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Original Article

Topical insulin accelerates cutaneous wound healing in insulin-resistant diabetic rats

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Abstract: Insulin signaling defects could lead to insulin resistance in insulin target organs: typically, in the muscler, liver, and adipose tissue. We have observed that insulin accelerated diabetic wound healing in our previous works; to further elucidate the mechanism, we investigated the expression and activation of insulin and insulin-like growth factor (IGF)-1 signaling, compared insulin sensitivity in skin tissue with that in liver tissue, and also observed the regulation of insulin on inflammatory response of wounds during the healing process. We found lower expression of insulin receptor, phos-AKT, IGF-1 in type II diabetic rat skin compared with that in normal rat skin. However, the level of phos-AKT in diabetic rat skin remarkably increased after systemic insulin injection, whereas no significant change of phos-AKT was observed in liver upon insulin stimulation. In insulin-treated wounds, we detected a significant increase in insulin signaling proteins and growth factor, as well as the phosphorylated insulin receptor substrate-1 and AKT. The increased Glut1 protein level and translocation of Glut1 from cytosol to cell membrane of the basal epidermal cells were also observed after insulin application. Insulin-treated wounds showed advanced infiltration and resolution of macrophages and a change pattern similar to that of inflammatory mediators, including TNF-α and IL-6. Our findings support that insulin is a valid agent for diabetic wound healing because of its effect on ameliorating defective insulin action and regulating inflammation response. Our results indicate the presence of subtle insulin responsiveness in diabetic skin tissue, regardless of the presence of impaired insulin sensitivity, which could be the cellular and molecular mechanism of insulin accelerating diabetic wound healing.

Keywords: Diabetic, insulin, insulin resistance, wound healing

Introduction

The effect of insulin on wound healing has been confirmed in various animal and wound models, including cutaneous ulcerations, incision wounds and fracture wounds [1]. The effectiveness of insulin on wound healing has also been observed in donor sites of burn patients [2]. Our previous studies found that insulin accelerates nondiabetic wound healing by stimulating the migration of keratinocytes and vascular endothelial cells, thus improving re-epithelialization and angiogenesis. Insulin regulating wound inflammation response is also responsible for insulin-induced wound healing [3-5]. Insulin receptor substrate (IRS) proteins serve as important signaling molecules for insulin, and insulin induced association of IRS-1 and PI-3 kinase have been found to be involved in insulin regulated growth-promoting effects in skin [6].

The wound healing process consists of sequential and overlapping phases, in which dysfunction of any stage could lead to improper wound healing [7]. Diabetes mellitus is one of the major diseases that causes pathological changes and leads to impaired wound healing. Twenty-five percent of diabetic patients will have a diabetic foot ulcer in his or her lifetime [8]. It has been widely accepted that diabetic impaired healing is not a singular etiology [9, 10]. Disturbance of glucose metabolism, dysregulation of inflammatory response, insufficient of growth factors secretion, and dysfunction of repairing cells and cell signaling are all involved in the morbidity of diabetic impaired wound healing [11-13].

Insulin resistance is one of the most typical and common pathological changes in diabetes mellitus. Defects that influence the process of insulin signal transmission across the plasma mem-

brane and those of biochemical pathways that allow glucose uptake and metabolism by the cells may lead to insulin resistance in non-insulin-dependent diabetes mellitus and obesity [14, 15]. As the primary regulator of blood glucose, insulin could increase glucose uptake in muscular and adipose tissues, and inhibit hepatic glucose production [16]. Insulin resistance that compromises the effectiveness of insulin on glucose regulation could lead to hyperglycemia. Experiments using insulin receptor null mouse model showed that insulin regulates the differentiation and glucose transportation for skin keratinocytes via insulin receptor (IR) [17]. Disruption of IRS-1, a major cytoplasmic substrate of IR, results in disrupted and thinner epidermis [18]. These results suggested insulin resistance might also occur in diabetic skin tissue, although skin tissue has not been classified as a typical insulin target organ.

