Reduced Insulin and IGF-I Signaling, not Hyperglycemia, Underlies the Diabetes-Associated Depletion of Interstitial Cells of Cajal in the Murine Stomach

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Damage to interstitial cells of Cajal (ICC), pacemakers, and mediators of neuromuscular neurotransmission in the gastrointestinal tract contributes to the pathogenesis of diabetic gastroenteropathy in both patients and animal models. ICC depletion in diabetes may result from chronic hyperglycemia or lost/ineffective insulin signaling. Because independent control of insulin and glucose concentrations is difficult in chronic in vivo studies, we used long-term organotypic cultures to address this problem. Murine gastric muscles were cultured in normoglycemic or hyperglycemic basal media with or without insulin or IGF-I for 1–3 months, the time required for gastroparesis and ICC damage to develop in diabetic mice. ICC were assessed by c-Kit immunohistochemistry and quantitative analysis of c-*kit* expression. Electrical pacemaking was studied by intracellular recording of slow waves. ICC survived for at least 34 days in unsupplemented normoglycemic media, but their networks, c-*kit* expression, and slow waves were profoundly reduced after 68 days. These changes could be entirely prevented by insulin or IGF-I supplementation. ICC networks were completely resistant to hyperglycemia for at least 72 days. Thus, hyperglycemia is unlikely to be responsible for the diabetes-associated depletion of ICC. In contrast, maintenance of ICC requires insulin or IGF-I, which are reduced or ineffective in diabetes. *Diabetes* 54:1528–1533, 2005

Diabetic gastropathy, termed broadly as gastric neuromuscular dysfunction, and gastroparesis, defined as symptomatic or asymptomatic gastric retention, occur in up to 50% of patients with type 1 diabetes and 30% of patients with type 2 diabetes (1,2). The spectrum of symptoms includes postprandial discomfort, bloating, fullness, abdominal pain, and the classic symptoms of gastroparesis: early satiation and recurring nausea and vomiting, which may be self-limiting, recurrent, or unrelenting (1). Asymptomatic gastroparesis may only manifest as poor glycemic control, including hypoglycemia (2). Although diabetic gastropathy is rarely life threatening, it seriously affects the patient's quality of life (1), a prime concern in an aging population.

Although generally considered a manifestation of irreversible autonomic or enteric neuropathy, diabetic gastropathy is likely multifactorial (1–3). Previously, we added a new dimension to the pathogenesis of diabetic gastropathy by showing that, in spontaneously diabetic NOD/LtJ mice, gastroparesis is associated with depletions of networks of interstitial cells of Cajal (ICC) in the distal stomach (4). Damages to ICC have since been demonstrated in the stomach (5,6), jejunum (7), and colon (8) of patients with gastroenteropathy due either to type 1 (7) or type 2 (8) diabetes. ICC are mesenchymal cells that play critical roles in gastric motility. First, networks of multipolar ICC located primarily in the myenteric region generate electrical pacemaker activity (9–11), provide a pathway for the corpus-to-antrum propagation of electrical slow waves that govern peristalsis (9,10), and thereby represent a key component of the "peristaltic pump" responsible for the emptying of solids from the stomach (12). We have also shown that depletion of pacemaker ICC can result in functional abnormalities (bradygastria, antral tachygastria, arrhythmias, and uncoupling) that are considered hallmarks of diabetic gastroparesis (4,9,10). Second, intramuscular ICC intercalated between nerve fibers and smooth muscle cells mediate neuromuscular neurotransmission (13). By relaying excitatory inputs to the fundus and mediating nitrergic relaxation of the pyloric sphincter (14), ICC also contribute to the "pressure pump" that regulates gastric emptying of liquids (15). Third, ICC have been reported to play a role in vagally mediated mechanoreception (16). Thus, disruptions of gastric ICC networks can potentially affect all aspects of gastric neuromuscular function and are likely to play a significant role in the pathogenesis of diabetic gastroenteropathy in both patients and animal models.

