

Presumed Apoptosis and Reduced Arcuate Nucleus Neuropeptide Y and Pro-Opiomelanocortin mRNA in Non-Coma Hypoglycemia

Nancy C. Tkacs, Ambrose A. Dunn-Meynell, and Barry E. Levin

Hypoglycemia reduces sympathoadrenal responses to subsequent hypoglycemic bouts by an unknown mechanism. To assess whether such hypoglycemia-associated autonomic failure is due to actual brain damage, male Sprague-Dawley rats underwent 1-h bouts of insulin-induced (5 U/kg i.v.) hypoglycemia (1.6–2.8 mmol/l) 1 or 3 times on alternate days. Rats remained alert and were rescued with intravenous glucose at 60–80 min. Plasma epinephrine and corticosterone responses were significantly reduced during the second and third bouts. Brains from these rats were processed by the terminal transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL) procedure as an index of apoptotic cell death at 24, 48, or 96 h after their first bout. At 48 h, but not 24 h, TUNEL⁺ cells were consistently seen only in the arcuate nucleus (arcuate hypothalamic nucleus [ARC]). Hypoglycemic rats had 188% more apoptotic ARC cells (1 bout 39 ± 5 ; 3 bouts 37 ± 4) than euglycemic controls (13 ± 3 ; $P = 0.001$). In situ hybridization for neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) mRNA was performed in sections of ARC containing maximal numbers of apoptotic cells as well as in other fresh frozen brains. After 1 bout, NPY (0.041 ± 0.003) and POMC (0.119 ± 0.022) mRNA were decreased, respectively, by 52 and 55% vs. controls (NPY 0.076 ± 0.007 ; POMC 0.222 ± 0.020 ; $P = 0.01$). NPY (0.029 ± 0.002) but not POMC (0.093 ± 0.013) fell 29% further after a third bout. NPY ($r = -0.721$; $P = 0.001$) and POMC ($r = -0.756$; $P = 0.001$) mRNA levels correlated negatively with the number of apoptotic ARC cells in the same sections. Thus, non-coma hypoglycemia produces apparent apoptotic cell death with reduced NPY and POMC expression selectively in the ARC. This may contribute to the reduced counterregulatory response following repeated bouts of hypoglycemia. *Diabetes* 49:820–826, 2000

With the advent of intensive insulin therapy for type 1 diabetes, there has been an increasing incidence of hypoglycemic episodes (1). Some of these are severe, resulting in coma and subsequent permanent brain damage and/or death (2–5). Other less severe hypoglycemic episodes do not impair consciousness. Regardless of degree, hypoglycemia is associated with a robust counterregulatory response (CRR) (6–9). Low levels of plasma glucose are sensed by selective neurons in the brain (10–14) as well as by glucosensors in the hepatic portal vein (15). Signals from these sensors provoke activation of the sympathoadrenal system with preferential release of epinephrine. In addition, cortisol is released from the adrenal cortex and glucagon from the pancreatic α -cells (6–9,16). This response maximizes the mobilization of glucose in the body. In addition to this CRR, subjects develop an awareness of their hypoglycemic state (6,9). Both the CRR and the hypoglycemia awareness can become blunted after only a single bout of hypoglycemia (9,17). Hypoglycemia awareness recovers fairly rapidly but the diminished CRR is often prolonged (6), suggesting that different mechanisms are responsible for the two phenomena. However, the mechanism underlying either of these remains unclear.

Here, we present a rat model of the dampened CRR and show that a single bout of moderate hypoglycemia produces highly site-specific apparent apoptotic response in cells of the hypothalamic arcuate nucleus (ARC). This apoptosis is associated with a significant reduction in the expression of both neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) mRNA in this same nucleus.

RESEARCH DESIGN AND METHODS

Animals and hypoglycemia. Male Sprague-Dawley rats were used at 300–400 g. They were housed at 22–23°C on a 12-h light-dark schedule and fed Purina rat chow (#5001) (PMI Nutrition International, Brentwood, MO) ad libitum. One group of rats ($n = 12$) had right atrial silastic catheters implanted via the right jugular vein under ketamine (45 mg/kg)/xylazine (9 mg/kg i.p.) anesthesia with buprenorphine (0.2 mg/kg) analgesia. Catheters were filled with a polyvinylpyrrolidone/heparin mix, which was replaced daily. The rats were allowed 4–5 days to recover and then were fasted overnight. Additional rats without venous catheters were also used ($n = 34$) and fasted overnight before testing. Between 0800 and 1000, a blood sample (0.5 ml) was withdrawn from the venous catheter for plasma catecholamines, glucose, lactate, and corticosterone, or from the tail tip for baseline glucose levels. Rats were then injected through the catheter or tail vein with 0.5 ml/kg of either 0.9% saline ($n = 16$) or 10 U/ml regular insulin in saline (5 U/kg; $n = 24$). Further, blood samples for glucose were taken at 30 and 60 min after injections. Saline was infused to maintain plasma volume, and washed red cells from the same rat were returned after the 30- and 60-min samples to maintain red cell mass. The

From the School of Nursing (N.C.T.), University of Pennsylvania, Philadelphia, Pennsylvania; Neurology Service (A.A.D.-M., B.E.L.), VA Medical Center, East Orange; and the Department of Neurosciences (A.A.D.-M., B.E.L.), New Jersey Medical School, Newark, New Jersey.

Address correspondence and reprint requests to Barry E. Levin, MD, Neurology Service (127C), VA Medical Center, 385 Tremont Ave., E. Orange, NJ 07018-1095. E-mail: levin@umdnj.edu.

Received for publication 18 August 1999 and accepted in revised form 1 February 2000.

ANOVA, analysis of variance; ARC, arcuate hypothalamic nucleus; CRR, counterregulatory response; HSP-72, heat shock protein 72; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; TUNEL, terminal transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling.

insulin infusions resulted in plasma glucose levels of 1.6–2.8 mmol/l (Fig. 1). Behavioral observations were made throughout, and all rats remained behaviorally alert and/or responsive to mild physical stimulation. At 70–80 min after insulin infusion, rats were infused with 1 g/kg glucose. This infusion brought plasma glucose levels to >6 mmol/l within 25–30 min. Rats were killed in 3 groups: 1) 24 h after insulin-induced hypoglycemia ($n = 5$) or saline ($n = 1$); 2) 48 h after hypoglycemia ($n = 16$) or saline ($n = 12$); and 3) after 3 bouts of insulin-induced hypoglycemia ($n = 7$) or saline injection ($n = 5$) on alternate days with death at 1–2 h after the third bout.

