glucose inhibits neurite outgrowth, activates caspase 3 activity and leads to apoptosis [49].

The model presented here examined several interrelated cellular targets for damage by "hyperglycemia", which are relevant for diabetic neuropathy: Phenomenologically, our study shows that elevated glucose levels inhibit cell proliferation and NGF-mediated neuronal differentiation (Figures 1 - 4). These findings might be related to reports that hyperglycemia induces damage to the nervous system in utero, during a time in embryonic development, when neuronal cells still proliferate [14,38,43,44] Mechanistically, a reduced rate of cell proliferation can be explained by enhanced apoptosis, as assessed by both early and late markers of programmed cell death (see Figures 6-9).

Recent reports indicate the occurrence of hyperglycemia- induced damage to the both the central and the peripheral nervous system, including apoptosis [14,43,49] . An enhanced rate of apoptosis will result in a reduced rate of cell proliferation, and this may be directly caused by enhanced ROS production [7]. In line with our findings, Russell et al.(1999) recently suggested that oxidative stress may lead to the activation of caspase mediated programmed cell death in neuronal cells cultured in elevated glucose [48,49].

In some respects, enhanced apoptosis in high glucose is reminiscent of the early steps in programmed cell death observed after NGF-withdrawal [33]. We, therefore, hypothesized that glucose -induced apoptosis might indicate an impairment of NGF signaling. In examining the effects of hyperglycemia on post-NGF signaling mechanisms, three distinct sub-classes of the MAP-kinase pathway were tested. High glucose had different effects on the activities (as assessed from the levels of tyrosine phosphorylation) of each of the three. Basal levels of all three MAP kinases were elevated in high glucose. In this system, p38 activity was little affected by hyperglycemia, while the levels of NGF-inducible phosphorylation of ERK and JNK/SAPK were decreased (Figure 12).

An interesting finding, with possible implications for the clinical relevance of this model (see below), is the observation that hyperglycemia leads to "hyperactive" neurotransmitter release. As seen in Figure 5, secretagogue (nicotine and K^+)-stimulated catecholamine release in cells exposed to elevated (0.5 %) glucose, was increased compared to cells maintained in normal (0.1 %) glucose. These results contradict previous in vivo reports on a decrease in catecholamine secretion in adrenal medullae of diabetic rats [58]. In this rat model, the decrease in catecholamine secretion is probably due to a defect of the afferent nerve, rather than in the catecholamine secreting cells. Our finding that hyperglycemia enhances neurotransmitter secretion from PC12 cells is in agreement with recent data that show that serum from diabetic patients increases the activity of certain calcium channels which are important in the process of neurotransmitter release [24]. Thus, the "hyperactivity" of the PC12 cells in high glucose might model, in part, some of the symptoms of diabetic neuropathy, namely the tingling sensation indicative of "hyperactive" nerves. Also, one might speculate that if hyperglycemia causes the nerves to become "hyperactive", they might eventually become refractive to subsequent stimulation. This, in turn, might explain another symptom of diabetic neuropathy, namely the feeling of numbness.

Recent studies by others have suggested a causal correlation between hyperglycemia and the generation of free oxygen radicals (reactive oxygen species, ROS) [45,46,54,55]. Thus, the chronic use of free radical scavengers can alleviate neurovascular dysfunction and prevent damage to nerves and endothelial cells in diabetic rats [9,45]. Based on our results, we present a model (Figure 13) which illustrates our hypothesize that ROS formation may be the common link that between impaired neuronal differentiation (trk signaling), hyperactivity (enhanced catecholamine secretion) and induction of apoptosis (p38 and JNK/SAPK).