To prevent or reverse ICC loss in diabetes, it is essential to understand its pathomechanisms. Similarly to other long-term complications, chronic or recurring hyperglycemia (3,17) and resultant oxidative damage, nonenzymatic glycation, and inappropriate activation of protein kinase C,

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ICC, interstitial cells of Cajal.

nuclear factor κB, and aldose reductase (18) may play a role. There is also ample evidence to indicate a significant role in diabetes complications for lost (type 1) or reduced and ineffective (type 2) insulin signaling (19,20), reduced levels of the proinsulin C-peptide (20), and abnormal levels of growth factors, e.g., IGF-I and possibly IGF-II (20). Indeed, in diabetic mice, insulin infusion for 1 week has been shown to improve reduced pyloric relaxation and delayed liquid emptying by stimulating neuronal nitric oxide synthesis independent of the concurrent normalization of glucose levels (21). However, ICC depletion and gastroparesis in NOD/LtJ mice occurs within 1.5 and 3 months after the onset of diabetes (4), and dissecting the relative significance of hyperglycemia and impaired insulin signaling in chronic in vivo studies has been notoriously difficult. Therefore, we developed an organotypic culture model that permits the independent control of insulin, glucose, and growth factor levels over several months to investigate the mechanism of diabetes-associated ICC depletion in the murine stomach. In this article, we report results obtained with chronic normo- and hyperglycemia in the presence or absence of insulin and IGF-I supplementation.

RESEARCH DESIGN AND METHODS

The 9- to 18-day-old BALB/c mice were obtained from breeder pairs purchased either from Harlan Sprague-Dawley (Indianapolis, IN) or Charles River Laboratories (Wilmington, MA). The animals were anesthetized with isoflurane (Baxter Healthcare, Deerfield, IL) before decapitation. Mice were maintained and the experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Use and Care Committee at the University of Nevada, Reno. Stomachs were opened along the lesser curvature. The fundus was cut away, and only the *tunica muscularis* of the entire gastric corpus and antrum was used after the removal of the mucosa and submucosa (10).

Organotypic cultures were prepared and maintained as described (9). Briefly, the intact continuous corpus and antrum *tunica muscularis* tissues (6–7 mg) were pinned, mucosal side up, onto the surface of 35-mm culture dishes coated with Sylgard 184 (Dow Corning, Midland, MI) and incubated at 37°C in normoglycemic (1 g/l; 5.55 mmol/l) M199 medium (Sigma, St. Louis, MO) containing 2% antibiotic-antimycotic and 2 mmol/l L-glutamine (Invitrogen, Carlsbad, CA). Other supplements (used alone or in combination; see RESULTS) included D-glucose (final concentration: 6 g/l, 33.3 mmol/l, or 10 g/l, 55.5 mmol/l), bovine insulin (5 μg/ml; Cambrex BioScience, Walkersville, MD, or Invitrogen), fetal bovine serum (5%; HyClone, Logan, UT), or murine IGF-I (100 ng/ml; Sigma). All treatments were started within 48 h after establishing the cultures and maintained throughout the entire experiment. Levels of the supplements were kept constant by changing the culture media every 48 h. This frequency was determined empirically by measuring glucose levels in the spent media with an Accu-Chek Complete blood glucose monitor (Roche Diagnostics, Indianapolis, IN) (4). After 48 h, glucose concentrations did not fall >15 mg/dl (0.8 mmol/l) in the normoglycemic cultures, and no measurable decline could be detected in the hyperglycemic cultures.

After acetone fixation of the cultured and freshly dissected tissues, ICC were identified with monoclonal (rat) c-Kit antibodies (ACK2; 5 μg/ml) and Alexa Fluor 488 anti-rat IgG (10 μg/ml; Molecular Probes, Eugene, OR) as previously described (4,9–11). Confocal images of the whole-mounts were taken with a Bio-Rad MRC 600 system (Hercules, CA). To control for the gradients in ICC network densities that occur along both the longitudinal axis and the circumference of the normal murine stomach, images were taken in three representative regions along the greater curve (orad corpus, corpus–antrum border, distal antrum) of each tissue (10). ICC network densities were analyzed quantitatively in superimposed two-dimensional projections of optical sections representing the entire thickness of the whole-mounts by a technique modified from He et al. (7) and validated in a previous study (10). Briefly, cellular and background fluorescence were separated by thresholding on the peak of the distribution of cellular fluorescence. To eliminate variations in brightness within and between images, fluorescence values above and below the threshold were assigned 1 (white) and 0 (black), respectively. ICC densities were expressed as the percent white pixels over a standard area (289 × 193 μm) in the two-dimensional projections and averaged for each tissue (10). Values obtained by this technique are numerical expressions of ICC network densities as they appear in the superimposed confocal sections (see for example the panels in Fig. 1), and neither represent the proportion of ICC nor the fraction of tissue volume occupied by these cells (10).

