

Glucose Effects on Skin Keratinocytes

Implications for Diabetes Skin Complications

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Altered skin wound healing is a common cause of morbidity and mortality among diabetic patients. However, the molecular mechanisms whereby diabetes alters skin physiology have not been elucidated. In this study, we investigated the relative roles of hyperglycemia, insulin, and IGF-I, all of which are abnormal in diabetes, in primary murine skin keratinocytes. These cells proliferate and differentiate in vitro in a manner similar to skin in vivo. It was found that in the presence of high glucose (20 mmol/l), the glucose transport rate of primary proliferating or differentiating keratinocytes was downregulated, whereas at 2 mmol/l glucose, the transport rate was increased. These changes were associated with changes in the GLUT1 expression and with changes in the affinity constant (K_m) of the transport. Exposure to high glucose was associated with changes in cellular morphology, as well as with decreased proliferation and enhancement of Ca^{2+} -induced differentiation of keratinocytes. Furthermore, in the presence of high glucose, ligand-induced IGF-I receptor but not insulin receptor (IR) autophosphorylation was decreased. Consequently, in high glucose, the effects of IGF-I on glucose uptake and keratinocyte proliferation were inhibited. Interestingly, lack of IR expression in IR-null keratinocytes abolished insulin-induced glucose uptake and partially decreased insulin- and IGF-I-induced proliferation, demonstrating the direct involvement of the IR in these processes. Our results demonstrate that hyperglycemia and impaired insulin signaling might be directly involved in the development of chronic complications of diabetes by impairing glucose utilization of skin keratinocytes as well as skin proliferation and differentiation. *Diabetes* 50:1627–1635, 2001

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dGlc, 2-deoxy-[³H]glucose; IGFR, IGF-I receptor; IR, insulin receptor; KO, knockout; NIH, National Institutes of Health; PBS, phosphate-buffered saline; PKC, protein kinase C; WT, wild-type.

Glucose represents a major fuel for most mammalian cells, and a wide range of factors regulates its utilization. However, abnormally high levels of glucose, as seen in diabetes, lead to the development of chronic complications. Several hypotheses were proposed to explain the various pathological changes induced by hyperglycemia, including glycation end products, hyperosmolarity, abnormal sorbitol and myoinositol metabolism, oxidant formation, and protein kinase C (PKC) activation by diacylglycerol (1–5). Furthermore, elevated levels of glucose were shown to affect insulin signaling in various ways. Hyperglycemia was shown to alter glucose-induced insulin secretion, a phenomenon referred to as glucose toxicity (6,7). Glucose is known to affect insulin action as well by regulating the expression of several genes, including the IGF-I receptor (IGFR) and insulin receptor (IR) genes, at both the transcriptional and translational levels (8–10). Moreover, hyperglycemia was shown to inhibit insulin action. This inhibition is thought to be a result of serine phosphorylation through a PKC-mediated mechanism (11,12) as well as by activation of protein tyrosine phosphatases, which deactivates the IR function (13).

In addition to its possible involvement in the development of complications of chronic diabetes, glucose was shown to downregulate its own transport and metabolism (14–20). As a result, high glucose levels create a vicious cycle in which even less glucose enters the cells, resulting in increased blood glucose levels, which in turn further disrupt the transport and metabolism of glucose into the cells. It is therefore clear that glucose per se, either directly or via changes in insulin signaling, is an important factor in both the regulation of its own transport and metabolism and in the pathogenesis of chronic complications of diabetes.

In the present study, we focused on the possible effects of hyperglycemia on skin keratinocytes. We have studied the direct effects of elevated glucose on skin cells as well as its possible interactions with the other factors contributing to the diabetes milieu, i.e., insulin and IGF-I. In our studies, we used a model system of primary skin keratinocytes in culture. In this model system, the differentiation state of the cells can be controlled by varying the calcium concentration in the medium (21,22). This differentiation process closely follows the maturation pattern of epidermis in vivo (22).

Using this cultured murine keratinocyte system, we

found that glucose downregulates its own transport into the cells. Furthermore, glucose was found to affect the proliferation and differentiation processes of keratinocytes, thereby altering their function. In addition, elevated glucose altered the effects of insulin and IGF-I on various cellular processes.

RESEARCH DESIGN AND METHODS

Cell culture. Primary mouse keratinocytes from newborn Balb/c mice or from newborn IR-null mice and their wild-type (WT) littermates (23) were prepared and maintained as described previously (22). Briefly, freshly isolated keratinocytes were cultured in Dulbecco's modified Eagle's medium containing 5.5 mmol/l D-glucose (Biological Industries, Beit Haemek, Israel), 10% chelexed fetal calf serum (Biological Industries), 1% antibiotics, and Ca^{2+} concentration adjusted to 0.05 mmol/l, as previously described (22). After a day in culture, the glucose concentrations were adjusted to the desired glucose concentrations (ranging from 2 to 20 mmol/l D-glucose) and were maintained in this growth medium, with medium changes every 2–3 days. Thus, in most experiments, unless indicated otherwise, the cells were maintained in the various glucose concentrations for 5 days. In experiments in which differentiation was induced in the cells, the Ca^{2+} levels were adjusted after 4 days in culture (3 of which were in the adjusted 2–20 mmol/l glucose concentration) to defined concentrations (0.05 mmol/l [low]; 0.12 mmol/l [medium]; 1.0 mmol/l [high] Ca^{2+}) for 24–48 h, as indicated in each experiment. The glucose concentrations, however, were further maintained as determined before (i.e., between 2 and 20 mmol/l D-glucose).

Protein lysate preparation. Cells were harvested by scraping into lysis buffer (phosphate-buffered saline [PBS] containing 1% Triton X-100, 1 mmol/l EDTA, 10 mmol/l sodium fluoride, 200 $\mu\text{mol/l}$ sodium orthovanadate, and a protease inhibitor cocktail). The lysate was spun down at maximal speed in a microcentrifuge, and the Triton-soluble supernatant was further analyzed by SDS-PAGE and immunoblotting. The Triton-insoluble pellet was kept for analysis of cytoskeletal proteins, as described below.

Preparation of cytoskeletal protein samples for analysis of keratin expression. The insoluble fraction (pellet), obtained as described above, was incubated for 30 min in a special lysis buffer of PBS containing β -mercaptoethanol (20%) and SDS (5%). The samples were centrifuged for 30 min at maximal speed in a microcentrifuge, and the lysate was further analyzed by SDS-PAGE and Western blot analysis.

Immunoblotting analysis. The protein samples were analyzed by SDS-PAGE and transferred by electroblotting onto nitrocellulose membranes. Immunoblotting was performed as described previously (24) using rabbit polyclonal antibodies against the IR and IGF1R (Santa Cruz Biotechnology, Santa Cruz, CA), GLUT1 (a gift from Dr. S. Cushman, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health [NIH]), keratin 1 (a gift from Dr. S.H. Yuspa, National Cancer Institute, NIH), or monoclonal antibodies recognizing phosphotyrosine residues (Upstate Biotechnology, Lake Placid, NY). Filters were then incubated with the appropriate secondary horseradish peroxidase-conjugated anti-rabbit (Bio-Rad, Hercules, CA) or anti-mouse (Amersham, Piscataway, NJ) antibodies. Proteins were detected using an enhanced chemiluminescence protein detection method (SuperSignal; Pierce, Rockford, IL).

Ligand-stimulated receptor phosphorylation in intact cells and immunoprecipitation. Confluent cultures were incubated in the presence or absence of 10^{-6} mol/l insulin (Eli Lilly, Indianapolis, IN) or 10^{-7} mol/l IGF-I (CytoLab, Tel Aviv, Israel) for 3 min at room temperature. The reaction was terminated by aspiration of the incubation medium followed by quick washes with ice-cold PBS. Cells were lysed in cold lysis buffer (PBS containing 1% Triton X-100, 1 mmol/l EDTA, 10 mmol/l sodium fluoride, 200 $\mu\text{mol/l}$ sodium orthovanadate, and a protease inhibitor cocktail). Receptors were immunoprecipitated with anti-phosphotyrosine antibodies (Upstate Biotechnology), followed by protein A/G-agarose beads (Santa Cruz Biotechnology) for 16–20 h at 4°C. The beads were then washed twice with cold lysis buffer. The immunoprecipitates were run on an SDS-PAGE, and phosphotyrosine-containing proteins were detected by Western blotting using appropriate antibodies, as described above.