We have observed that low dose of a topical application of insulin stimulates wound healing in rats with type II diabetes [19]. To explore the mechanism of insulin-induced diabetic wound healing, especially in the presence of insulin resistance, we compared insulin sensitivity in skin tissue with that in liver tissue by analyzing the expression of IR, IRS-1, phosphorylated-AKT (phos-AKT), insulin-like growth factor-1 (IGF-1), IGF-1 receptor (IGF-1R), and glucose transporter-1 (Glut1). We also examined the responsiveness of skin to insulin treatment and studied the effect of insulin on the inflammatory response of wounds during the healing process. We found that: (1) a lower expression of IR, phos-AKT, and IGF-1 as well as a higher expression of tumor necrosis factor-alpha (TNF-α) and phos-ERK were detected in skin of rats with type II diabetes, (2) systemic insulin application induced a remarkably increase of phos-AKT in diabetic rat skin but not liver tissue, (3) in insulin-treated diabetic wounds, we detected a significant increase of insulin-signaling proteins and growth factors, including IR and IGF-1 as well as phosphorylated IRS-1 and AKT. Furthermore, increased Glut1 protein level and increased translocation of Glut1 from cytosol to cell membrane of the basal cells of epidermal were also observed after insulin application. Our results suggested that diabetic skin tissue showed potent insulin responsiveness although a certain extent of insulin resistance exists, which could be the cellular and molecular mechanism of insulin accelerating diabetic wound healing.

Materials and methods

Reagents

The following antibodies were obtained from various suppliers: anti-phos-ERK1/2 (catalog no. 8544), anti-phos-AKT (catalog no. 5012), anti-AKT (catalog no. 9272), anti-phos-IRS-1 (catalog no. 2385), anti-β-actin were purchased from Cell Signaling Technology. Anti-IR (catalog no. ab69508), anti-IGF-1 (catalog no. 182408), anti-IGF-1R (catalog no. 39675) were from Abcam. Anti-Glut1 (Millipore), anti-CD68 (Abdserotec), anti-keratin14 (Pierce, Rockford, IL), and anti-TNF-α (Genetex) also were used.

Induction of diabetes

Forty Wistar rats, pathogen free, were purchased from Shanghai Laboratory Animal Center and housed at the Animal Science Center of Shanghai Jiao Tong University, School of Medicine (SJTUSM). The animal procedures were performed in accordance with the rules of the Animal Care Committee of SJTUSM, and all experimental protocols were approved by the SJTUSM Institutional Animal Care and Use Committee. Animals at age 8~12 weeks were randomly assigned into two groups (diabetic group, n=30; control group, n=10). The induction of diabetes mellitus was performed with multiple streptozotocin (STZ) injections after 2 months of a 60% high-fat diet (HFD) feeding. STZ was dissolved in citrate buffer (pH 4.5) and was given to rats by intraperitoneal (I.P.) injection at a dose of 10 mg/kg body weight (BW) for 4 consecutive days. The HFD was maintained for the next 5 weeks after injections. The control group received I.P. injection of saline and was fed a normal diet. Blood was collected from the tail vein and the glucose level was determined using a glucometer (One Touch Ultra, Lifescan, America). The blood glucose measurement was performed 3 weeks after STZ injection. A blood glucose level >13.9 mmol/L was indicative of diabetes.

In vivo ischemic diabetic wounds

Experiments were performed on 30 diabetic rats induced by HFD feeding with multiple I.P. injections of STZ (10 mg/kg BW) as described previously. Blood glucose and BW measure-

ments were performed 1 week after rats received STZ injections. All rats were checked for symptoms of polydipsia or polyuria. Experiments were carried out after the rats were stable and maintained a diabetic state. Rats (n=20) were anaesthetized with a single I.P. injection of thiopental sodium (40 mg/kg BW). The backs of the rats were shaved, and hairs thoroughly removed with a depilatory. The model was made as we previously described [20]. The outline of the flap was drawn using a surgical marker pen. The spine was set as the long axis and the flap extended from the scapulae to the iliac crests. Next, a 12- × 4-cm double pedicle rectangular flap was n created by excising two sides parallel to the spine. The panniculus carnosus underneath the flap was then thoroughly removed. Four 0.9-cm full-thickness excision wounds were made on the flap at 5 cm and 7 cm from the top pedicle of the flap and 5 mm to the excision edge. The flap was then repositioned and sutured with incisional-edge normal skin. The wounds were covered with sterile gauze. The rats were kept separately, and supplied with unrestricted food and water. Human isophane insulin suspension (Humulin N, Lilly USA, 0.1 U/20 μL saline) or saline (20 μL) were dropped into the wounds every 24 h until the wounds healed.