Assay for apoptosis. The terminal transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL) assay was performed on fresh frozen tissue using an ApopTag kit (Intergen, Purchase, NY). This procedure enzymatically labels the DNA 3'-OH strand breaks on both single- and double-stranded DNA with unlabeled and digoxigenin-labeled nucleotides by terminal deoxynucleotidyl transferase (18). The reaction was carried out on 5- μ m frozen brain sections. Labeled fragments were identified by incubation with anti-digoxigenin antibody conjugated to peroxidase. The complex was then visualized using diaminobenzidine reaction, and positive cells were counted by an observer who was unaware of the experimental group of the animal. Four control and 6 insulin-treated brains were examined throughout the entire neuraxis for TUNEL⁺ cells. When it became apparent that positive cells were found only in the ARC and rarely in the medulla, the remaining brains were examined in only these areas.

Histologic markers of cell injury. Detection of heat shock protein 72 (HSP-72) immunoreactivity and cellular necrosis by acid fuchsin staining were carried out by previously described methods (19) in brains of 4 control rats and 4 rats 48 h after insulin-induced hypoglycemia. Additional brains from 4 control rats and 6 insulin-induced hypoglycemic rats were perfused 48 h later with paraformaldehyde, paraffin-embedded, sectioned, and stained with bis-benzimide (33258; Hoechst, Frankfurt, Germany), which binds to poly[d(A-T)] sequences in nuclear DNA (20). These sections were observed by fluorescent microscopy at an excitation/emission spectrum of 350/460 nm.

In situ hybridization for prepro-NPY and POMC mRNA. Brains were processed for in situ hybridization by minor modifications of previously described methods (21–23) using ³⁵S-labeled riboprobes for NPY (24) and POMC (25) on 5- μ m frozen brain sections post-fixed in 4% paraformaldehyde. Of all the brains run, 24 (14 hypoglycemic and 10 controls) had been run previously for the TUNEL procedure. In these brains, sections ($n = 3$ per rat) were selected for in situ hybridization based on the finding of a maximal number of apoptotic cells in that section. An additional 5 hypoglycemic and 5 controls were run for in situ hybridization fresh, i.e., without prior TUNEL assay. In these brains, 10 sections per brain through the ARC were run to assure that that no selection bias occurred. The hybridized sections were exposed to SB-5 X-ray film (Kodak, Rochester, NY) for 3 days and 12 h for NPY and POMC, respectively. The resulting autoradiograms were read using computer-assisted densitometry (Drexel, Philadelphia, PA) by an individual unaware of the experimental treatment groups. Area and optical density measures were made in sections with maximal numbers of apoptotic cells or at the mid-portion of the ARC where NPY and POMC are most affected by metabolic perturbations (26,27). Expression of NPY and POMC mRNA was taken as the product of the area (mm^2) \times optical density readings (arbitrary units).

Assays of plasma constituents. Plasma catecholamines were assayed by high-performance liquid chromatography with electrochemical detection (28). Plasma corticosterone assays were done by radioimmunoassay (ICN Biomedicals, Costa Mesa, CA). Glucose and lactate were assayed by automated glucose oxidase and lactate oxidase methods (Yellow Springs Instruments, Yellow Springs, OH).

Statistics. Intergroup comparisons for each parameter related to cell counting or neuropeptide mRNA expression were made by 1-way analysis of variance (ANOVA). Plasma constituents were analyzed by repeated-measures design. When significant intergroup differences were found by ANOVA ($P \leq 0.05$), post hoc comparisons were carried out using Scheffe multiple comparison tests. Correlations were carried out with Pearson's coefficient of correlation.

RESULTS

Plasma constituents during insulin-induced hypoglycemia.

After insulin injections at 5 U/kg i.v., plasma glucose levels fell to 1.6–2.8 mmol/l within 30 min and remained at that level through 60 min (Fig. 1). There was no significant change in this pattern across 3 bouts of hypoglycemia. Commensurate with the decline in plasma glucose during the first bout of hypoglycemia, plasma epinephrine levels (baseline 38 \pm 8 pg/ml) rose by 150-fold at 30 min and declined to 85 times

baseline at 60 min. However, the rise in plasma epinephrine levels was significantly reduced to 79- and 73-fold at 30 min and 82- and 73-fold after the second and third bouts of hypoglycemia, respectively (Fig. 1). Similarly, plasma corticosterone levels (baseline 113 \pm 32 ng/ml) rose to 7.8 and 8.9 times those at baseline at 30 min and 60 min after the first bout of hypoglycemia, but the 30-min increases were significantly depressed to 5.5 and 5.2 times those at baseline during the second and third bouts, respectively. Plasma norepinephrine levels (baseline 167 \pm 29 pg/ml) rose to 2.5 and 2.3 times those at baseline at 30 and 60 min during the first bout of hypoglycemia, respectively, and this did not change significantly during the second and third bouts (Fig. 1). Finally, plasma lactate levels (baseline 6.09 \pm 0.47 mmol/l) rose continuously from 2.8 to 4.2 times baseline at 30 and 60 min during the first hypoglycemic bout. This pattern and magnitude of lactate rise did not change significantly during the second or third hypoglycemic bout (Fig. 1).