2-Deoxy- ^3H glucose uptake. Glucose transport was evaluated by measuring 2-deoxy- ^3H glucose (dGlc) according to the method described previously (14). Briefly, on the day of the experiment, cells were washed three times with PBS. Then, 0.1 mmol/l 2-deoxy-glucose ("cold") PBS with tracer amounts of dGlc (1 μCi per plate; American Radiolabeled Chemicals, St. Louis, MO) was added to the cells. Uptake was continued for 10 min at room temperature. The reaction was stopped by three quick washes with 1.0 ml cold PBS on ice, and cells were lysed in 1% Triton X-100. The samples were counted in the

^3H -window of a Tricarb scintillation counter. Uptake was linear under these conditions for up to 15 min (data not shown). Furthermore, it was established that when uptake was carried out at 37°C, the results were similar, though of a higher magnitude, to those obtained when uptake was performed at room temperature. However, since carrying out the uptake at room temperature was more consistent, the experiments were further performed in room temperature. Mean values were determined from measurements of triplicate samples under each experimental condition for each experiment.

Measurements of affinity (K_m) and maximal velocity (V_{max}) constants.

Determination of the K_m and V_{max} constants for glucose uptake in the presence of 2–20 mmol/l D-glucose was carried out as previously described (14). Briefly, cells were incubated for 5 days in various glucose concentrations, and uptake was carried out as described above, in the presence of increasing concentrations of 0.1–10 mmol/l 2-deoxy-glucose (cold) PBS with tracer amounts of dGlc (1 μCi per plate; ARC). Uptake was continued for 10 min at room temperature. Data were analyzed according to the Lineweaver-Burk equation. From the resulting curves, the values of K_m (intercept on the horizontal axis is equal to $-1/K_m$) and V_{max} (the intercept of the line on the vertical axis is equal to $1/V_{max}$) can be determined.

Thymidine incorporation. Cell proliferation was evaluated by measuring ^3H thymidine incorporation into DNA. Cells were pulsed with ^3H thymidine (1 $\mu\text{Ci/ml}$; ICN, Irvine, CA) for 1 h at 37°C. After incubation, cells were washed three times with PBS, incubated for 20 min at room temperature in 5% trichloroacetic acid, and solubilized in 1% Triton X-100. The radioactivity incorporated into the cells was counted in the ^3H -window of a Tricarb liquid scintillation counter. Mean values were determined from measurements of triplicate samples under each experimental condition for each experiment.

RESULTS

Autoregulation of glucose transport in proliferating keratinocytes. Glucose was shown to autoregulate its own transport in many cell systems (14–19). This effect has been claimed to further deteriorate the metabolic regulation in diabetic patients by creating a vicious cycle: as less glucose enters the cells, the extra cellular glucose concentration further increases, thereby further downregulating the entry of glucose into the cells. We initially wished to determine whether such an autoregulatory mechanism exists in murine keratinocytes as well. Proliferating keratinocytes were incubated in various glucose concentrations for 5 days, after which the glucose transport rate was estimated by measuring dGlc uptake (Fig. 1A). As can be seen, at high glucose concentrations, the cells decrease their glucose uptake rate; reducing the glucose concentration in the medium is associated with an increase in the uptake rate. This autoregulation process is present in all stages of differentiation. As can be seen in Fig. 1B, induction of keratinocyte differentiation is associated with a decrease in the basal glucose uptake rate. However, incubating the cells in high-glucose medium further decreases the uptake rate proportionate to the glucose concentrations in the growth medium. Having demonstrated (Fig. 1A and B) that the autoregulatory process is present in all glucose concentrations tested, we chose to perform the studies from this point on by comparing the two extreme glucose concentrations of 2 and 20 mmol/l. However, in most experiments we included a more physiological concentration of glucose as well.

The autoregulatory effect is reversible. When cells incubated in 2 or 20 mmol/l of glucose were switched to 20 or 2 mmol/l of glucose, respectively, their uptake rate adjusted to the new glucose concentration (Fig. 1C). Interestingly, the downregulation process in response to high glucose concentration was more rapid than the upregulation of the uptake in response to low glucose levels in the growth medium (Fig. 1C).

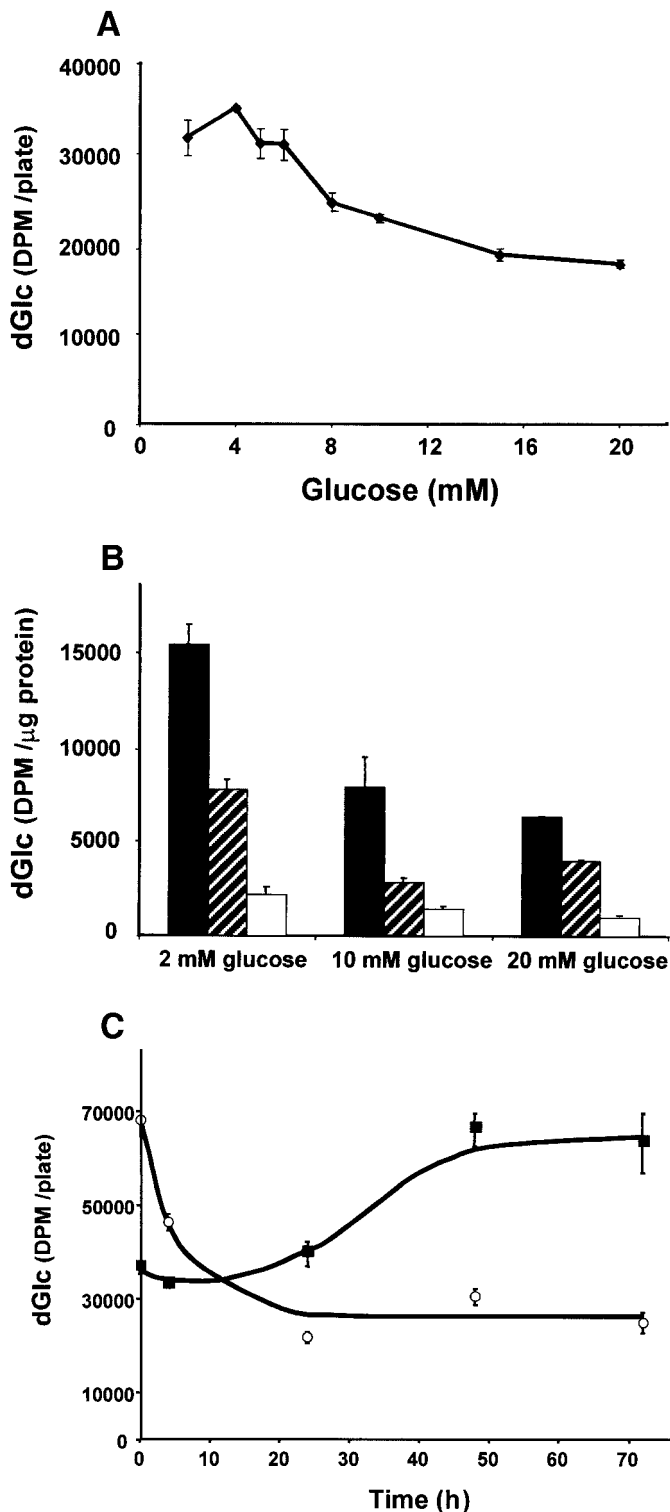


FIG. 1. Autoregulation of glucose transport in keratinocytes. Keratinocytes were isolated and plated as described in RESEARCH DESIGN AND METHODS. **A:** Proliferating cells were grown for 5 days in different glucose concentrations (2, 4, 5, 6, 8, 10, 15, and 20 mmol/l). **B:** Keratinocytes were allowed to differentiate for 48 h in different Ca²⁺ concentrations of 0.05 mmol/l (■, low), 0.12 mmol/l (▨, medium), or 1.0 mmol/l (□, high) in the presence of three glucose concentrations (2, 10, and 20 mmol/l). **C:** Proliferating cells grown for 5 days in 2 mmol/l (○) and 20 mmol/l (■) of glucose were switched to medium containing 20 and 2 mmol/l of glucose, respectively, for different time periods (4, 24, 48, and 72 h). In all experiments (A–C), dGlc uptake rate was measured for 10 min at room temperature. The experiments were performed in triplicate. A representative experiment of three different experiments is shown.