Histological observations

The skin tissues were collected (n=6) for histological analysis at day 3, 7, and 13 after wounding and the day healing was completed. The wounds were harvested and fixed as described previously and the sections were then deparaffinized, rehydrated, and washed with distilled water. The sections were placed in 95~98°C antigen retrieval citrate buffer for 10~15 min. Endogenous peroxidase activity was blocked by placing the sections in 3% hydrogen peroxide in methanol for 30 min. Nonspecific staining was blocked with normal bovine serum, and the sections were incubated with monoclonal mouse anti-rat Glut1, phos-AKT, Keratin14, and CD68 overnight at 4°C. After washing, a horseradish peroxidase-labeled secondary antibody was applied for 1 h at room temperature (RT) and then stained with diaminobenzidine, and counterstained with hematoxylin.

Acute insulin responsiveness assay

The rats fasted overnight and were challenged with insulin at a dose of 1 U/kg BW. Two full-thickness 9-mm punch wounds were made on the symmetric sites of the rats' backs after they were anesthetized. Insulin was then injected through the tail vein. Wound tissue and liver tissues were collected 15 min after injection and flash frozen in liquid nitrogen. Wound tissue and liver tissues without insulin injection served as control and are referred to as 'basal'. Collected tissues were used for western blot analysis and immunofluorescence staining according to standard protocol.

Immunoblotting

On day 1, 3, 7, and 13 after wounding, rats were anesthetized and skin samples consisting of the wounded area in addition to 5 mm of surrounding skin were collected. Skin tissues were lysed on ice with a radioimmunoprecipitation assay buffer with 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl (pH 7.4), 0.25% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride for 1 h. The tissue lysates were repeatedly pipetted to shear DNA, and incubated on ice for additional 30 min before centrifugation at 12,000 rpm for 15 min to remove the insoluble precipitation. Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). Equal amounts of protein in the skin extracts were mixed with sample buffer, boiled, and analyzed using 12% or 10% acrylamide SDS-PAGE. The membrane was blocked by 5% nonfat powdered milk in Tris-buffered saline with Tween-20 and then incubated with the appropriate primary antibodies including anti-IR, phos-IRS-1, phos-AKT, phos-ERK1/2, TNF-α, IGF-1, IGF-1R, and Glut1, followed with HRP-conjugated secondary antibodies. Bands were visualized with enhanced chemiluminescence (Millipore). Relative quantities of protein were determined using a densitometer and presented in comparison with β-actin expression.

Real-time PCR

A total mRNA extraction was performed using Trizol. complementary DNA (cDNA) synthesis was performed using PrimeScript® RT reagent Kit Perfect Real Time using 1 mg RNA. The cDNA sample was subjected to PCR analyses using SYBR® Premix Ex Taq(Takara)™. The primers for the genes and the internal control (β-actin) are as follows: TNF-α: forward primer: 5'-GCTCCCTCTCATCAGTTCCA-3', reverse prim-