TUNEL⁺ cells in hypoglycemic brains. A survey of sections through the entire brain showed only 2 areas in which there were cells exhibiting the TUNEL reaction (Fig. 2). The results were similar for animals subjected to either 1 or 3 bouts of hypoglycemia (Table 1) and for those given insulin by intravenous catheter or tail vein. At 48 h, but not 24 h after a single hypoglycemic episode, TUNEL⁺ cells were seen consistently in the ARC and sporadically at the border zone between the area postrema and the brainstem at the level of the nucleus tractus solitarius (subpostrema). In the subpostrema zone, 1–4 TUNEL⁺ cells were seen in 4 of 6 hypoglycemic brains examined. No TUNEL⁺ cells were ever seen in this area in the 4 control brains. The most consistent TUNEL reactivity was found in the ARC, where hypoglycemic rats had 188% more TUNEL⁺ cells than euglycemic controls 48 h after a single bout of hypoglycemia ($P = 0.001$). An approximately equal number of TUNEL⁺ cells were seen both medially and laterally in the ARC. There was no increase in the number of positive cells seen after 3 bouts of hypoglycemia as compared with a single bout in either the medial or lateral part of the ARC. There was also no correlation between the number of TUNEL⁺ cells seen in the ARC and plasma glucose levels during any bout of hypoglycemia. Specific examination for TUNEL⁺ cells was made in each brain in the hippocampus, cerebral cortex, basal ganglia, and cerebellum. In no case were any positive cells found in any of these areas in either hypoglycemic or control brains.

Histologic evidence of cell damage. There were only 2 cells that expressed HSP-72 immunoreactivity seen in 2 of 4 hypoglycemic brains run for this reaction. One cell was found in the ventromedial nucleus of the hypothalamus in 1 brain and 1 cell was present in the perifornical hypothalamus of the other (data not shown). No other cells were seen in any other hypoglycemic brain area or in 4 control brains. There were also no cells that showed patterns of nuclear DNA clumping characteristic of apoptosis in the hypothalamus or other brain areas. Staining with acid fuchsin for necrotic cells also failed to reveal any positive cells in either hypoglycemic or control brains in any area.

In situ hybridization for NPY and POMC mRNA. This procedure was carried out in sections from brains that had been processed first for TUNEL assay (using sections showing the maximum number of positive cells) as well as from fresh brains that were not previously manipulated (Table 2).

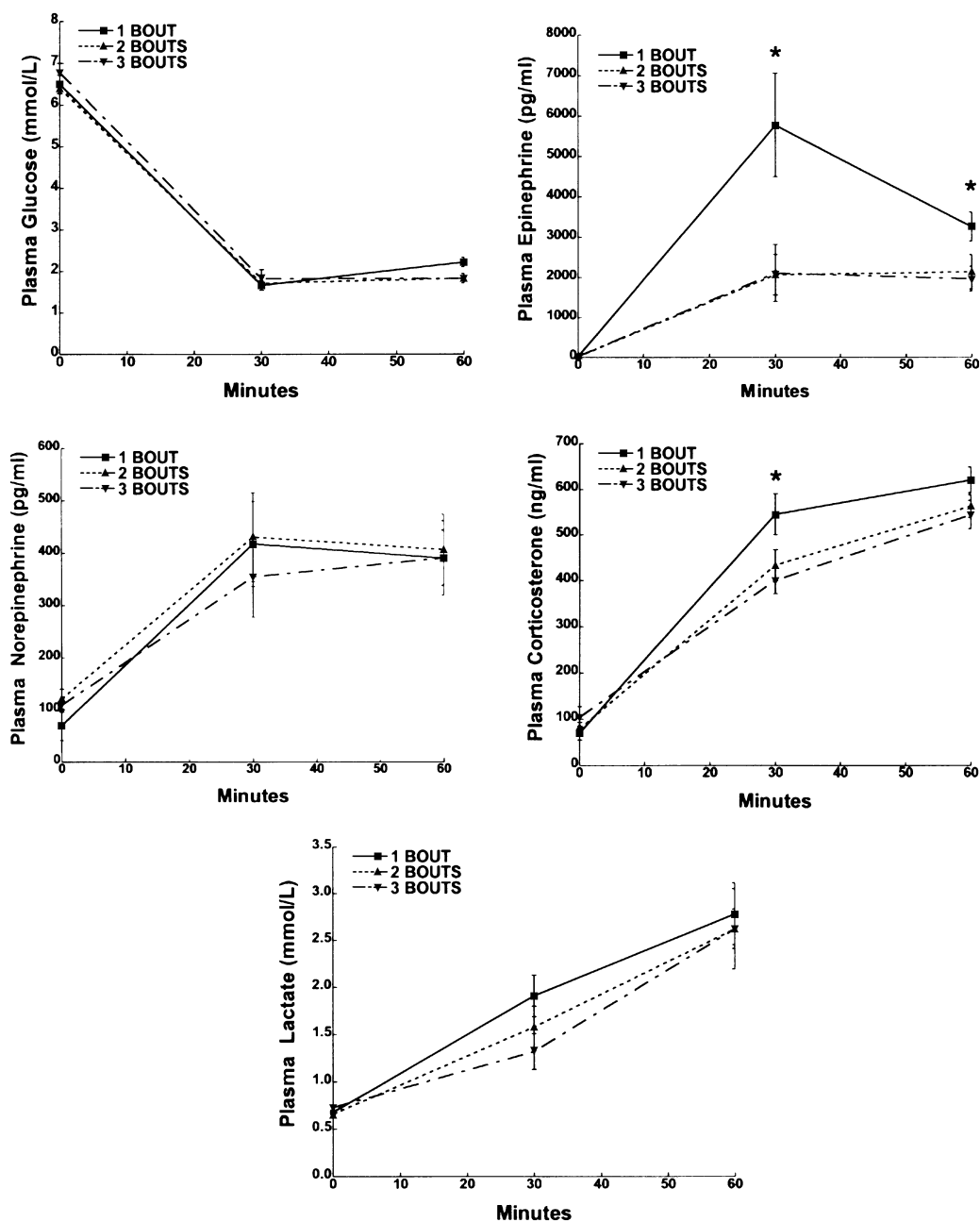


FIG. 1. Response of plasma catecholamines, corticosterone, and lactate to 3 bouts of hypoglycemia. Overnight fasted rats ($n = 10$) were given insulin (5 U/kg i.v.) after a baseline blood sample was taken. Further samples were taken at 30 and 60 min after insulin and the rats were given glucose (1 g/kg i.v.) at 70–80 min after the insulin dose. The second and third bouts were 48 h apart. Data are mean \pm SE. * $P < 0.05$ or less by post hoc analysis where there was an overall significant group difference by repeated-measures ANOVA, and a given value at a specific time point during the first bout differed significantly from respective values during the second and third hypoglycemic bouts.