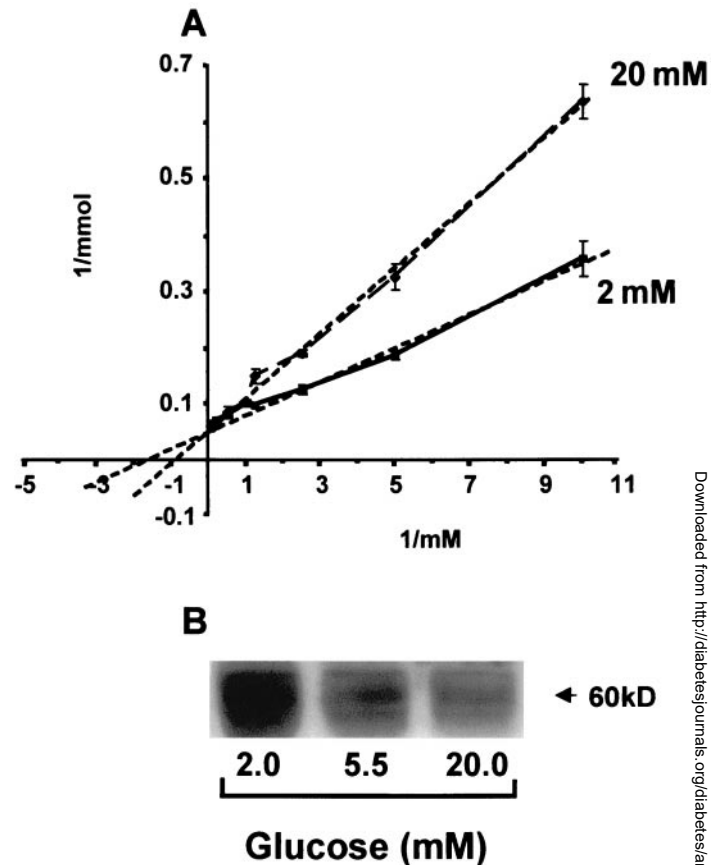


FIG. 2. High glucose levels decrease glucose transport affinity and GLUT1 expression in keratinocytes. **A:** Proliferating keratinocytes were grown for 5 days in 2 or 20 mmol/l D-glucose concentrations. 2dGlc uptake was measured for 10 min, as described in RESEARCH DESIGN AND METHODS, in the presence of increasing concentrations of dGlc (cold) (0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 5.0, 7.5, and 10 mmol/l). The resulting uptake values were analyzed according to the Lineweaver-Burk equation, and the K_m and V_{max} constants were determined as detailed in RESEARCH DESIGN AND METHODS. Values shown are means \pm SD of triplicate determinations from a representative experiment of three different experiments. **B:** Proliferating cells were grown for 5 days in the presence of 2, 5.5, and 20 mmol/l glucose. Total lysates were analyzed by Western blot analysis using anti-GLUT1 antibody. A representative experiment of five different experiments is shown.

Glucose effects on affinity and maximal velocity of glucose uptake. Glucose could decrease the transport rate by either decreasing the affinity of the glucose transporters or decreasing the maximal velocity of the process. Values of K_m and V_{max} were calculated by Lineweaver-Burk plotting and are shown in Fig. 2A and Table 1. Incubating the cells in high glucose concentrations was associated with a decrease in the affinity of the transporters to glucose, whereas the maximal velocity of the uptake process, representing the number of active glucose transporters present, was unchanged.

TABLE 1
Lineweaver-Burk values of K_m and V_{max}

	V_{max}	K_m
2 mmol/l	12.2 \pm 4.7	0.5 \pm 0.11
20 mmol/l	11.1 \pm 7.07	1.1 \pm 0.32

Data are means \pm SE. Experiments were carried out as detailed in Fig. 2A. The mean of three independent experiments was calculated. * $P < 0.05$.

Surprisingly, although there was no change in the maximal velocity of the transport, exposure to high glucose concentrations resulted in a decrease in the total expression of GLUT1 (Fig. 2B), the main glucose transporter in proliferating keratinocytes. Expression of GLUT1 was evident, as expected, in the plasma membrane fraction (data not shown). Although there are other glucose transporter isoforms expressed in keratinocytes, their expression is low, and there was no detectable change in their expression in response to glucose (data not shown). Thus, our data suggest that even though the total number of transporters present at the plasma membrane decreased, the number of active transporters did not change. However, the affinity of these active transporters decreased, resulting in the lower transport rate measured.

Glucose effects are not mediated via the IR. Growth in high-glucose conditions models a hyperglycemic situation in vivo. However, in diabetes, hyperglycemia appears in the presence of a partial or complete deficiency of insulin action. Thus, we next wanted to identify possible interactions between the effects of insulin resistance and elevated glucose; furthermore, we wanted to find out whether the glucose autoregulation process is mediated via the IR. To this end, we studied the effects of elevated glucose in keratinocytes lacking IR expression, which were isolated from IR knockout (KO) mice (23). As can be seen in Fig. 3, despite the absence of IR expression, the autoregulation by glucose of its transport (Fig. 3A) was similar in the cells isolated from the IR-KO mice and cells isolated from WT IR mice. Interestingly, the downregulation of GLUT1 expression in the presence of glucose was lower in the null cells (Fig. 3B and C).

Glucose affects cellular morphology of keratinocytes. After establishing that glucose downregulates its own transport, we studied the possible effects that glucose might have on cellular functions. The first observation made was the change in cellular morphology after cells are incubated in different glucose concentrations. As seen in Fig. 4, cells incubated in low (2 mmol/l) glucose levels are small and organized, whereas cells maintained at high (20 mmol/l) glucose concentrations were larger and flattened and had lost some of their orientation toward each other. **High glucose concentrations inhibit keratinocyte proliferation.** The striking effects of glucose on cellular morphology encouraged us to examine the effects of glucose on cellular proliferation. Two phases of cellular growth can be identified in cultured nonimmortalized keratinocytes. An early rapid proliferation rate, sustained for up to 5 days in culture, follows exhaustion of the cellular replication capacity after fixed cycles in vitro. Proliferation of the cells was estimated by measuring the [³H]thymidine incorporation into cells grown at low Ca²⁺ concentration (0.05 mmol/l), in which they maintain their proliferating characteristics, and in the presence of 2 or 20 mmol/l D-glucose. Cells maintained in either low or high glucose had similar proliferation rates during the first days in culture (Fig. 5A). However, it was found that increasing the glucose concentration resulted in keratinocytes ceasing to proliferate and going through an earlier and deeper crisis than that seen in cells incubated in 2 mmol/l glucose (Fig. 5A and B).