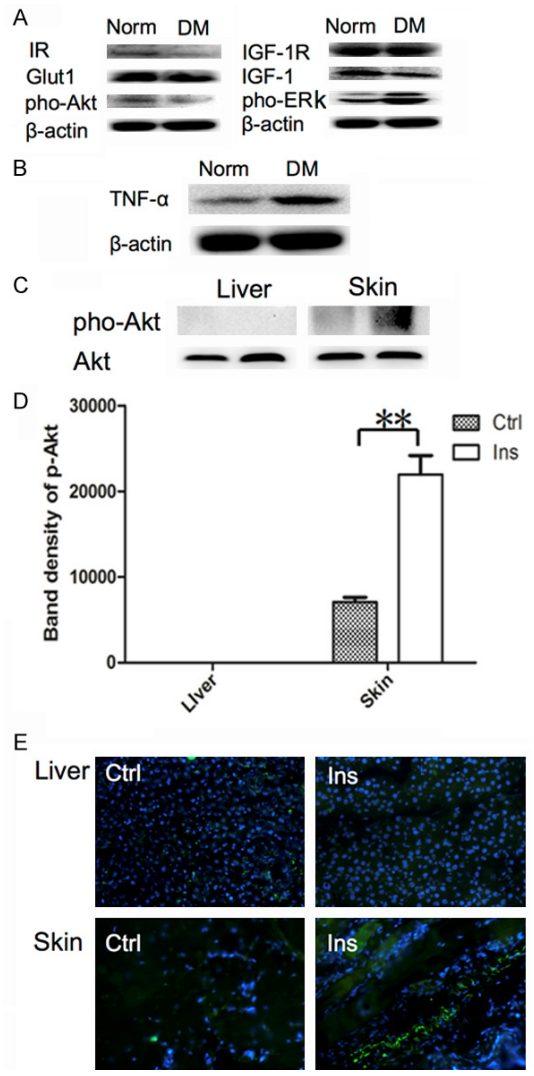

Figure 1. Diabetic skin showed vigorous responsiveness to insulin although impaired insulin sensitivity was noted. After the induction of diabetes of wistar rats, excision wounds (9-mm diameter) were made in control and diabetic rats; full-thickness skin samples were obtained from the back of diabetic and normal rats for analysis. (A) Western blot analysis was performed to detect protein expression of insulin receptor, IGF-1 receptor, IGF-1, Glut1, phos-AKT, phos-ERK, and (B) TNF-α. β-actin was detected as a loading control. (C) Western blot analysis of phos-AKT in the liver and skin of diabetic rats before (-Ins) or after (+Ins; tissue harvested at 15 min) acute insulin injection. A submaximal insulin dose (0.1 U/kg) was used; liver samples were obtained at basal (-Ins) and 15 min after insulin injection. (D) The quantified expression of the proteins were determined using Image J. Data are shown as the mean ± SD. Significant differences between means were determined by analysis of variance followed by unpaired t-test. *$p < 0.05$, **$p < 0.01$, $n=6$. (E) Represented images of immunofluorescence staining of phos-AKT were detected. Original magnification was 200x and scales bar =50 um.

er: 5'-GCTTGGTGGTTTGCTACGAC-3', β-actin: forward primer: 5'-CTAAGGCCAACCGTGAAAAG-3', reverse primer: 5'-CTAAGGCCAACCGTGAAAAG-3', IL-6: forward primer: 5'-GTCAACTCCATCTGCCCTTC-3', reverse primer: 5'-TGTGGGTGGTATCCTCTGTG-3'. The samples were run on ABI 7500 Real-time PCR System (Applied Biosystems) according to the following program: 95°C 30 s; 95°C 5 s × 40; 62°C 34 s. For data analysis, the DDC(T) method was applied as described previously.

Immunofluorescence staining

The slides were incubated with paraformaldehyde for 20 min. The phos-AKT antibodies were then applied at an optimal concentration overnight in a wet chamber after blocking with 5% bovine serum albumin for 1 h at RT. The slides then rinsed in phosphate buffered saline and incubated with the secondary antibody conjugated to Alexa Fluor 488 for 1 h at RT. The nuclei were counterstained with 4',6-diamidino-2-phenylindole.

Statistical analysis

Values were presented are mean ± standard deviation (SD). Data were analyzed by SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Significant differences between means were determined by analysis of variance followed by unpaired t-test. Statistical significance was set at $P < 0.05$ or $P < 0.01$.

Results

Diabetic skin showed vigorous responsiveness to insulin although impaired insulin sensitivity was noticed

To clarify the state of insulin resistance in diabetic skin and to explore the possible pathological influence of diabetic state on wound healing, we first took skin samples from normal and diabetic rats and compared the expression of their insulin signaling related proteins. Diabetic skin showed a decreasing of IR expression but not IGF-1R. Decreased expression of IGF-1 and Glut1 were observed in diabetic skin as well (**Figure 1A**). We also observed downregulated phos-AKT and substantial upregulated phos-ERK in diabetic skin (**Figure 1A**), which suggested the presence of impaired insulin signaling in diabetic skin tissue. Furthermore, the increased

TNF-α expression in diabetic skin suggested a chronic inflammatory state in diabetic skin, which could be related to the morbidity of insulin resistance (**Figure 1B**).