At 48 h after a single bout of hypoglycemia, there was a 46% decrease in ARC NPY mRNA compared with control brains in which sections were run first for the TUNEL procedure. In sections from fresh brains, there was a 30% decrease in ARC NPY mRNA at 48 h after a single hypoglycemic bout compared with controls. After a third bout of hypoglycemia, there was a further decrease in NPY mRNA to 62% of control. ARC POMC mRNA was decreased by 47% at 48 h after a single bout of hypoglycemia in sections previously run for TUNEL assay. There was a 33% decrease in the ARC of fresh brains. There was a tendency for a further reduction in POMC mRNA after

3 bouts of hypoglycemia, but this did not reach statistical significance.

DISCUSSION

The mechanism underlying the decreased CRR after a prior bout of hypoglycemia has eluded explanation to date. A clue to the possible underlying pathology is provided by the observation that hypoglycemia unawareness seems more resilient and recovers sooner from a bout of hypoglycemia than the impaired CRR (6,29,30). This discrepancy implies a selective vulnerability of pathways

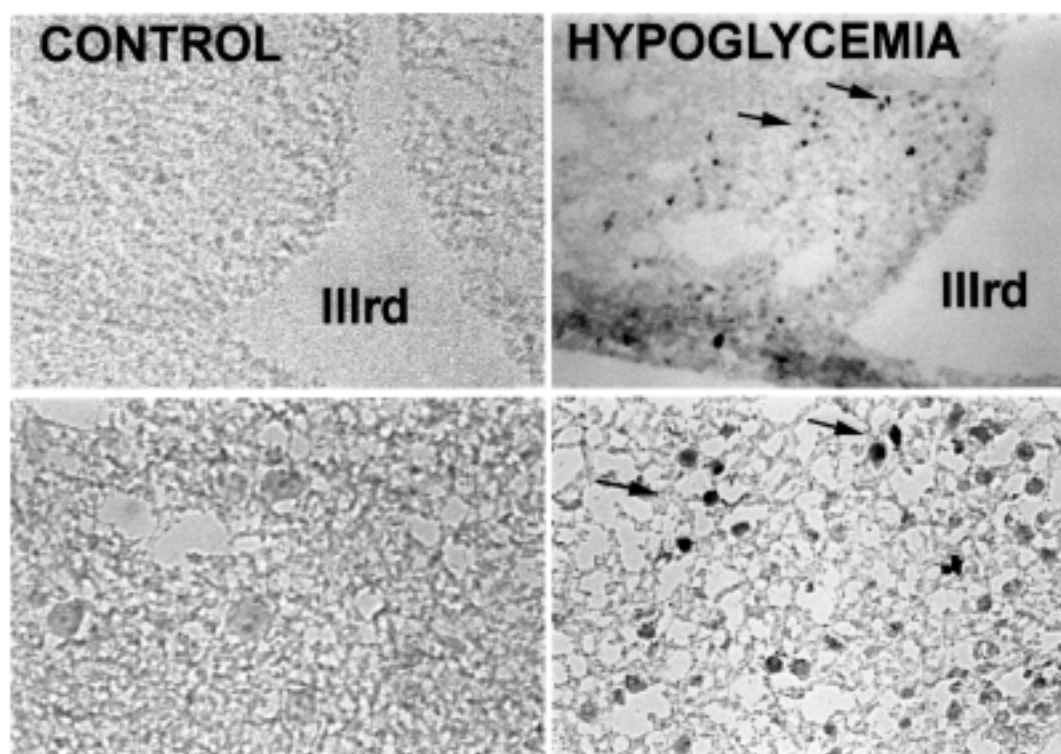


FIG. 2. TUNEL⁺ cells in the arcuate nucleus (ARC) at 48 h after an injection of saline (CONTROL: left panels) or a single bout of hypoglycemia (2 mmol/l) (HYPOGLYCEMIA: right panels). Upper panels are low power views of the region of the ARC and IIIrd cerebral ventricle (IIIrd). Arrows in the low power view of the hypoglycemic brain point to TUNEL⁺ neurons that are shown in the high power view of the same neurons in the lower panel. The left lower panel is a high power view of the comparable area in the control rat.

involved in the CRR. But it also implies an element of functional plasticity in this pathway. The finding of a selective induction of apparent apoptosis in ARC neurons after a single bout of hypoglycemia suggests that cell death in this area might underlie this phenomenon. Here, we show that such a single bout of non-coma hypoglycemia in rats results in a significant decrease in the plasma epinephrine and corticosterone responses to subsequent bouts of hypoglycemia. These reduced responses are similar to those seen in humans (6,8,9,30) and were associated with apparent apoptosis of cells in the ARC occurring between 24 and 48 h after the first episode. No consistent evidence of apoptotic or necrotic cell death was seen in any other brain area, including the cerebral cortex, hippocampus, basal ganglia, and cerebellum. Reported pathological data in humans has come predominantly from subjects who experienced severe, repeated bouts of hypoglycemia that produced coma. In those individuals, the cerebral cortex and portions of the hippocampus and basal ganglia showed neuronal necrosis (3,4). Cerebral atrophy (5) and demonstrable lesions on magnetic resonance imaging in these areas (2) appear to be consequences of such repeated severe hypoglycemic insults (5). In rats, cortical electrical silence induced by severe insulin-induced hypoglycemia produces neuronal necrosis in many of those same areas (31). Interestingly, there was no mention of hypothalamic pathology in any of these reports. However, such a severe, repeated degree of hypoglycemia is not required to reduce the CRR to hypoglycemia and produce apoptosis in ARC neurons.

The conclusion that a relatively mild degree of hypoglycemia produces ARC apoptosis is based on the labeling of DNA fragments by the TUNEL reaction (18). Such DNA degradation is part of the process of apoptosis and is not seen in neuronal necrosis (32). On the other hand, histologic evidence of nuclear DNA fragmentation with chromatin condensation (33) was not demonstrable in the ARC or any other brain area. However, this may have been a technical problem because cells are densely packed and not morphologically homogeneous in this area. These features make identification of small condensed nuclei extremely difficult. Importantly,

TABLE 1
Effect of hypoglycemia on TUNEL⁺ cells in the arcuate nucleus

	Control	1 Bout	3 Bouts
Medial	6 ± 1 ^a	24 ± 4 ^b	21 ± 3 ^b
Lateral	7 ± 1 ^a	15 ± 2 ^b	16 ± 2 ^b
Total	13 ± 3 ^a	39 ± 5 ^b	37 ± 4 ^b

Data are means + SE of the number of TUNEL⁺ cells seen in the medial, lateral, and total arcuate nucleus. Rats were subjected to 1 bout ($n = 7$) or 3 bouts of hypoglycemia for 60–80 min on alternate days ($n = 7$). They were killed 48 h later after a single bout or within 1–2 h after the third bout, and their brains were processed for TUNEL assay. They were compared with comparable saline-injected controls ($n = 5$ per time period). Data with differing superscript letters differ from each other at the $P < 0.05$ or less level by post hoc t test after significant intergroup differences were found by ANOVA.