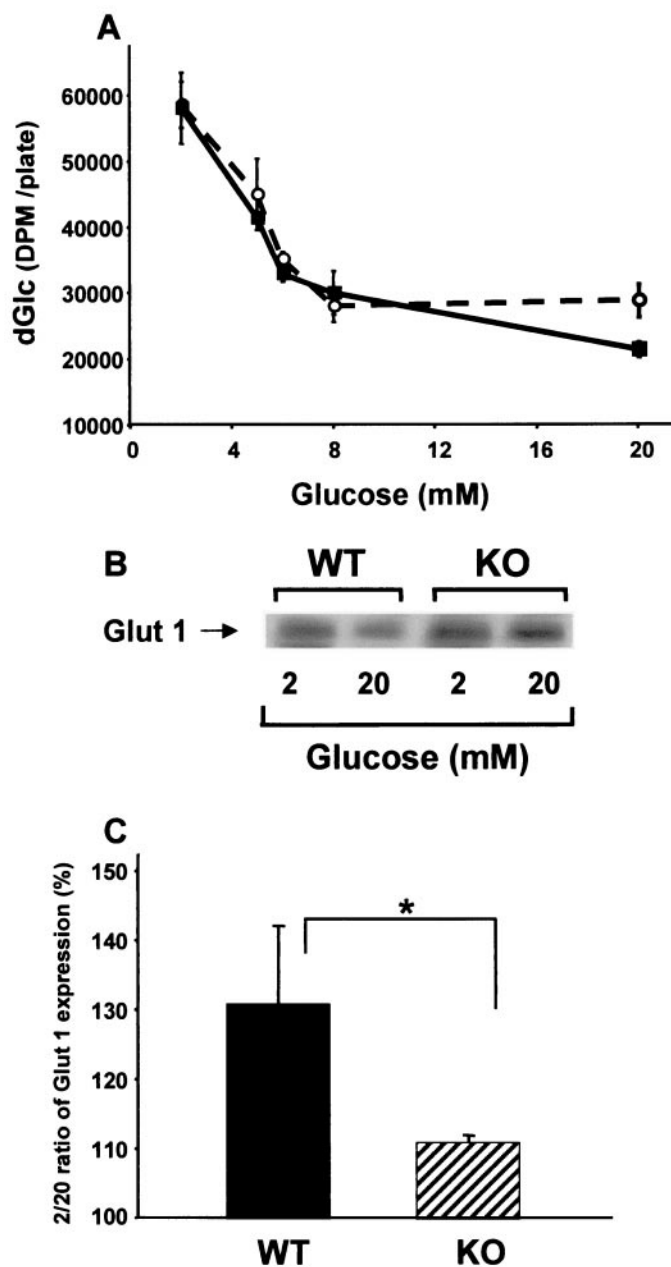


FIG. 3. Lack of IR does not affect glucose transport system in cultured keratinocytes. Keratinocytes were isolated and plated as described in RESEARCH DESIGN AND METHODS. **A:** Proliferating cells isolated from IR-KO (■, KO) pups or WT (○) littermates were grown for 5 days in different glucose concentrations (2, 4, 5, 6, 8, 10, 15, and 20 mmol/l), and dGlc uptake rate was measured for 10 min at room temperature. The experiments were carried out in triplicate. A representative experiment of three different experiments is shown. **B:** Proliferating cells isolated from WT and IR-KO (KO) pups were grown for 5 days in the presence of 2 or 20 mmol/l glucose. Total lysates were analyzed by Western blot analysis using anti-GLUT1 antibody. A representative experiment of four different experiments is shown. **C:** Statistical analysis of the densitometry reading of glucose-induced downregulation of GLUT1 expression in WT and IR-KO (KO) cells in four different experiments. The quantitation is expressed as the ratio of the densitometry reading of GLUT1 expression in the presence of 2 mmol/l D-glucose in the growth medium to the reading of GLUT1 expression in the presence of 20 mmol/l D-glucose. **P* < 0.05.

High glucose concentrations enhance Ca²⁺-induced differentiation of keratinocytes. The fact that the IR-KO cells ceased to proliferate in the presence of high glucose concentrations could result from induction of differentiation. Thus, we next followed the effects of glucose

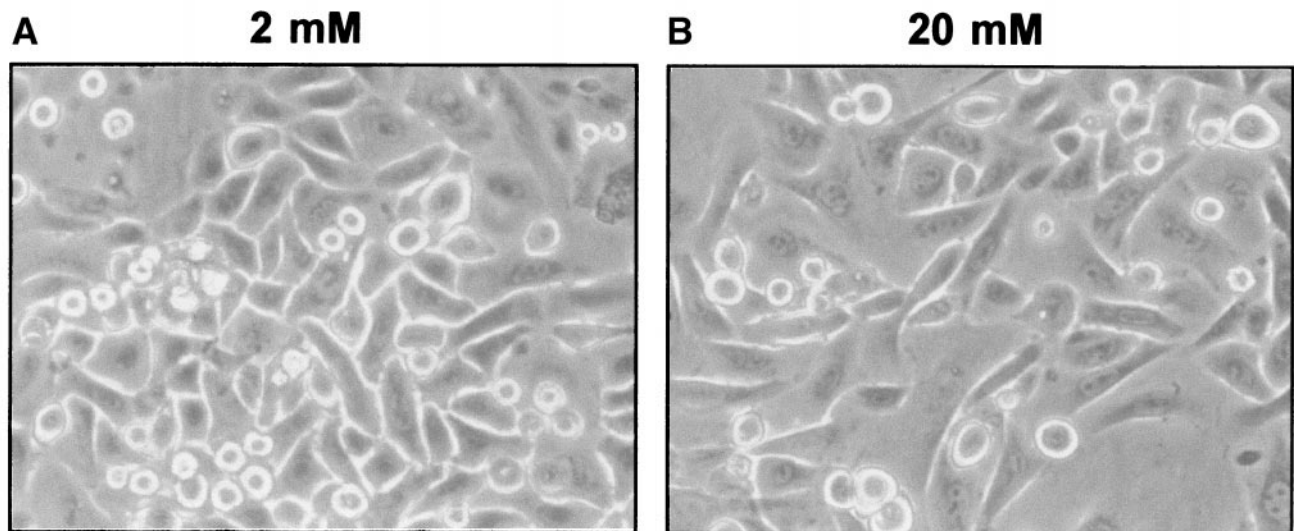


FIG. 4. Hyperglycemia affects morphology of cultured keratinocytes. Proliferating cells were grown in 2 mmol/l (A) and 20 mmol/l (B) of glucose for 5 days. Photographs of representative fields were taken ($\times 20$).

on the Ca^{2+} -induced differentiation of keratinocytes. Skin keratinocytes can be induced to differentiate in culture by elevating the concentration of Ca^{2+} in the growth medium (21,22). This in vitro differentiation process follows closely the differentiation process of skin in vivo. One of the known characteristics of this process is a change in the expression of keratins, which are cytoskeletal proteins, during the differentiation process. Proliferating keratinocytes, grown in 0.05 mmol/l Ca^{2+} , express keratins 5 and 14. Induction of differentiation by increasing the level of Ca^{2+} to >0.1 mmol/l is associated with an increased expression of keratins 1 and 10. This increase is followed by a later increase in the expression of loricrin, filaggrin, and transglutaminase that coincides with terminal differentiation of the cells (22).

Cells were induced to differentiate with calcium in the presence of either 2 or 20 mmol/l D-glucose, and the expression of various differentiation markers was followed. As can be seen in Fig. 6, Ca^{2+} -induced differentiation in the presence of high glucose concentrations was associated with an enhanced expression of keratin 1. These glucose effects, however, were dependent on the degree of confluency of the cells (data not shown), being more pronounced when cells were induced to differentiate after reaching 80% confluency. There was no change in markers of the granular layer, such as filaggrin (data not shown). **Glucose inhibits the phosphorylation of the IGFR.** We have shown so far that exposure of keratinocytes to high glucose concentrations, mimicking the hyperglycemic state, has effects on skin cells, resulting in inhibition of proliferation and an abnormal differentiation process. However, in diabetic patients, development of hyperglycemia also results in changes in insulin and IGF-I signaling. Indeed, we have previously shown that both insulin and IGF-I participate in the proliferation and differentiation of murine skin keratinocytes (25), and it is plausible that the direct effects of high glucose on the proliferation and differentiation processes might be further augmented by its effects on insulin and IGF-I signaling. Therefore, we next chose to study insulin and IGF-I signaling in the presence

of high glucose concentrations. Exposure of the cells to high glucose concentrations did not lead to a change in the expression levels of either IR or IGFR (Fig. 7A). However, the autophosphorylation state of both receptors was altered. As can be seen in Fig. 7B, the autophosphorylation of the IGFR was decreased in cells grown in 20 mmol/l D-glucose for 5 days, compared with cells grown in low glucose concentrations of 2 mmol/l. The decrease in autophosphorylation was seen in response to either IGF-I or insulin stimulation. Autophosphorylation of the IR, on the other hand, was slightly increased under similar conditions. **Glucose downregulates IGFR-mediated effects**
Glucose transport. As we have just shown, incubating cells under high glucose conditions decreases mainly the autophosphorylation of IGFR. We therefore wished to determine which IGFR-mediated effects are abolished as a result of this decreased response. To this end, we followed two of the most important known roles of the IGFR in skin keratinocytes: the regulation of both glucose uptake and the proliferation process.