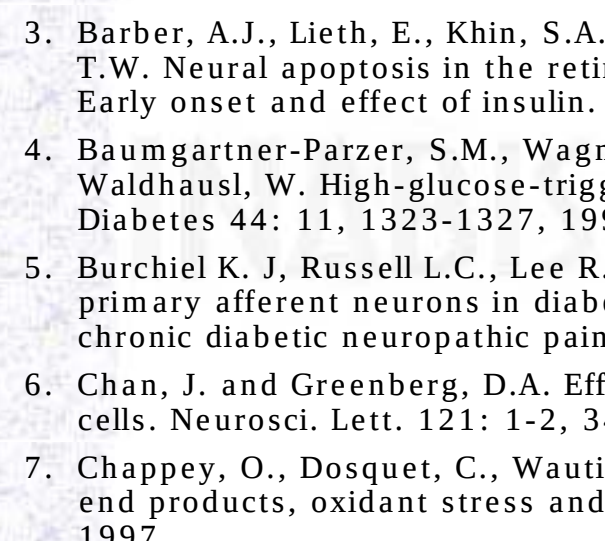

Figure 13:Model for Hyperglycemia - Induced Biochemical and Functional Alterations in PC12 Cells: Glucose - enhanced ROS formation may provide a common mechanism, which may result in the observed - induced alterations of MAP - Kinase activity and enhanced apoptosis (as seen by nucleic DNA fragmentation and by increased CPP32 activation,) which in turn might contribute to decreased PC12 cell proliferation and impaired neuronal differentiation. Enhanced oxidative stress might also increase catecholamine release. The system may be useful for modeling some of the cellular and molecular manifestations of diabetic neuropathy.

Clinical manifestations of diabetic neuropathy include both loss of nerves as well as their malfunction, including initial hyperactivity followed by subsequent loss of response by desensitization [13]. The PC12 cell model does not claim to "solve the problem" of diabetic neuropathy. This rather simple model, which uses PC12 cells cultured in various glucose levels, does not address hormonal/paracrine signaling that may be of importance in diabetic neuropathy [18]. But this *in vitro* model can reproduce at the cellular level some of the phenomena associated with the clinical picture, such as "neuronal atrophy" -apoptosis and decreased neurotrophin signaling (neurotrophin is not only a differentiation factor but, in established nerves, it is first and foremost a "survival factor") - and altered neuronal activity, such as hyper-reactive neurotransmitter secretion. Therefore this model is suitable for further characterization of the effects of elevated glucose (hyperglycemia) on the molecular biology and physiology of cultured nerve cells

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Invited Symposium: Reactive Oxygen Species and Neurodegenerative Diseases *(7 Presentations in this Symposium)*

Reactive Oxygen Species, Apoptosis and Altered NGF-Signaling in PC12 Pheochromocytoma Cells Cultured in Elevated Glucose: An *in vitro* Cellular Model for Diabetic Neuropathy

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[ABSTRACT] [INTRODUCTION] [MATERIALS AND METHODS] [RESULTS] [IMAGES] [IMAGES-2] [DISCUSSION] [REFERENCES] [Discussion Board]

IMAGES

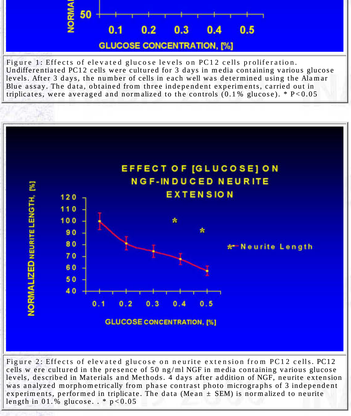

Figure 1: Effects of elevated glucose levels on PC12 cells proliferation. Undifferentiated PC12 cells were cultured for 3 days in media containing various glucose levels. After 3 days, the number of cells in each well was determined using the Alamar Blue assay. The data, obtained from three independent experiments, carried out in triplicates, were averaged and normalized to the controls (0.1% glucose). * P<0.05