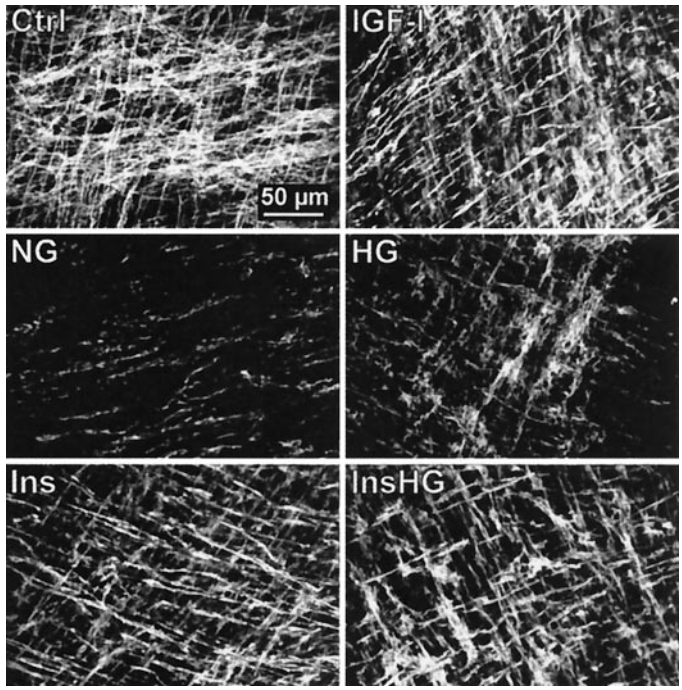

FIG. 1. Long-term effects of hyperglycemia, insulin, and IGF-I on ICC networks in organotypic cultures of gastric corpus and antrum *tunica muscularis*. Representative confocal images of c-Kit–like immunofluorescence in freshly dissected controls (Ctrl) and in tissues cultured for 68–72 days with one of the following media are shown: unsupplemented normoglycemic (NG) or hyperglycemic (HG) media, normoglycemic media containing insulin (Ins), hyperglycemic media supplemented with insulin (InsHG), or normoglycemic media containing IGF-I (IGF-I). The images show all ICC that occur within the entire thickness of the tissues projected onto a two-dimensional plane. The scale bar in the top left panel applies to all panels. Note the depletion of ICC in normoglycemic cultures and the partial or complete prevention of ICC loss by elevated glucose concentrations, insulin, or IGF-I.

ICC in cultured and freshly dissected corpus and antrum muscles obtained from 14-day-old mice were also assessed by quantitative analysis of c-*kit* expression. Quantitative RT-PCR was performed using SYBR Green chemistry on a GeneAmp 5700 sequence detector (Applied Biosystems, Foster City, CA) as described (22). Total RNA was isolated from the tissues containing the entire anatomically defined distal stomach (corpus + antrum). The cDNA was amplified (40 cycles) with specific primers (Qiagen, Valencia, CA) for c-*kit* and the housekeeping gene β-actin (22). Transcriptional quantification of gene products was obtained relative to the β-actin standard curve and expressed in β-actin units as transcript per corpus + antrum *tunica muscularis*. We tested for genomic DNA contamination by PCR with cytoglobin primers that span an intron (22). Nonspecific amplification and primer dimers were controlled for by omitting the template from the PCR amplification.

Electrical slow-wave activity in cultured and freshly dissected corpus + antrum tissues obtained from 14-day-old mice was analyzed by intracellular recording as described (4,9,10). Transmembrane potential of circular muscle cells impaled with KCl-filled glass microelectrodes was recorded at 37.5 ± 0.5°C using an Intra 767 amplifier (World Precision Instruments, Sarasota, FL) and a BIOPAC (Santa Barbara, CA) MP100 data acquisition system.

Data are expressed as means ± SE. Percentage data were transformed [arcsin($\sqrt{x}$)] before statistical analysis. One-way ANOVA or Kruskal-Wallis one-way ANOVA on ranks followed by all-pairwise multiple comparison (Tukey test or Dunn's method, respectively) were used for statistical comparisons. A probability value of $P < 0.05$ was used as a cutoff for statistical significance in all statistical procedures.