First, we studied the effects of insulin and IGF-I on glucose transport into skin keratinocytes. As we have previously shown, insulin and IGF-I do not acutely affect the transport rate into cells (26). However, chronic stimulation by these hormones (especially IGF-I) upregulates the transport rates into the cells. Growing the cells in high glucose concentrations led to a dampening of the IGF-I-induced increase in dGlc uptake rate; however, uptake was not completely abolished (Fig. 8A). In contrast, no effect was seen on the insulin-induced increase in the uptake rate. These results suggest that IGF-I's effects on glucose transport are mediated via the IGFR. The fact that the effects of IGF-I were not completely abolished in the presence of high glucose could be because IGFR phosphorylation under these conditions was not completely eliminated, or it could result from transactivation of the IR by IGF-I. In contrast, insulin effects were not affected by the glucose-mediated change in IGFR phosphorylation, suggesting that insulin effects on glucose transport are mediated via its own receptor and not via the IGFR.

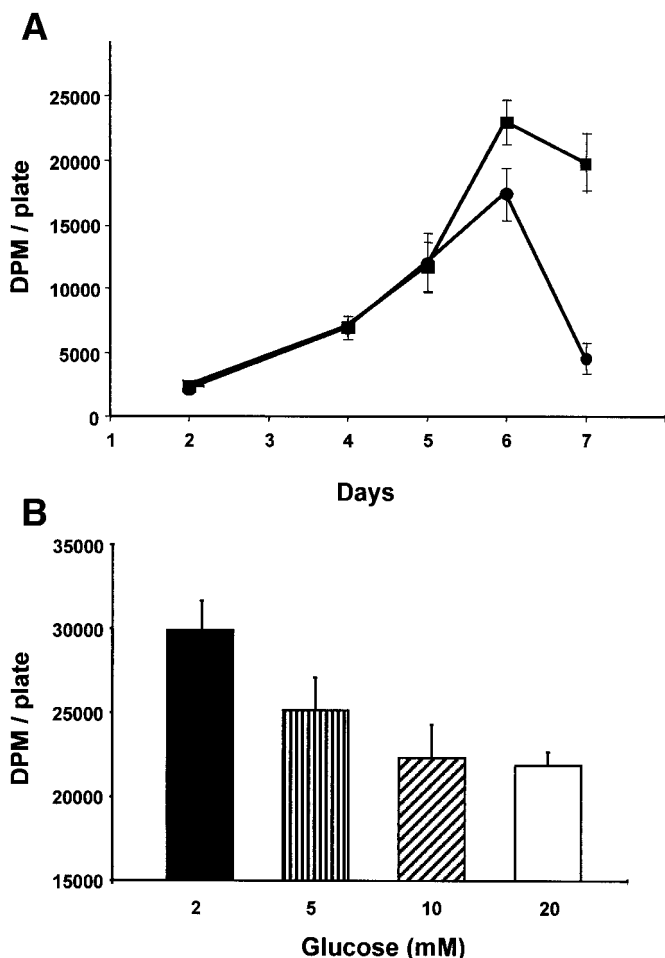


FIG. 5. High glucose-induced loss of proliferative capacity. Proliferating cells were grown in different glucose concentrations as indicated in the figures. Thereafter, the cells were incubated with [³H]thymidine for 1 h. Values represent means ± SD of triplicate determinations. A representative experiment of three different experiments is shown in each panel. **A:** Cells were grown for several days in 2 mmol/l (■) or 20 mmol/l (●) D-glucose. Each day, thymidine incorporation was measured as described above and in RESEARCH DESIGN AND METHODS. **B:** Cells were grown for 6 days in 2, 5, 10, or 20 mmol/l D-glucose, and thymidine incorporation was measured as described above.

To further investigate this issue, we performed similar studies in keratinocytes that were lacking the expression of the IR and isolated from IR-KO mice. Insulin-induced glucose transport is completely abolished in the IR-KO keratinocytes (Fig. 8B), demonstrating that insulin effects on glucose transport are indeed mediated solely via the IR. Lack of IR expression also resulted in a decreased IGF-I-induced glucose transport rate, thus suggesting, as indicated above, the involvement of IR transactivation in the IGF-I regulation of glucose transport.

Cellular proliferation. As mentioned earlier, another effect of insulin and IGF-I on keratinocytes is an increase in cellular proliferation (25). Therefore, we evaluated the proliferation rate of keratinocytes in response to chronic insulin or IGF-I stimulation in the presence of 2 or 20 mmol/l D-glucose. As can be seen in Fig. 9, both insulin and IGF-I induced an increase in the proliferation rate of the cells (142 and 155% above control, respectively). However, in the presence of high glucose concentrations, the effects of both hormones—but mainly of IGF-I—were reduced (129 and 123% above control, respectively). Glucose ef-

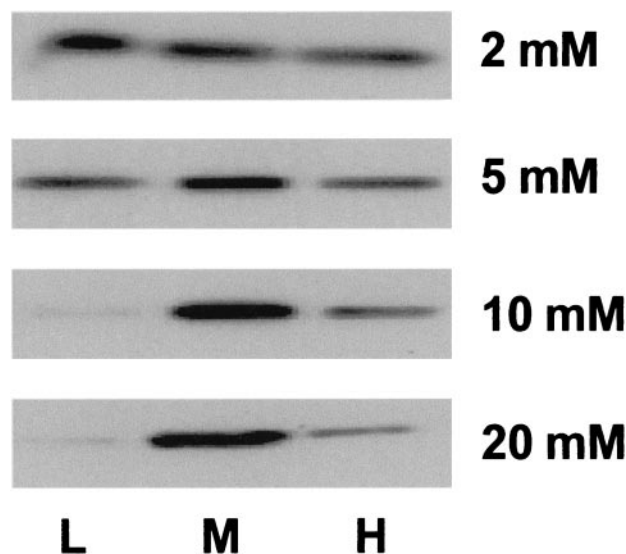


FIG. 6. High glucose level stimulates keratinocyte differentiation. Keratinocytes were allowed to differentiate for 48 h in different Ca²⁺ concentrations of 0.05 mmol/l (L), 0.12 mmol/l (M), or 1.0 mmol/l (H) in the presence of various glucose concentrations (2, 5, 10, and 20 mmol/l). Cytoskeletal fraction was isolated as described in RESEARCH DESIGN AND METHODS. The induction of differentiation markers was identified by Western blot analysis, using antibodies against keratin 1. A representative experiment of four different experiments is shown.

fects were specific, as there was no effect on the activity of keratinocyte growth factor on glucose transport (Fig. 9).

DISCUSSION

Development of hyperglycemia is associated with exacerbation of diabetes because it leads to increased insulin resistance as well as a reduction in general glucose utilization. Indeed, achieving euglycemia is associated with reversal of these two pathological findings. Several processes have been suggested to participate in the pathophysiology of these effects, including the following: downregulation of IR phosphorylation, PKC activation (11,12), activation of protein tyrosine phosphatases leading to dephosphorylation of the IR (13) or downstream elements in insulin signaling (27), and a decrease in glucose transport from the bloodstream into the cells by a direct autoregulatory mechanism (14–20). In addition, high glucose levels have other effects on cellular physiology that could lead to abnormal function of these tissues. In the present study, we focused on the effects of glucose in skin keratinocytes and determined the role of glucose in the abnormal skin physiology found in diabetic patients.