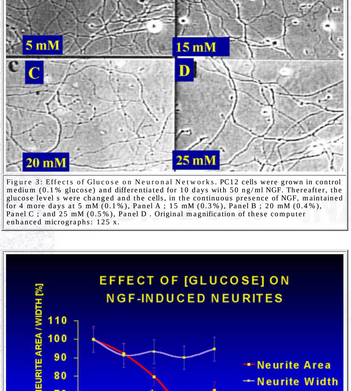

Figure 2: Effects of elevated glucose on neurite extension from PC12 cells. PC12 cells w ere cultured in the presence of 50 ng/ml NGF in media containing various glucose levels, described in Materials and Methods. 4 days after addition of NGF, neurite extension was analyzed morphometrically from phase contrast photo micrographs of 3 independent experiments, performed in triplicate. The data (Mean ± SEM) is normalized to neurite length in 0.1 % glucose. , * p<0.05

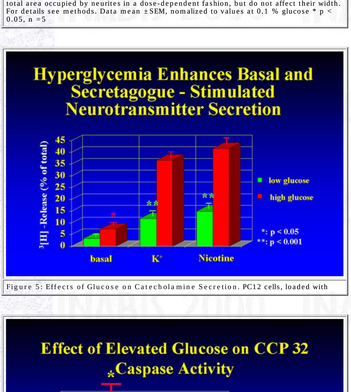

Figure 3: Effects of Glucose on Neuronal Networks. PC12 cells were grown in control medium (0.1 % glucose) and differentiated for 10 days with 50 ng/ml NGF. Thereafter, the glucose level s were changed and the cells, in the continuous presence of NGF, maintained for 4 more days at 5 mM (0.1 %), Panel A ; 15 mM (0.3%), Panel B ; 20 mM (0.4 %), Panel C ; and 25 mM (0.5%), Panel D . Original magnification of these computer enhanced micrographs: 125 x.

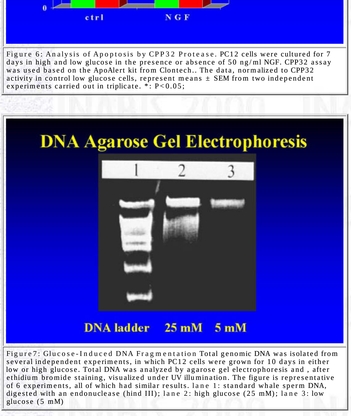

Figure4: Effects of Various Glucose Levels on Neuronal Networks: Quantitative Evaluation by Computer-Aided Image Analysis. Elevated glucose levels decrease the total area occupied by neurites in a dose-dependent fashion, but do not affect their width. For details see methods. Data mean ±SEM, nomalized to values at 0.1 % glucose * p < 0.05, n =5

Figure 5: Effects of Glucose on Catecholamine Secretion. PC12 cells, loaded with

Figure 6: Analysis of Apoptosis by CPP32 Protease. PC12 cells were cultured for 7 days in high and low glucose in the presence or absence of 50 ng/ml NGF. CPP32 assay was used based on the ApoAlert kit from Clontech.. The data, normalized to CPP32 activity in control low glucose cells, represent means ± SEM from two independent experiments carried out in triplicate. *: P<0.05;

Figure7: Glucose-Induced DNA Fragmentation Total genomic DNA was isolated from several independent experiments, in which PC12 cells were grown for 10 days in either low or high glucose. Total DNA was analyzed by agarose gel electrophoresis and , after ethidium bromide staining, visualized under UV illumination. The figure is representative of 6 experiments, all of which had similar results. lane 1: standard whale sperm DNA, digested with an endonuclease (hind III); lane 2: high glucose (25 mM); lane 3: low glucose (5 mM)

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[ABSTRACT] [INTRODUCTION] [MATERIALS AND METHODS] [RESULTS] [IMAGES] [IMAGES-2] [DISCUSSION] [REFERENCES] [Discussion Board]

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Reactive Oxygen Species, Apoptosis and Altered NGF-Signaling in PC12 Pheochromocytoma Cells Cultured in Elevated Glucose: An *in vitro* Cellular Model for Diabetic Neuropathy