RESULTS

Consistent with previous results (9), culturing juvenile corpus + antrum muscles for 34 days in unsupplemented

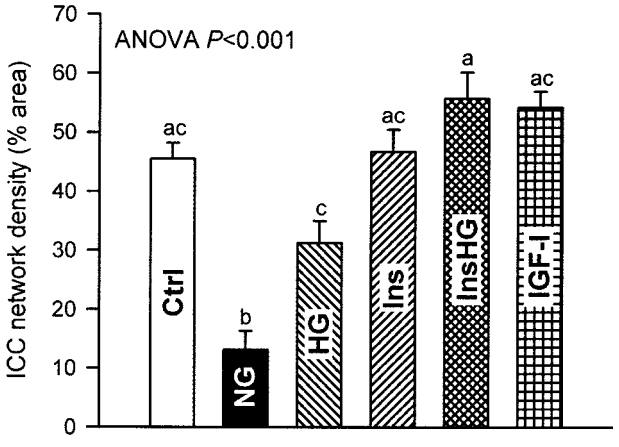

FIG. 2. Long-term effects of hyperglycemia, insulin, and IGF-I on ICC networks in organotypic cultures of gastric corpus and antrum *tunica muscularis*. ICC network densities in the two-dimensional confocal composite images (e.g., those shown in Fig. 1) obtained by quantitative image analysis are shown. Labels correspond to labels in Fig. 1. The number of cultures in the different treatment groups were as follows: Ctrl: 11, NG: 9, HG: 5, Ins: 8, InsHG: 7, IGF-I: 4. Groups not sharing the same superscript are significantly different by multiple comparisons. ICC networks were depleted in long-term unsupplemented normoglycemic cultures, and this reduction could be prevented by insulin (regardless of glucose levels) or IGF-I. Note partial prevention of ICC loss by high glucose concentrations.

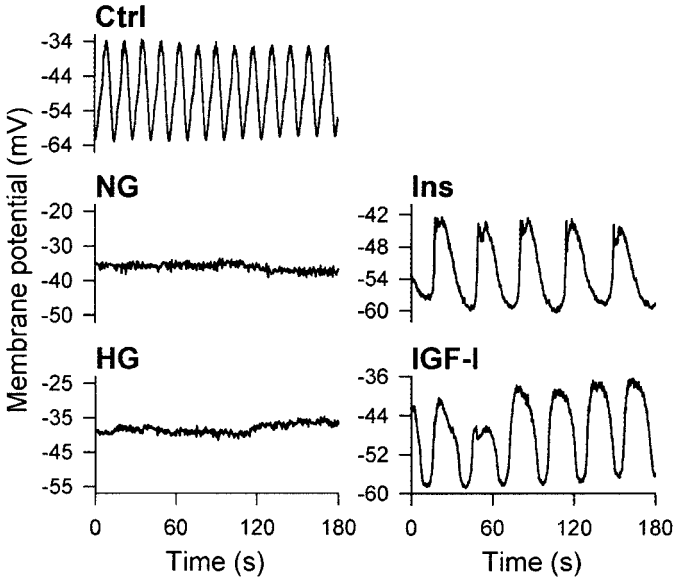

FIG. 3. Long-term effects of hyperglycemia, insulin, and IGF-I on gastric slow waves in organotypic cultures. Representative recordings made in freshly dissected controls (Ctrl) and in tissues cultured for 83–86 days in unsupplemented normoglycemic (NG) or hyperglycemic (HG) media or in normoglycemic media containing insulin (Ins) or IGF-I (IGF-I) are shown. Note the lack of slow waves and depolarization of resting membrane potentials in the unsupplemented, normoglycemic, or hyperglycemic cultures. Both depolarization and the loss of slow wave activity were prevented by insulin or IGF-I.