TABLE 2
Effect of hypoglycemia on the expression of arcuate nucleus NPY and POMC mRNA

	Control	1 Bout	3 Bouts
NPY			
TUNEL	0.076 ± 0.007 ^a	0.041 ± 0.003 ^b	0.029 ± 0.002 ^c
Fresh	0.096 ± 0.009 ^a	0.067 ± 0.006 ^b	
POMC			
TUNEL	0.222 ± 0.020 ^a	0.119 ± 0.022 ^b	0.093 ± 0.012 ^b
Fresh	0.248 ± 0.013 ^a	0.166 ± 0.012 ^b	

Data are means + SE of the area × optical density reading (arbitrary units). Rats were subjected to 1 bout ($n = 7$) or 3 bouts of hypoglycemia for 60–80 min on alternate days ($n = 7$). They were killed 48 h after a single bout and within 1–2 h of the third bout. Brains were processed for TUNEL assay, and sections through the arcuate nucleus of each brain showing the highest number of TUNEL⁺ cells were then processed for in situ hybridization for either NPY or POMC mRNA. These were compared with comparably treated sections from euglycemic controls ($n = 5$ per time period). An additional group of rats was subjected to a single bout of hypoglycemia ($n = 5$) or injection of saline ($n = 5$) and was killed 48 h later. Sections through the arcuate nucleus were run for in situ hybridization without previously being run for TUNEL assay (fresh). Data with differing superscript letters differ from each other at the $P < 0.05$ or less level by post hoc t test after significant intergroup differences were found by ANOVA (for TUNEL) or by t test for unpaired samples (for fresh).

there was no evidence of neuronal necrosis in any brain area using acid fuchsin (19). This lack of neuronal necrosis was not surprising. The transition to necrosis requires sustained levels of reduced glucose (and oxygen) to decrease intracellular ATP levels well below those required to produce apoptosis (34). Finally, there was no evidence that the cells in this or any other brain area induced HSP-72 expression, which is often found in damaged cells that are destined to survive their injury (35). The induction of HSP-72 is thought to confer a degree of protection and it is rarely seen in cells undergoing apoptosis or necrosis (36,37). However, severe hypoglycemia does induce HSP-72 in both neurons (35) and astrocytes (36). Interestingly, prior bouts of hypoglycemia may actually confer some degree of protection on certain aspects of cognitive function during subsequent hypoglycemic episodes (29). The lack of HSP-72 expression in the hypoglycemic brains here is in keeping with previous studies showing that such expression occurs only after severe bouts of hypoglycemia, which produce burst suppression and an isoelectric electroencephalogram (35).

Additional evidence for hypoglycemia-induced loss of ARC neurons comes from the reduction in levels of both ARC NPY and POMC mRNA seen 48 h after a prior bout of hypoglycemia. Induction of intracellular glucopenia with the glucose antimetabolite 2-deoxyglucose produces either no change (38,39) or an increase (40) in ARC NPY and no change in ARC POMC (38) mRNA or peptide levels. It is likely that increased ARC NPY expression in such circumstances is due to reduced energy availability. A fasting-induced reduction in energy availability increases ARC NPY mRNA (23,27,41) and may decrease POMC mRNA levels transiently (42). However, these changes are reversed after a few hours of refeeding (43). It is highly unlikely that brief

episodes of hypoglycemia would produce a prolonged decrease in either ARC NPY or POMC mRNA expression after 48 h of refeeding. Thus, the reduction of ARC NPY and POMC mRNA seen at 48 h after a single bout or within 1–2 h of a third bout of hypoglycemia is consistent with a loss of neurons containing NPY and POMC. In humans, the CRR is eventually restored when further hypoglycemic episodes are avoided (6,30). This point was not examined here, but a recovery of the CRR could be due to neural plasticity in which remaining ARC NPY, POMC, or other neurons compensate for those lost during the hypoglycemic episodes by increasing their expression of peptide and/or by making new neural connections. Here, further bouts of hypoglycemia led to additional loss of ARC NPY mRNA expression and a trend toward decreased POMC expression suggesting additional damage. But repeated bouts led to no further increase in the number of TUNEL⁺ ARC cells. This result is not necessarily contradictory, since positive cells at 48 h after the first bout may well have died and been removed by the time brains were assessed after the third bout. Thus, no absolute increase in the number of positive cells would be evident, whereas there would be a progressive loss of NPY and possibly POMC expressing neurons with each subsequent bout as this pool of neurons was removed. Therefore, while definitive proof of apoptotic cell death in the ARC is lacking, the presence of TUNEL⁺ cells in this area, where there was a progressive decrease in neuropeptide message, supports the contention that there was neuronal loss associated with relatively mild degrees of hypoglycemia.

Loss of ARC NPY and POMC neurons might well underlie the reduced CRR seen after a single bout of hypoglycemia. These neurons project both rostrally and caudally to brain areas involved in the autonomic and pituitary components of the CRR as well as responses to changes in energy homeostasis. ARC NPY and/or POMC neurons project to both the paraventricular nucleus (44) and lateral hypothalamic areas (45,46). The paraventricular nucleus has efferent projections to both brainstem and spinal cord autonomic centers (10,47,48) as well as the pituitary for release of ACTH (47). The lateral hypothalamic area also projects to downstream autonomic areas (48,49). In addition, ARC POMC neurons project directly to spinal cord areas that give rise to sympathoadrenal efferents (50). Several studies have demonstrated that manipulations of the ventromedial hypothalamic area have major effects on the CRR to hypoglycemia (51–54). Given the anatomical location of the ARC within this area, most of these results could be explained by involvement of the ARC glucosensing neurons.