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IMAGES-2

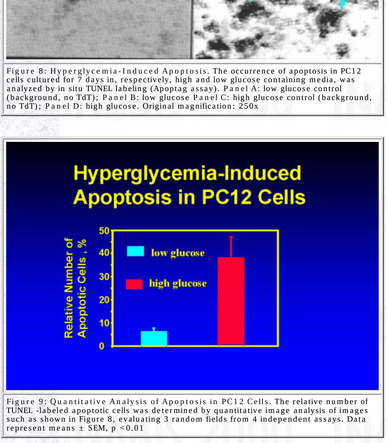

Figure 8: Hyperglycemia-Induced Apoptosis. The occurrence of apoptosis in PC12 cells cultured for 7 days in, respectively, high and low glucose containing media, was analyzed by in situ TUNEL labeling (Apoptag assay). Panel A: low glucose control (background, no TdT); Panel B: low glucose Panel C: high glucose control (background, no TdT); Panel D: high glucose. Original magnification: 250x

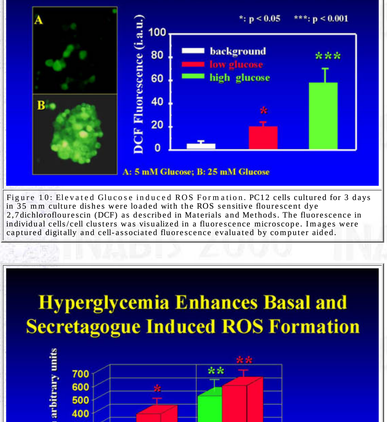

Figure 9: Quantitative Analysis of Apoptosis in PC12 Cells. The relative number of TUNEL -labeled apoptotic cells was determined by quantitative image analysis of images such as shown in Figure 8, evaluating 3 random fields from 4 independent assays. Data represent means ± SEM. p <0.01

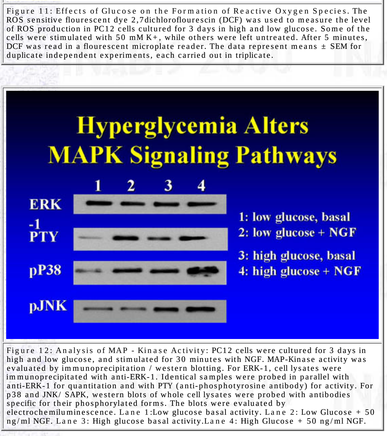

Figure 10: Elevated Glucose induced ROS Formation. PC12 cells cultured for 3 days in 35 mm culture dishes were loaded with the ROS sensitive flouresecent dye 2,7dichlorofluorescin (DCF) as described in Materials and Methods. The fluorescence in individual cells/cell clusters was visualized in a fluorescence microscope. Images were captured digitally and cell-associated fluorescence evaluated by computer aided.

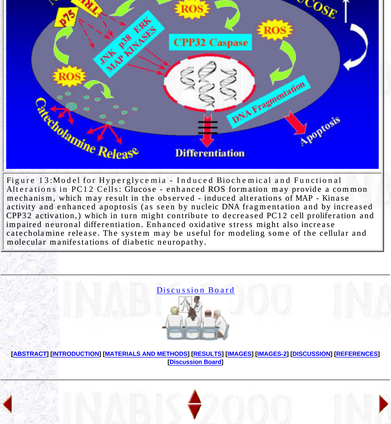

Figure 11: Effects of Glucose on the Formation of Reactive Oxygen Species. The ROS sensitive flourescent dye 2,7dichlorofluorescin (DCF) was used to measure the level of ROS production in PC12 cells cultured for 3 days in high and low glucose. Some of the cells were stimulated with 50 mM K+, while others were left untreated. After 5 minutes, DCF was read in a flourescent microplate reader. The data represent means ± SEM for duplicate independent experiments, each carried out in triplicate.