normoglycemic media did not significantly affect the density of ICC networks: c-Kit–like immunoreactivity in the superimposed binarized confocal sections reflecting ICC occurring throughout the entire thickness of the whole-mounts occupied 45.5 ± 2.7% of the image area in the freshly dissected controls (n = 11) and 37.6 ± 5.1% in the normoglycemic cultures (n = 7; NS). ICC network densities also did not decrease in cultures maintained under hyperglycemic conditions (33.3 mmol/l for the first 17 days and 55.5 mmol/l for the second 17 days: 44.0 ± 6.0%, n = 4; NS). Therefore, in the next set of experiments, we extended the culture period to 68–72 days (Figs. 1 and 2). Relative to freshly dissected controls, ICC networks were significantly depleted in the unsupplemented normoglycemic cultures (Figs. 1 and 2, unsupplemented normoglycemic media [NG]). Both intramuscular and myenteric ICC were affected. Surprisingly, hyperglycemia (55.5 mmol/l throughout the culture period) partially, but significantly, prevented the reduction of both classes of ICC (Figs. 1 and 2, hyperglycemic media [HG]). Supplementation of culture media with insulin completely prevented the loss of ICC (Figs. 1 and 2, insulin [Ins]), and the networks maintained with the aid of insulin remained unaffected by chronic hyperglycemia (Figs. 1 and 2, hyperglycemic media supplemented with insulin [InsHG]). Measurements of glucose concentrations in 2-day spent media indicated that they were essentially unaffected by tissue utilization. Thus, the lack of effect was not due to a fall of glucose levels by the end of the culture period between media changes. Addition of 5% fetal bovine serum to insulin-supplemented normoglycemic (n = 3) or hyperglycemic (n = 3) media did not influence the results (not shown). IGF-I supplementation mimicked the effects of insulin and completely prevented the depletion of ICC networks under normoglycemic conditions (Figs. 1 and 2, IGF-I).

In a parallel experiment, we used quantitative RT-PCR analysis of c-*kit* expression to assess ICC in corpus + antrum *tunica muscularis* tissues cultured for 75 days. The results were similar to those obtained by c-Kit immunohistochemistry: Total c-*kit* expression was significantly reduced in unsupplemented normoglycemic cultures relative to freshly dissected controls (3.0 ± 1.2 β-actin units, n = 5; controls: 64.7 ± 12.6, n = 8; P = 0.011), and hyperglycemia did not cause a further decrease in c-*kit* mRNA (8.9 ± 5.3, n = 5). Both insulin (n = 5) and IGF-I (n = 5) treatment prevented the reduction seen in the unsupplemented cultures, although the degree of protection varied greatly (insulin 46.2 ± 19.1; IGF-I 70.7 ± 35.1; NS vs. freshly dissected controls).

Finally, we examined the effects of hyperglycemia, insulin, and IGF-I on electrical slow-wave activity recorded from circular smooth muscle cells in tissues cultured for 83–86 days (Figs. 3 and 4). In cultures not supplemented with insulin or IGF-I, resting membrane potentials were depolarized and slow waves could not be recorded, except for a single impalement made in a tissue maintained under hyperglycemia, where arrhythmic activity of very low amplitude was detected. Both insulin and IGF-I prevented depolarization and the loss of slow waves. However, while slow-wave amplitudes were efficiently maintained by either insulin or IGF-I, their frequencies remained at ~50% of those detected in freshly dissected controls, an effect likely caused by culturing per se (9). The slower activity was accompanied by a noticeable but statistically insignificant increase in slow-wave duration.

DISCUSSION

In this study, we used organotypic cultures of gastric corpus and antrum *tunica muscularis* to dissect the contributions of hyperglycemia and insulinopenia to diabetes-associated ICC loss (4–8). Our results demonstrate