It has been suggested that reduced glucose uptake to tissues, as seen in diabetes, can further exacerbate hyperglycemia in the circulation. Since skin composes 15% of an individual's total body weight, a reduction of the glucose uptake into skin cells could indeed contribute to the increased glucose levels in the blood (28). We therefore examined the mechanism of the autoregulation by glucose of its transport. We first demonstrated that in skin keratinocytes, glucose downregulates its own transport. The decrease is achieved via a decrease in the affinity of the glucose transporters for glucose. Interestingly, we did observe a decrease in the expression levels of GLUT1, the major glucose transporter isoform in keratinocytes. However, there was no change in the maximal velocity of the

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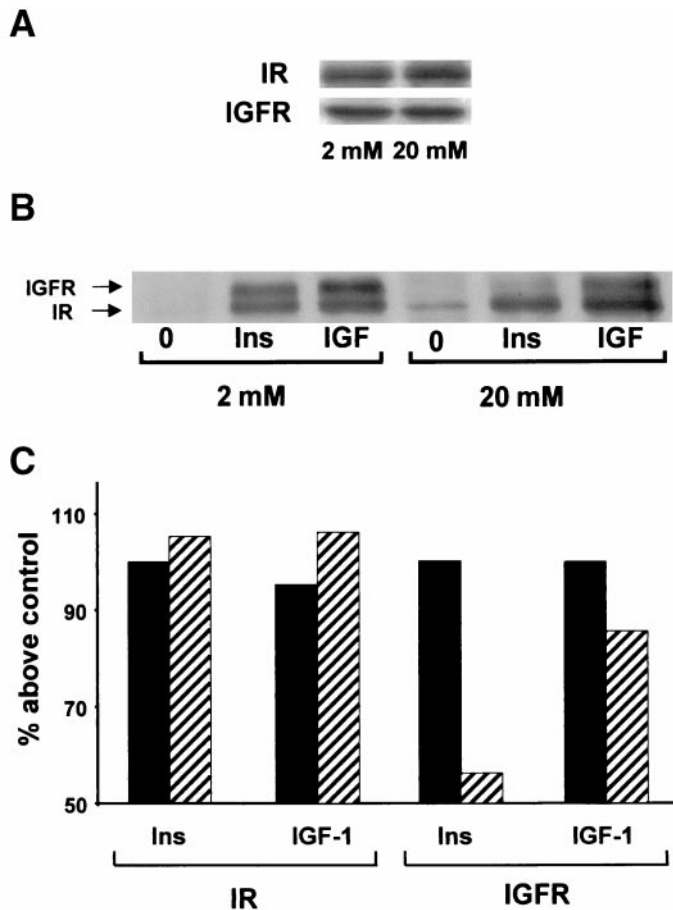


FIG. 7. High glucose level decreases phosphorylation of IGFR. Proliferating keratinocytes were grown for 5 days in different glucose concentrations (2 mmol/l [■] and 20 mmol/l [▨]). **A:** Total lysates were analyzed by Western blot analysis with anti-IR and anti-IGFR antibodies. **B:** The cells were acutely stimulated for 3 min at room temperature with insulin (10^{-6} mol/l) or IGF-I (10^{-7} mol/l), and total lysates were immunoprecipitated with anti-phosphotyrosine antibody. The filter was blotted with anti-IR antibody and thereafter reblotted with an anti-IGFR antibody. A representative experiment of three different experiments is shown. **C:** Densitometry quantitation of the experiment shown in **B**. For each receptor, the densitometry reading of the insulin-induced phosphorylation at 2 mmol/l was set as 100%, and the results for each receptor are expressed as a percent above this control.

transport system, as evaluated by Lineweaver-Burk plotting, indicating that despite the reduced number of GLUT1 molecules, the total number of active glucose transporters was unchanged. Indeed, recent studies have demonstrated (29,30) a discrepancy between the expression of the glucose transporters present at the plasma membrane and their activity, suggesting that transporters must be activated after their synthesis.

In studying IR-null cells, we found that the effects of glucose on glucose transport rate are not mediated via the IR. The basal uptake as well as the glucose-induced downregulation in uptake rate were not affected by lack of IR expression. Interestingly, the glucose-induced decrease in GLUT1 protein expression, seen in WT cells, was smaller in the IR-null keratinocytes. This might be an attempt of the IR-null cells to compensate for the lack of the receptor, or it may suggest the involvement of the receptor in the glucose-induced regulation of GLUT1 transcription or translation. However, as mentioned before, despite the change in the glucose-induced downregulation of GLUT1

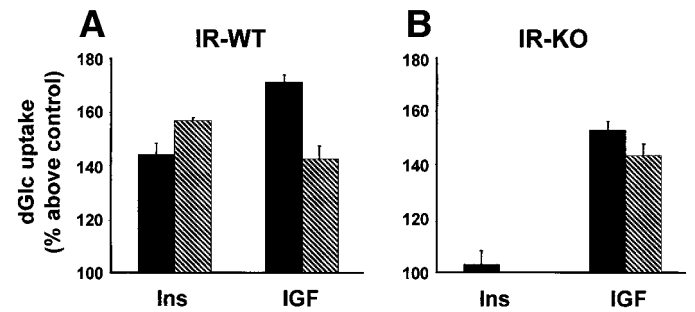


FIG. 8. Insulin and IGF-I regulation of dGlc uptake in the presence of high glucose and insulin resistance. Proliferating cells isolated from WT (**A**) or IR-KO (**B**) mice were grown in different glucose concentrations (■, 2 mmol/l; ▨, 20 mmol/l) for 5 days. Thereupon, the cells were chronically stimulated with insulin (10^{-6} mol/l) or IGF-I (10^{-7} mol/l) overnight. dGlc uptake was measured as described in RESEARCH DESIGN AND METHODS. Values represent means \pm SD of triplicate determinations and are shown as a percentage relative to the value for nonstimulated cells. A representative experiment of three different experiments is shown.

expression, there was no change in the level of active transporters, as the uptake rate of the IR-null cells was not altered.

Glucose was found to have effects on the proliferation and differentiation of cells. We found that cells grown in the presence of high glucose concentrations demonstrated a decreased proliferation rate. A review of the literature demonstrates that glucose was reported in some studies to increase cellular proliferation, whereas in other studies it was found to inhibit the process. Glucose was reported to increase proliferation in vascular smooth muscle (31,32), rabbit coronary smooth muscle (33), MCF7 human breast cells (34), renal cortical fibroblasts (35), and SV40-transformed human corneal epithelium (36). However, glucose was shown to inhibit proliferation in other cell types, including dermal fibroblasts (37), monkey kidney cell line (38), bovine carotid artery endothelial cells (39), human mesangial cells (40), cultured vascular endothelial cells, and human fetal cells (41). There are a few possible explanations for the differences among the various studies. It is clear that there is a dependence on cell type. For exam-

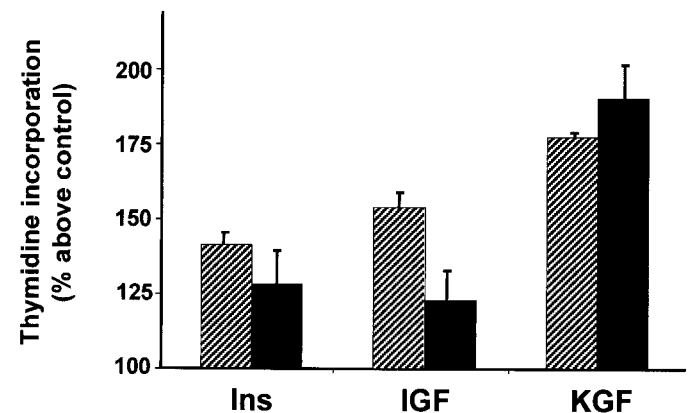


FIG. 9. High glucose reduces keratinocyte proliferation response to insulin and IGF-I. Proliferating cells grown for 5 days in two glucose concentrations (2 mmol/l [▨] and 20 mmol/l [■]) were chronically stimulated with insulin (Ins; 10^{-6} mol/l), IGF-I (IGF; 10^{-7} mol/l), or keratinocyte growth factor (KGF; 2 ng/ml) overnight. At that point, the cells were incubated with [3 H]thymidine for 1 h. Values represent means \pm SD of triplicate determinations and are shown as a percentage relative to the value for nonstimulated cells. A representative experiment of three different experiments is shown.