Figure 12: Analysis of MAP - Kinase Activity: PC12 cells were cultured for 3 days in high and low glucose, and stimulated for 30 minutes with NGF. MAP-Kinase activity was evaluated by immunoprecipitation / western blotting. For ERK-1, cell lysates were immunoprecipitated with anti-ERK-1. Identical samples were probed in parallel with anti-ERK-1 for quantitation and wit PTY (anti-phosphotyrosine antibody) for activity. For p38 and JNK/ SAPK, western blots of whole cell lysates were probed with antibodies specific for their phosphorylated forms. The blots were evaluated by electrochemiluminescence. Lane 1:Low glucose basal activity. Lane 2: Low Glucose + 50 ng/ml NGF. Lane 3: High glucose basal activity.Lane 4: High Glucose + 50 ng/ml NGF.

Figure 13:Model for Hyperglycemia - Induced Biochemical and Functional Alterations in PC12 Cells: Glucose - enhanced ROS formation may provide a common mechanism, which may result in the observed - induced alterations of MAP - Kinase activity and enhanced apoptosis (as seen by nucleic DNA fragmentation and by increased CPP32 activation,) which in turn might contribute to decreased PC12 cell proliferation and impaired neuronal differentiation. Enhanced oxidative stress might also increase catecholamine release. The system may be useful for modeling some of the cellular and molecular manifestations of diabetic neuropathy.

Discussion Board

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Efrat Lelkes, Brian R. Unsworth, Peter I. Lelkes

Last update: 19/01/00

DISCUSSION BOARD

Reactive Oxygen Species and Neurodegenerative Diseases

New Comment Presentation # 30 *Reactive Oxygen Species, Apoptosis and Altered NG...*

(354) Wilma Starke Buzetti Date: 25/02/2000 17:32:56
Good presentation.
How did you get PC12 rat pheochromocytoma cells?

RE: - New Comment

(390) Efrat Lelkes Date: 26/02/2000 6:01:35
Thanks for your interest. The PC12 cells were isolated from a rat chromaffin cell tumor (Tischler and Greene, PNAS, 1978?) You may want to consult our published references for where we actually got the cells from. We also could supply you with cells if you need them. Further, the cells are commercially available through ATCC.

New Comment Presentation # 41 *Discovery and History of 6-Hydroxydopamine...*

(152) Cyrus Creveling Date: 20/02/2000 23:51:34
While the present evidence has not substantiated the formation of 6-hydroxydopamine in vivo has yet to demostrated their are hints that this may actually occur especially in area of relatively high dopamine concentation. There was a report that the plasma of of Parkinsonian patients interacted with BSA-6HO. Certainly not definative but curious.

New Comment Presentation # 41 *Discovery and History of 6-Hydroxydopamine...*

(151) Cyrus Creveling Date: 20/02/2000 23:45:22
Old, primative by todays methods, but a very significant step in neuroscience--but we all stand on someones shoulders.
--dedicated to Dr. Sidney Udenfried who recently passed away.

New Comment Presentation # 41 *Discovery and History of 6-Hydroxydopamine...*

(146) Duska Meh Date: 20/02/2000 18:02:04
I am sorry; your topic is interesting but for asking something wise I will need more time!

dose vs chronic treatment Presentation # 197 *Free radicals and drug-induced neurodegeneration...*

(297) Henry Szechtman Date: 25/02/2000 2:57:10
Thank you for an excellent presentation. I was wondering whether one would expect the same neurodegerative processes to operate if rather than a high dose exposure to the METH, there were repeated exposures to small doses of the drug.

New Comment Presentation # 197 *Free radicals and drug-induced neurodegeneration...*

(17) Vicente Felipo Date: 15/02/2000 9:06:51
I can not see the Figures, which are essential to understand the mechanism of neurotoxicity proposed by the author. How can I find the Figures ?

RE: - New Comment

(18) Vicente Felipo Date: 15/02/2000 9:08:32
You indicate that you can see reactive gliosis in the cultured cells in vitro. Do you see an increase in the number of GFAP-positive cells or only an alteration in the morphology ?

RE: - New Comment

(19) Vicente Felipo Date: 15/02/2000 9:10:37
Could you discuss briefly the possible role of glutamate receptors in the mediation of metamphetamine neurotoxicity ?

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