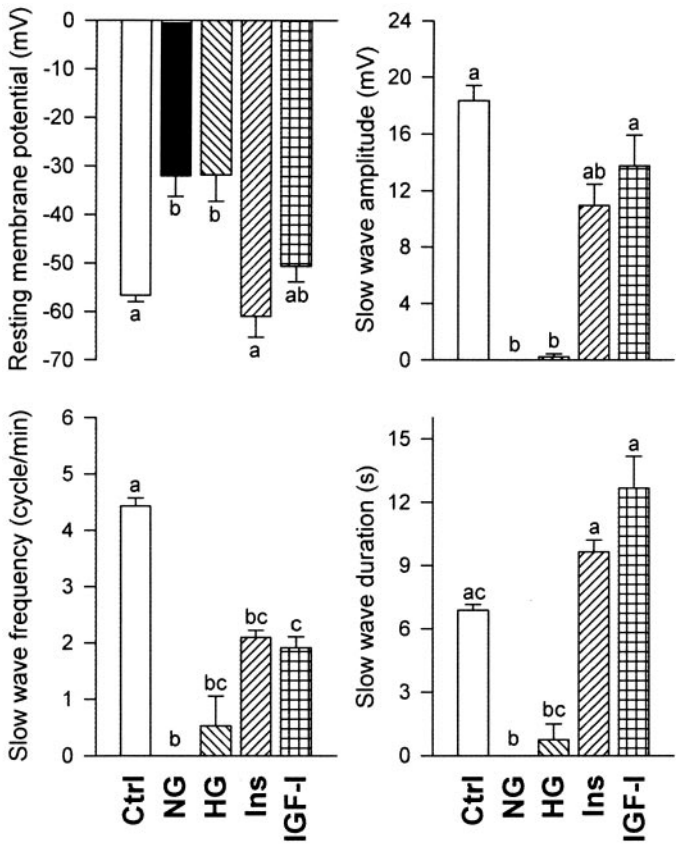

FIG. 4. Long-term effects of hyperglycemia, insulin, and IGF-I on gastric slow waves in organotypic cultures. Slow-wave parameters obtained by intracellular impalements (n = 9–16; two tissues per treatment) are shown. Labels correspond to labels in Fig. 3. Slow-wave duration was measured at one-half amplitude. ANOVA $P < 0.001$ in all panels; groups not sharing the same superscript are significantly different by multiple comparisons. Slow waves were largely missing in unsupplemented normoglycemic and hyperglycemic cultures. Both insulin and IGF-I prevented the loss of slow-wave activity.

that normal ICC network morphology is completely unaffected by long-term hyperglycemia. This observation was surprising considering that chronic complications of diabetes are generally attributed to recurring episodes of hyperglycemia and resultant oxidative damage, nonenzymatic glycation, and activation of protein kinase C, nuclear factor κB, and aldose reductase (18). Hyperglycemia has been shown to induce apoptosis via activation of caspase-3 in cultured superior cervical ganglia, dorsal root ganglion neurons, Schwann cells, and neuroblastoma cells, and only a relatively small proportion of these effects can be attributed to hyperosmolarity (20,23). It is unclear why ICC are not damaged by hyperglycemia, but it is remarkable that they contain an abundance of mitochondria and rely on oxidative metabolism for electrical pacemaking (11) and thus likely possess efficient mechanisms for elimination of superoxide and other reactive oxygen species (18). Even more surprising was the finding that hyperglycemia can actually limit the damage to ICC networks that occurs between 34 and 68 days of culture. High levels of glucose (up to 100 mmol/l) have been reported to have paradoxical neuroprotective effects against glutamate and free radical neurotoxicity and oxygen-glucose deprivation by enhancing mitochondrial transmembrane potentials (24). Glucose can also activate members of the mitogen-activated protein kinase family (25) and may stimulate the production of tissue growth factors required for ICC survival and function. Whether any of these mechanisms contributes to the observed effects remains to be investigated. It is also important to note that hyperglycemia could not prevent the loss of electrical slow waves, suggesting an inhibition of pacemaking independent of ICC depletion. This observation is consistent with previous findings that even acute elevations in blood glucose levels can have deleterious effects on gastrointestinal motility by eliciting gastric dysrhythmias, impaired antroduodenal motor activity, delayed gastric emptying, altered visceral sensation, and impaired colonic response to feeding (1–3,17). It is therefore likely that acute hyperglycemic episodes in diabetes may interact with ICC loss precipitated by other mechanisms to cause acute exacerbations of chronic symptoms in various manifestations of diabetic gastroenteropathies (1,17).