ple, in one laboratory, differences were found between endothelial cells, the proliferation of which was inhibited by glucose, and smooth muscle cells, which responded to high glucose by increasing the proliferation rate (42). In another report, MCF7 human breast cells were sensitive to glucose, whereas MCF7 multidrug-resistant variant cells were not (34). Another possible explanation is the incubation time. There are reports showing that the effects of glucose on proliferation are time dependent—a result similar to our findings. In both peritoneal fibroblasts (43) and murine mesangial cell line (44), glucose initially stimulated cell proliferation and thereafter inhibited its proliferation. Finally, there is dependence of glucose effects on the presence of other cellular factors. For example, glucose increased proliferation of vascular endothelial cells in the presence of low serum concentration, whereas it inhibited proliferation in high serum concentration (45).

Glucose was also found to alter the differentiation of keratinocytes. We found that high glucose concentrations directly enhanced the differentiation process, further augmenting the induction of differentiation in the presence of glucose. Indeed, involvement of glucose in cellular differentiation was seen in several cell types; in some it resulted in loss of differentiation, whereas in others it resulted in enhanced differentiation (25,46–48). Thus, the effects of glucose on differentiation vary depending on the tissue.

In addition to its direct effects on cellular physiology, glucose downregulated IGF-R phosphorylation. Interestingly, high glucose levels had only minimal effects on IR phosphorylation. In most studies conducted to evaluate the effects of glucose on phosphorylation, the emphasis was on its effects on insulin signaling. In those studies, glucose was found to inhibit IR tyrosine phosphorylation, whereas IGF-R phosphorylation was either unchanged or, in most cases, not examined (11,13,49,50). Furthermore, these studies were conducted on either cells overexpressing the IR or on cells, such as muscle cells, that are classic targets for insulin action. In these classic insulin target cells, insulin acts mainly to regulate glucose transport and metabolism, whereas in the nonclassical target cells (such as skin keratinocytes), it has been demonstrated to play different roles (25,26). Thus, it is conceivable that glucose may have different targets depending on the role of the hormones and their receptors in specific cells.

Next, we evaluated the physiological importance of the glucose-induced changes in insulin and IGF-I signaling by following its effects on insulin- and IGF-I-mediated processes. We have previously shown that in skin keratinocytes, IR and IGF-R have different roles in skin proliferation that are mediated via distinct signaling pathways (25). In addition, we have shown in the present study that high glucose levels, in the absence of any additional perturbation, are associated with decreased cellular proliferation. Thus, glucose inhibits proliferation by both direct effects as well as by reducing the stimulatory effect of IGF-I on proliferation.

In conclusion, the consequence of high glucose inhibition on the proliferation of skin keratinocytes and its enhancement of their differentiation is obvious. By changing the proliferation-differentiation balance, which is one of the essential steps in the healing process, as well as by decreasing other possible local effects of IGF-I on wound

healing, high glucose levels might indeed contribute to impaired wound healing in diabetes.

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REFERENCES

- Friedman EA: Advanced glycosylated end products and hyperglycemia in the pathogenesis of diabetic complications. *Diabetes Care* 22 (Suppl. 2):B65–B71, 1999
- Wautier JL, Guillausseau PJ: Diabetes, advanced glycation endproducts and vascular disease. *Vasc Med* 3:131–137, 1998
- Yki-Jarvinen H: Toxicity of hyperglycaemia in type 2 diabetes. *Diabetes Metab Rev* 14 (Suppl. 1):S45–S50, 1998
- Koya D, King GL: Protein kinase C activation and the development of diabetic complications. *Diabetes* 47:859–866, 1998
- Porte DJ, Schwartz MW: Diabetes complications: why is glucose potentially toxic? [Published erratum appears in *Science* 1996 Jun 28;272(5270):1861]. *Science* 272:699–700, 1996
- Rossetti L: Glucose toxicity: the implications of hyperglycemia in the pathophysiology of diabetes mellitus. *Clin Invest Med* 18:255–260, 1995
- Flatt PR, Abdel-Wahab YH, Boyd AC, Barnett CR, O'Harte FP: Pancreatic B-cell dysfunction and glucose toxicity in non-insulin-dependent diabetes. *Proc Nutr Soc* 56:243–262, 1997
- Lowe WLJ, Adamo M, Werner H, Roberts CTJ, LeRoith D: Regulation by fasting of rat insulin-like growth factor I and its receptor: effects on gene expression and binding. *J Clin Invest* 84:619–626, 1989
- Oemar BS, Foellmer HG, Hodgdon-Anandant L, Rosenzweig SA: Regulation of insulin-like growth factor I receptors in diabetic mesangial cells. *J Biol Chem* 266:2369–2373, 1991
- Hauguel-de-Mouzon S, Mrejen C, Alengrin F, Van Obberghen E: Glucose-induced stimulation of human insulin-receptor mRNA and tyrosine kinase activity in cultured cells. *Biochem J* 305:119–124, 1995
- Berti L, Mosthaf L, Kroder G, Kellerer M, Tippmer S, Mushack J, Seffer E, Seedorf K, Haring H: Glucose-induced translocation of protein kinase C isoforms in rat-1 fibroblasts is paralleled by inhibition of the insulin receptor tyrosine kinase. *J Biol Chem* 269:3381–3386, 1994
- Muller HK, Kellerer M, Ermel B, Muhlhofer A, Obermaier-Kusser B, Vogt B, Haring HU: Prevention by protein kinase C inhibitors of glucose-induced insulin-receptor tyrosine kinase resistance in rat fat cells. *Diabetes* 40:1440–1448, 1991
- Ide R, Maegawa H, Kikkawa R, Shigeta Y, Kashiwagi A: High glucose condition activates protein tyrosine phosphatases and deactivates insulin receptor function in insulin-sensitive rat 1 fibroblasts. *Biochem Biophys Res Commun* 201:71–77, 1994
- Wertheimer E, Sasson S, Cerasi E: Regulation of hexose transport in L8 myocytes by glucose: possible sites of interaction. *J Cell Physiol* 143:330–336, 1990
- Ogura K, Sakata M, Yamaguchi M, Kurachi H, Murata Y: High concentration of glucose decreases glucose transporter-1 expression in mouse placenta in vitro and in vivo. *J Endocrinol* 160:443–452, 1999
- Howard RL: Down-regulation of glucose transport by elevated extracellular glucose concentrations in cultured rat aortic smooth muscle cells does not normalize intracellular glucose concentrations. *J Lab Clin Med* 127:504–515, 1996
- Ciaraldi TP, Abrams L, Nikoulina S, Mudaliar S, Henry RR: Glucose transport in cultured human skeletal muscle cells: regulation by insulin and glucose in nondiabetic and non-insulin-dependent diabetes mellitus subjects. *J Clin Invest* 96:2820–2827, 1995
- Simmons RA, Flozak AS, Ogata ES: Glucose regulates Glut 1 function and expression in fetal rat lung and muscle in vitro. *Endocrinology* 132:2312–2318, 1993
- Kaiser N, Sasson S, Feener EP, Boukoubza-Vardi N, Higashi S, Moller DE, Davidheiser S, Przybylski RJ, King GL: Differential regulation of glucose transport and transporters by glucose in vascular endothelial and smooth muscle cells. *Diabetes* 42:80–89, 1993
- Greco-Perotto R, Wertheimer E, Jeanrenaud B, Cerasi E, Sasson S: Glucose