Glucosensing neurons use glucose as a signaling molecule to alter their firing rate when ambient glucose levels change (11,12,14,55). These same neurons also contain receptors for leptin (56) and insulin (57). Thus, they are equipped with signaling mechanisms that should allow them to monitor the status of peripheral energy stores and glucose metabolism. An interesting feature of the reduced CRR after hypoglycemia in humans is that the diminished response is selective for hypoglycemia. Sympathoadrenal responses to both postural change and cognitive stressors are preserved (7,58). Selective loss of ARC glucosensing neurons could explain this specific decrease in responding to hypoglycemia. Because brain areas mediating the sympathoadrenal responses to other stressors did not show cell damage after hypoglycemia, only

the hypoglycemic CRR would be expected to be diminished because of the selective loss of ARC neurons. Finally, in our rat model and in humans (6,8,9), neither a single nor repeated bouts of hypoglycemia completely ablated the CRR to subsequent hypoglycemic bouts. This is in keeping with the fact that only a portion of ARC neurons are damaged. Further, other brain areas (59,60) and/or peripheral sites (61) that contribute to the CRR are likely to remain intact after recurrent hypoglycemia.

A critical question posed by these studies is why ARC neurons should be selectively vulnerable to relatively mild degrees of hypoglycemia. It is assumed that the apoptotic response occurred predominantly in neurons as opposed to glia because of the reduction in both POMC and NPY mRNA. ARC neurons can use glucose as a signaling molecule to regulate the rate of cell firing. Here, ATP produced by intracellular glucose metabolism acts to inactivate an ATP-sensitive K^+ channel leading to increased cell firing (10,12,14,62–64). Other undefined mechanisms for glucoregulation of cell firing have also been proposed (65). But this property is not unique to ARC neurons. A subpopulation of neurons in the adjacent ventromedial nucleus is also capable of such glucosensing (10,12,14,62–64). By necessity, all glucosensing neurons must be sensitive to relatively small variations in ambient glucose levels. This heightened sensitivity to changes in glucose levels might also make them selectively vulnerable to pathologically low levels of glucose. One feature that distinguishes ARC from ventromedial nucleus neurons is that ARC neurons contain insulin receptors (57). The presence of these receptors on ARC glucosensing neurons might be the critical element making them more vulnerable to hypoglycemia accompanied by hyperinsulinemia. Of course this is exactly the condition that occurs in hypoglycemic diabetics. The mechanism for such an enhanced vulnerability remains unclear because insulin actually hyperpolarizes glucosensing neurons by its actions on the ATP-sensitive K^+ channel (55). This hyperpolarization should protect them from glucopenic damage (14). Also, there is evidence that high insulin levels might actually protect against the reduced CRR seen in hypoglycemia (66). Another possibility is that the sequence of hypoglycemia induced by hyperinsulinemia followed by acute elevation of glucose during the rescue phase might damage glucosensing ARC neurons as it does glucosensing pancreatic β -cells under certain circumstances (67). This could result when glucose-sensitive neurons containing insulin receptors are damaged during the hyperinsulinemic glucopenic phase and then driven to high rates of firing with rapid re-exposure to high glucose levels.

In conclusion, a single bout of moderate insulin-induced hypoglycemia, which does not produce coma in rats, results in a reduced CRR to subsequent hypoglycemic challenges (16). This occurrence is associated with a highly selective apoptotic response in ARC neurons, which is accompanied by decreased expression of both NPY and POMC mRNA. Whether these 2 findings are causally related remains to be seen. However, the fact that such a moderate and short-lived reduction in glucose levels can lead to apparent cell death poses an important cautionary note. It suggests that the repeated bouts of hypoglycemia suffered by diabetic subjects treated with insulin might have permanent adverse effects on brain function that previously have not been appreciated.

ACKNOWLEDGMENTS

This work was funded by the University of Pennsylvania Research Foundation, National Institute of Diabetes and Digestive and Kidney Diseases Grant R01DK53181, and the Research Service of the Department of Veterans Affairs.

We thank Karen Brown, Antoinette Moralishvili, Harriet Terodemos, and Joseph Menona for expert technical assistance.