When gastric or small intestinal muscles are isolated from newborn mice and cultured in normoglycemic unsupplemented basal media, ICC and electrical slow waves continue to develop for at least 10 days (26) and are maintained for about a month (present data; 9). These findings indicate that the *tunica muscularis* has intrinsic reserves from which ICC can draw for normal function. However, ICC networks begin to deteriorate around 6 weeks (9) and, as we demonstrate herein, undergo significant depletion by the end of the 10th week of culture, following a time course similar to the ICC loss that occurs in untreated diabetic NOD/LtJ mice (4). The decline in ICC is paralleled by a similar disruption of electrical slow waves (4,9). These changes can be accelerated by continuous blockade of c-Kit signaling with the neutralizing antibody ACK2, indicating that stem cell factor, the natural ligand for c-Kit, plays a critical role in the maintenance of the ICC phenotype not only in vivo (27,28), but also in culture (9,26), and that endogenous production of this cytokine may require additional (likely serum-born) factors beyond a certain period of time. This dependence on extrinsic factors is more evident in canine (29) and mouse colon tissues placed in culture (T.Ö., unpublished data), where ICC and smooth muscle cells require serum supplementation for survival. In this study, we demonstrate that insulin is an important serum-born factor required for the long-term maintenance of ICC. The depletion of ICC networks could be completely prevented by insulin treatment, even in the presence of 55.5 mmol/l glucose, and these effects were not enhanced any further by the administration of 5% fetal bovine serum. In fact, we have shown that c-Kit–like immunoreactivity, c-*kit* expression, and electrical slow-wave activity in ICC can be maintained for at least 86 days under these circumstances, except for an ~50% reduction in slow-wave frequency, which is likely to be an effect of culturing per se (9). Recently, we have found that insulin treatment can also prevent ICC loss in gastric antrum tissues obtained from adult BALB/c mice (unpublished data). These results strongly suggest that insulinopenia may be the underlying cause of ICC loss and resultant deterioration of electrical pacemaking and gastroparesis in diabetic NOD/LtJ mice (4) and, possibly, in patients with diabetes (5–8). Although chronic complications of diabetes have been traditionally attributed to

hyperglycemia (18), it is now well established that reduced or ineffective insulin signaling and abnormal levels of other growth factors also play important roles (20), even in type 2 diabetes, where blunted glucose-induced insulin secretion accompanies insulin resistance in target tissues (19,30). The lower prevalence of gastropathy in type 2 diabetes (up to 30% vs. 50% in type 1 diabetes) (2) also supports a role for absolute or relative insulinopenia in this complication. Furthermore, 1-week insulin treatment has been reported to restore delayed liquid emptying in NOD mice by stimulating neuronal nitric oxide synthase expression, an effect that could not be replicated by acute normalization of blood glucose levels (21). Thus, insulin may have multiple beneficial effects on diabetes-associated gastric motor dysfunction. At present, the primary target for insulin in the gastric *tunica muscularis* is unclear. Recent unpublished work in our laboratory suggests that ICC may not express insulin receptors, and effects of insulin on these cells may be mediated by other growth factors (possibly stem cell factor) produced by smooth muscle cells and enteric neurons (31,32). Whether these primary insulin target cells develop resistance to this hormone in type 2 diabetes remains to be investigated.

The disruption of ICC networks in our studies could also be prevented by IGF-I supplementation. Circulating IGF-I levels do not appear to correlate with the degree of glycemic control and are reduced in both type 1 and type 2 diabetes, although the changes are less pronounced in the latter (20). IGF-I may mediate, at least in part, the effects of insulin, which is known to stimulate IGF-I expression (20). Because both insulin and IGF-I and their receptors are structurally related, cross-talk between the two systems may also occur at pharmacological concentrations (20,33). Insulin may also be able to activate signaling through the IGF-I receptor β subunit at physiological concentrations by binding to hybrid insulin/IGF-I receptors containing the A isoform of insulin receptor (33). However, it is unclear whether such hybrid receptors can be found in the gastrointestinal *tunica muscularis*. In any case, the finding that both insulin and IGF-I can prevent the loss of ICC suggests that these effects are more likely to be mediated by genomic, rather than metabolic, actions of insulin (20). The intracellular pathways involved in the long-term maintenance of the ICC phenotype by insulin and IGF-I remain to be investigated.

Finally, it is important to emphasize that in addition to insulin and IGF-I, other factors may also contribute to the long-term maintenance of ICC and electrical slow waves. For example, IGF-II, another natural ligand for the IGF-I receptor, may have effects similar to IGF-I (rev. in 20). In addition, the proinsulin C-peptide has been shown to enhance or mimic the effects of insulin and to normalize the expression of insulin receptor, IGF-I, and IGF-I receptor in peripheral nerves of diabetic rats (rev. in 20). Elucidation of the role of these factors on ICC requires further investigation.

In summary, our results indicate that the diabetes-associated depletion of ICC, a significant component of diabetic gastropathy and gastroparesis, is unlikely to be caused by chronic or recurrent hyperglycemia. In contrast, maintenance of ICC requires insulin or IGF-I, which are reduced or ineffective in diabetes. These findings could form the basis of novel treatment options to restore function that is lost in patients suffering from this frequent and potentially debilitating complication of diabetes.

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