- regulates its transport in L8 myocytes by modulating cellular trafficking of the transporter GLUT-1. *Biochem J* 286:157-163, 1992
21. Watt FM, Green H: Stratification and terminal differentiation of cultured epidermal cells. *Nature* 295:434-436, 1982
 22. Yuspa SH, Kilkenny AE, Steinert PM, Roop DR: Expression of murine epidermal differentiation markers is tightly regulated by restricted extracellular calcium concentrations in vitro. *J Cell Biol* 109:1207-1217, 1989
 23. Accili D, Drago J, Lee EJ, Johnson MD, Cool MH, Salvatore P, Asico LD, Jose PA, Taylor SI, Westphal H: Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. *Nat Genet* 12:106-109, 1996
 24. Wertheimer E, Taylor SI, Tennenbaum T: Insulin receptor regulation of cell surface integrins: a possible mechanism contributing to the development of diabetic complications. *Proc Assoc Am Physicians* 110:333-339, 1998
 25. Wertheimer E, Trebic M, Eldar T, Gartsbein M, Nofeh-Moses S, Tennenbaum T: Differential roles of insulin receptor and insulin-like growth factor-1 receptor in differentiation of murine skin keratinocytes. *J Invest Dermatol* 115:24-29, 2000
 26. Sampson SR, Shen S, Bak A, Wertheimer E, Tennenbaum T: Different PKC isoenzymes are involved in the insulin and IGF-I mediated proliferation of mouse keratinocytes (Abstract). *Diabetes* 48 (Suppl. 1):A233, 1999
 27. Caruso M, Miele C, Oriente F, Maitan A, Bifulco G, Andreozzi F, Condorelli G, Formisano P, Beguinot F: In L6 skeletal muscle cells, glucose induces cytosolic translocation of protein kinase C- α and trans-activates the insulin receptor kinase. *J Biol Chem* 274:28637-28644, 1999
 28. Gherzi R, Melioli G, de Luca M, D'Agostino A, Distefano G, Guastella M, D'Anna F, Franzini AT, Cancedda R: HepG2/erythroid/brain type glucose transporter (GLUT 1) is highly expressed in human epidermis: keratinocyte differentiation affects GLUT1 levels in reconstituted epidermis. *J Cell Physiol* 150:463-474, 1992
 29. Sweeney G, Somwar R, Ramlal T, Volchuk A, Ueyama A, Klip A: An inhibitor of p38 mitogen-activated protein kinase prevents insulin-stimulated glucose transport but not glucose transporter translocation in 3T3-L1 adipocytes and L6 myotubes. *J Biol Chem* 274:10071-10078, 1999
 30. Czech MP, Corvera S: Signaling mechanisms that regulate glucose transport. *J Biol Chem* 274:1865-1868, 1999
 31. James DE, Hiken J, Lawrence JCJ: Isoproterenol stimulates phosphorylation of the insulin-regulatable glucose transporter in rat adipocytes. *Proc Natl Acad Sci U S A* 86:8368-8372, 1989
 32. Avena R, Mitchell ME, Carmody B, Arora S, Neville RF, Sidaway AN: Insulin-like growth factor-1 receptors mediate infragenicular vascular smooth muscle cell proliferation in response to glucose and insulin not by insulin receptors. *Am J Surg* 178:156-161, 1999
 33. Yasunari K, Kohno M, Kano H, Yokokawa K, Minami M, Yoshikawa J: Antioxidants improve impaired insulin-mediated glucose uptake and prevent migration and proliferation of cultured rabbit coronary smooth muscle cells induced by high glucose. *Circulation* 99:1370-1378, 1999
 34. Yamamoto M, Patel NA, Taggart J, Sridhar R, Cooper DR: A shift from normal to high glucose levels stimulates cell proliferation in drug sensitive MCF-7 human breast cancer cells but not in multidrug resistant MCF-7/ADR cells which overproduce PKC- β II. *Int J Cancer* 83:98-106, 1999
 35. Han DC, Isono M, Hoffman BB, Ziyadeh FN: High glucose stimulates proliferation and collagen type I synthesis in renal cortical fibroblasts: mediation by autocrine activation of TGF- β . *J Am Soc Nephrol* 10:1891-1899, 1999
 36. McDermott AM, Kern TS, Murphy CJ: The effect of elevated extracellular glucose on migration, adhesion and proliferation of SV40 transformed human corneal epithelial cells. *Curr Eye Res* 17:924-932, 1998
 37. Hehenberger K, Heilborn JD, Brismar K, Hansson A: Inhibited proliferation of fibroblasts derived from chronic diabetic wounds and normal dermal fibroblasts treated with high glucose is associated with increased formation of l-lactate. *Wound Repair Regen* 6:135-141, 1998
 38. Kannan K, Jain SK: Effect of high glucose on cellular proliferation and lipid peroxidation in cultured Vero cells. *Horm Metab Res* 26:322-325, 1994
 39. Nakao-Hayashi J, Ito H, Kawashima S: An oxidative mechanism is involved in high glucose-induced serum protein modification causing inhibition of endothelial cell proliferation. *Atherosclerosis* 97:89-95, 1992
 40. Nahman NS Jr, Leonhart KL, Cosio FG, Hebert CL: Effects of high glucose on cellular proliferation and fibronectin production by cultured human mesangial cells. *Kidney Int* 41:396-402, 1992
 41. Nelson DM, Curran EM: High glucose levels decrease proliferation of cultured human fetal cells from placenta. *Am J Obstet Gynecol* 161:1553-1558, 1989
 42. Graier WF, Grubenthal I, Dittrich P, Wascher TC, Kostner GM: Intracellular mechanism of high D-glucose-induced modulation of vascular cell proliferation. *Eur J Pharmacol* 294:221-229, 1995
 43. Higuchi C, Sanaka T, Sato T, Omata M, Watanabe M, Mine S, Inuzuka N, Nihei H: The effect of glucose on the proliferation of peritoneal fibroblasts. *Adv Perit Dial* 13:253-256, 1997
 44. Wolf G, Sharma K, Chen Y, Erickson M, Ziyadeh FN: High glucose-induced proliferation in mesangial cells is reversed by autocrine TGF- β . *Kidney Int* 42:647-656, 1992
 45. Hayashi JN, Ito H, Kanayasu T, Asuwa N, Morita I, Ishii T, Murota S: Effects of glucose on migration, proliferation and tube formation by vascular endothelial cells. *Virchows Arch B Cell Pathol Incl Mol Pathol* 60:245-252, 1991
 46. Hugl SR, White MF, Rhodes CJ: Insulin-like growth factor I (IGF-I)-stimulated pancreatic beta-cell growth is glucose-dependent: synergistic activation of insulin receptor substrate-mediated signal transduction pathways by glucose and IGF-I in INS-1 cells. *J Biol Chem* 273:17771-17779, 1998
 47. Jonas JC, Sharma A, Hasenkamp W, Ilkova H, Patane G, Laybutt R, Bonner-Weir S, Weir GC: Chronic hyperglycemia triggers loss of pancreatic beta cell differentiation in an animal model of diabetes. *J Biol Chem* 274:14112-14121, 1999
 48. Shigematsu S, Yamauchi K, Nakajima K, Iijima S, Aizawa T, Hashizume K: D-Glucose and insulin stimulate migration and tubular formation of human endothelial cells in vitro. *Am J Physiol* 277:E433-E438, 1999
 49. Pillay TS, Xiao S, Olefsky JM: Glucose-induced phosphorylation of the insulin receptor: functional effects and characterization of phosphorylation sites. *J Clin Invest* 97:613-620, 1996
 50. Kroder G, Bossenmaier B, Kellerer M, Capp E, Stoyanov B, Muhlhofer A, Berti L, Horikoshi H, Ullrich A, Haring H: Tumor necrosis factor- α - and hyperglycemia-induced insulin resistance: evidence for different mechanisms and different effects on insulin signaling. *J Clin Invest* 97:1471-1477, 1996