REFERENCES

1. The DCCT Research Group: Hypoglycemia in the Diabetes Control and Complications Trial. *Diabetes* 46:271–286, 1997
2. Fujioka M, Okuchi K, Hiramatsu K-I, Sakaki T, Sakaguchi S, Ishii Y: Specific changes in human brain after hypoglycemic injury. *Stroke* 28:584–587, 1997
3. Perros P, Frier BM: The long-term sequelae of severe hypoglycemia on the brain in insulin-dependent diabetes mellitus. *Horm Metab Res* 29:197–202, 1997
4. Patrick AW, Campbell IW: Fatal hypoglycaemia in insulin-treated diabetes mellitus: clinical features and neuropathological changes. *Diabet Med* 7:349–354, 1990
5. Perros P, Deary IJ, Sellar RJ, Best JJK, Frier BM: Brain abnormalities demonstrated by magnetic resonance imaging in adult IDDM patients with and without a history of recurrent severe hypoglycemia. *Diabetes Care* 20:1013–1018, 1997
6. Dagogo-Jack S, Rattarasarn C, Cryer PE: Reversal of hypoglycemia unawareness, but not defective glucose counterregulation, in IDDM. *Diabetes* 43:1426–1434, 1994
7. Robinson AM, Parkin HM, Macdonald IA, Tattersall RB: Antecedent hypoglycemia in non-diabetic subjects reduces the adrenaline response for 6 days but does not affect the catecholamine response to other stimuli. *Clin Sci* 89:359–366, 1995
8. Amiel SA, Tamborlane WV, Simonson DC, Sherwin RS: Defective glucose counterregulation after strict glycaemic control of insulin-dependent diabetes mellitus. *N Engl J Med* 316:1376–1383, 1987
9. Heller SR, Cryer PE: Reduced neuroendocrine and symptomatic responses to subsequent hypoglycemia after 1 episode of hypoglycemia in nondiabetic humans. *Diabetes* 40:223–226, 1991
10. Dunn-Meynell AA, Govek E, Levin BE: Intracarotid glucose infusions selectively increase Fos-like immunoreactivity in paraventricular, ventromedial and dorsomedial nuclei neurons. *Brain Res* 748:100–106, 1997
11. Oomura Y, Ono T, Ooyama H, Wayner MJ: Glucose and osmosensitive neurons of the rat hypothalamus. *Nature* 222:282–284, 1969
12. Dunn-Meynell AA, Routh VH, McArdle JJ, Levin BE: Low affinity sulfonylurea binding sites reside on neuronal cell bodies in the brain. *Brain Res* 745:1–9, 1997
13. Levin BE, Levy R, Govek E: The ATP-sensitive potassium (K_{ATP}) channel and glucose-induced nigrostriatal dopamine release (Abstract). *Soc Neurosci Abst* 23:576, 1998
14. Levin BE, Dunn-Meynell AA, Routh VH: Brain glucosensing and body energy homeostasis: role in obesity and diabetes. *Am J Physiol* 276:R1223–R1231, 1999
15. Hevener AL, Bergman RN, Donovan CM: Novel glucosensor for hypoglycemic detection localized to the portal vein. *Diabetes* 46:1521–1525, 1997
16. Tkacs NC, Levin BE: Reduced counterregulation after repeated insulin injections in rats: CNS activation patterns (Abstract). *Soc Neurosci Abst* 24:1121A, 1998
17. Pipeleers DG: Heterogeneity in pancreatic beta-cell population. *Diabetes* 41:777–781, 1992
18. Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 119:493–501, 1992
19. Dunn-Meynell AA, Levin BE: Histological markers of neuronal, axonal and astrocytic changes after lateral rigid impact traumatic brain injury. *Brain Res* 761:25–41, 1997
20. Eggleston AK, Rahim NA, Kowalczykowski SC: A helicase assay based on the displacement of fluorescent, nucleic acid-binding ligands. *Nucleic Acids Res* 24:1179–1182, 1996
21. Levin BE, Dunn-Meynell A: Regulation of growth-associated protein 43 (GAP-43) messenger RNA associated with plastic change in the adult rat barrel receptor complex. *Mol Brain Res* 18:59–70, 1993
22. White JD, Olchovsky D, Kershaw M, Berelowitz M: Increased hypothalamic content of preproneuroptide-Y messenger ribonucleic acid in streptozotocin-diabetic rats. *Endocrinology* 126:765–772, 1990
23. Levin BE, Dunn-Meynell AA: Dysregulation of arcuate nucleus preproneuroptide Y mRNA in diet-induced obese rats. *Am J Physiol* 272:R1365–R1370, 1996

24. Higuchi H, Yang H-YT, Sabol SL: Rat neuropeptide Y precursor gene expression. *J Biol Chem* 263:6288–6295, 1988
25. Kim EM, Welch CC, Grace MK, Billington CJ, Levine AS: Chronic food restriction and acute food deprivation decrease mRNA levels of opioid peptides in arcuate nucleus. *Am J Physiol* 270:R1019–R1024, 1996
26. Sipols AJ, Baskin DG, Schwartz MW: Effect of intracerebroventricular insulin infusion on diabetic hyperphagia and hypothalamic neuropeptide gene expression. *Diabetes* 44:147–151, 1995
27. Marks JL, Li M, Schwartz MW, Porte D Jr, Baskin DG: The effect of fasting on neuropeptide Y mRNA and insulin receptors in the rat brain. *Mol Cell Neurosci* 3:199–205, 1992
28. Levin BE: Reduced paraventricular nucleus norepinephrine responsiveness in obesity-prone rats. *Am J Physiol* 270:R456–R461, 1996
29. Ovalle F, Fanelli CG, Paramore DS, Hershey T, Craft S, Cryer PE: Brief twice-weekly episodes of hypoglycemia reduce detection of clinical hypoglycemia in type 1 diabetes mellitus. *Diabetes* 47:1472–1479, 1998
30. Cranston I, Lomas J, Maran A, Macdonald I, Amiel SA: Restoration of hypoglycaemia awareness in patients with long-duration insulin-dependent diabetes. *Lancet* 344:283–287, 1994
31. Auer RN, Wieloch T, Olsson Y, Siesjo BK: The distribution of hypoglycemic brain damage. *Acta Neuropathol* 64:177–191, 1984
32. Perry SW, Epstein LG, Gelbard HA: Simultaneous in situ detection of apoptosis and necrosis in monolayer cultures by TUNEL and trypan blue staining. *Biotechniques* 22:1102–1106, 1997
33. Ben-Sasson SA, Sherman Y, Gavrieli Y: Identification of dying cells: in situ staining (Review). *Methods Cell Biol* 46:29–39, 1995
34. Nicotera P, Leist M, Ferrando-May E: Intracellular ATP, a switch in the decision between apoptosis and necrosis (Review). *Toxicol Lett* 102–103:139–142, 1998
35. Bergstedt M, Hu B-H, Wieloch T: Initiation of protein synthesis and heat-shock-72 expression in the rat brain following severe insulin-induced hypoglycemia. *Acta Neuropathol* 86:145–153, 1993
36. Xu L, Giffard RG: HSP70 protects murine astrocytes from glucose deprivation injury. *Neurosci Lett* 224:9–12, 1997
37. States BA, Honkanieni J, Weinstein PR, Sharp FR: DNA fragmentation and HSP70 protein induction in hippocampus and cortex occurs in separate neurons following permanent middle cerebral artery occlusions. *J Cereb Blood Flow Metab* 16:1165–1175, 1996
38. Giraudo SQ, Kim EM, Grace MK, Billington CJ, Levine AS: Effect of peripheral 2-DG on opioid and neuropeptide Y gene expression. *Brain Res* 792:136–140, 1998
39. Corrin SE, McCarthy HD, McKibbin PE, Williams G: Unchanged hypothalamic neuropeptide Y concentrations in hyperphagic, hypoglycemic rats: evidence for specific metabolic regulation of hypothalamic NPY. *Peptides* 12:425–430, 1991
40. Akabayashi A, Zaia CT, Silva I, Chae HJ, Leibowitz SF: Neuropeptide Y in the arcuate nucleus is modulated by alterations in glucose utilization. *Brain Res* 621:343–348, 1993
41. Levin BE: Arcuate NPY neurons and energy homeostasis in diet-induced obese and resistant rats. *Am J Physiol* 276:R382–R387, 1998
42. Nichols CG, Shyng SL, Nestorowicz A, Glaser B, Clement JP IV, Gonzalez G, Aguilar-Bryan L, Permutt MA, Bryan J: Adenosine diphosphate as an intracellular regulator of insulin secretion. *Science* 272:1785–1787, 1996
43. Pages N, Orosco M, Rouch C, Yao O, Jacquot C, Bohuon C: Refeeding after 72 hour fasting alters neuropeptide Y and monoamines in various cerebral areas. *Comp Biochem Physiol* 106:845–849, 1995
44. Baker RA, Herkenham M: Arcuate nucleus neurons that project to the hypothalamic paraventricular nucleus: neuropeptidergic identity and consequences of adrenalectomy on mRNA levels in the rat. *J Comp Neurol* 358:518–530, 1995
45. Elias CF, Saper CB, Maratos-Flier E, Tritos NA, Lee C, Kelly J, Tatro JB, Hoffman GE, Ollmann MM, Barsh GS, Sakurai T, Yanagisawa M, Elmquist JK: Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area. *J Comp Neurol* 402:442–459, 1998
46. Horvath TL, Diano S, van den Pol AN: Synaptic interaction between hypocretin (orexin) and neuropeptide Y cells in the rodent and primate hypothalamus: a novel circuit implicated in metabolic and endocrine regulations. *J Neurosci* 19:1072–1087, 1999
47. Sawchenko PE, Swanson LW: Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. *J Comp Neurol* 205:260–272, 1982
48. Jansen ASP, Wessendorf MW, Loevy AD: Transneuronal labeling of CNS neuropeptide and monoamine neurons after pseudorabies virus injections into stellate ganglion. *Brain Res* 683:1–24, 1995
49. van den Pol AN: Hypothalamic hypocretin (orexin): robust innervation of the spinal cord. *J Neurosci* 19:3171–3181, 1999
50. Elias CF, Lee C, Kelly J, Aschkenasi C, Ahima RS, Couceyro PR, Kuhar MJ, Saper CB, Elmquist JK: Leptin activates CART neurons projecting to the spinal cord. *Neuron* 21:1375–1385, 1998
51. Borg WP, Sherwin RS, Doring MJ, Borg MA, Shulman GI: Local ventromedial hypothalamic glucopenia triggers counterregulatory hormone release. *Diabetes* 44:180–184, 1995
52. Borg WP, Doring MJ, Sherwin RS, Borg MA, Brines ML, Shulman GI: Ventromedial hypothalamic lesions in rats suppress counterregulatory responses to hypoglycemia. *J Clin Invest* 93:1677–1682, 1994
53. Borg MA, Sherwin RS, Borg WP, Tamborlane WV, Shulman GI: Local ventromedial hypothalamus glucose perfusion blocks counterregulation during systemic hypoglycemia in awake rats. *J Clin Invest* 99:361–365, 1997
54. Borg WP, Tamborlane WV, Brines ML, Shulman GI, Sherwin RS: Chronic hypoglycemia and diabetes impair counterregulation induced by localized 2-deoxy-glucose perfusion of the ventromedial hypothalamus in rats. *Diabetes* 48:584–587, 1999
55. Routh VH, McArdle JJ, Spanswick DC, Levin BE, Ashford MLJ: Insulin modulates the activity of glucose responsive neurons in the ventromedial hypothalamic nucleus (VMN) (Abstract). *Soc Neurosci Abst* 23:577A, 1997
56. Baskin DG, Breininger JF, Schwartz MW: Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hypothalamus. *Diabetes* 48:828–833, 1999
57. Marks JL, Porte D Jr, Stahl WL, Baskin DG: Localization of insulin receptor mRNA in rat brain by in situ hybridization. *Endocrinology* 127:3234–3236, 1990
58. Rattarasarn C, Dagogo-Jack S, Zachwieja JJ, Cryer PE: Hypoglycemia-induced autonomic failure in IDDM is specific for stimulus of hypoglycemia and is not attributable to prior autonomic activation. *Diabetes* 43:809–818, 1994
59. Frizzel RT, Jones EM, Davis SN, Biggers DW, Myers SR, Connolly CC, Neal DW, Jaspas JB, Cherrington AD: Counterregulation during hypoglycemia is directed by widespread brain regions. *Diabetes* 42:1253–1261, 1993
60. Sanders NM, Ritter S: Recurrent hypoglycemia reduces fos expression in rat hindbrain (Abstract). *Soc Neurosci Abst* 23:2054A, 1997
61. Cane P, Haun CK, Evered J, Youn JH, Bergman RN: Response to deep hypoglycemia does not involve glucose receptors in carotid perfused tissue. *Am J Physiol* 255:E680–E687, 1988
62. Levin BE, Brown KL, Dunn-Meynell AA: Differential effects of diet and obesity on high and low affinity sulfonylurea binding sites in the rat brain. *Brain Res* 739:293–300, 1996
63. Dunn-Meynell AA, Rawson NE, Levin BE: Distribution and phenotype of neurons containing the ATP-sensitive K⁺ channel in rat brain. *Brain Res* 814:41–54, 1998
64. Levin BE, Govek EK, Dunn-Meynell AA: Reduced glucose-induced neuronal activation in the hypothalamus of diet-induced obese rats. *Brain Res* 808:317–319, 1998
65. Niimi M, Sato M, Tamaki M, Wada Y, Takahara J, Kawanishi K: Induction of Fos protein in the rat hypothalamus elicited by insulin-induced hypoglycemia. *Neurosci Res* 23:361–364, 1995
66. Fruehwald-Schultes B, Kern W, Deininger E, Wellhoener P, Kerner W, Born J, Fehm HL, Peters A: Protective effect of insulin against hypoglycemia-associated counterregulatory failure. *J Clin Endocrinol Metab* 84:1551–1557, 1999
67. Efanova IB, Zaitsev SV, Zhivotovsky B, Kohler M, Efendic S, Orrenius S, Berggren PO: Glucose and tolbutamide induce apoptosis in pancreatic beta-cells: a process dependent on intracellular Ca²⁺ concentration. *J Biol Chem* 273:33501–33507, 1998