To compare the responsiveness of diabetic skin to insulin with that of liver tissue, we measured the phosphorylation/activation of AKT in these two tissues by utilizing 1U of insulin systemic injection. No obvious phos-AKT was detected in liver tissue after insulin application. However, a significant increase of phos-AKT in skin tissue was detected after insulin treatment, using western blot analysis (**Figure 1C**, **1D**). These results were confirmed by immunofluorescence staining of phos-AKT, and more phos-AKT positive staining cells were found in skin rather than liver tissue after insulin treatment (**Figure 1E**).

The aforementioned results suggest that although insulin sensitivity is impaired in diabetic skin, compelling responsiveness to insulin still exists, which might be the molecular foundation for the regulative effect of insulin on diabetic skin homeostasis and diabetic wound healing.

Topical insulin applications improved diabetic wound healing by regulating wound inflammatory cells and repairing cell function

To investigate the effect of insulin on diabetic wound healing, we utilized a type II diabetic rat ischemic wound model, which we established before [20], and treated wounds with either topical saline or insulin application. Larger unhealed wounds were found in saline-treated rats, compared with insulin-treated rats, at day 3, 7, 9, 11, 13, and 15 after wounding (**Figure 2A**). Wound area analysis confirmed these findings; insulin application led to a substantial decrease in unhealed wound area during the healing process (**Figure 2B**). Keratinocyte migration was estimated by the length of migration tongue: at day 7 after wounding, the length of migration tongue in insulin-treated wounds was much longer than in control wounds, which suggested a faster healing rate induces by insulin (**Figure 2C**). Histological examination shortly after wound closure showed that insulin induced more mature "skin" formation, demonstrated by more reticular ridges and more blood vessels in wounds area (**Figure 2C**). Maturation and differentiation of epidermis were evaluated using Keratin 14 immunolabeling staining. We found extensive and stronger staining for Keratin14 in all epidermal layers and thinner and well-organized epidermis in insulin-treated wounds, which suggested more mature and differentiated epidermis induced by insulin (**Figure 2D**).

We then analyzed wound inflammatory response by monitoring the expression of CD68, a specific marker of macrophage, and by measuring TNF-α and IL-6 levels on wound area. Compared with saline-treated wounds, insulin-treated wounds showed more CD68 positive cells infiltrating at day 3 and day 7 after wounding, which suggested an early initiation of wound inflammatory response. At day 13 after wounding, wound macrophages were substantially decreased in insulin-treated wounds, whereas large amounts of macrophages were still present in the saline-treated wound area, which suggested an earlier and prompt resolution of wound inflammatory response induced by insulin (**Figure 2E**). The expression pattern of wound inflammatory mediators TNF-α and IL-6 was similar to that of macrophages infiltration of wounds, and the mRNA levels of TNF-α and IL-6 in insulin-treated wounds were substantially higher than that in saline-treated wounds at day 3 and day 7 after wounding and substantially lower than that in saline-treated wounds at day 11 and day 24 after wounding (**Figure 2F**, **2G**).

Topical insulin application regulated expression of insulin signaling related proteins on wound area

We collected and homogenized the saline- and insulin-treated wound tissue at day 1, 3, 7, and 13 after wounding, and immunoblotting the proteins with relevant antibodies of insulin signaling related proteins. Both saline- and insulin-treated wounds showed similar IR and IGF-1R expression pattern: the highest expression of IR and IGF-1R were noticed immediately after wounding and then gradually decreased along with the progress of healing. However, insulin induced more IR expression at all time points and more IGF-1R expression at day 1 and day 3 after wounding (**Figure 3A**, **3B**). We also noticed that insulin induced an enormous expression of IGF-1 at day 1 and day 3 after wounding and a higher level of IGF-1 at day 7 and day 13, although no statistical significance was determined (**Figure 3A**, **3C**). Similarly,

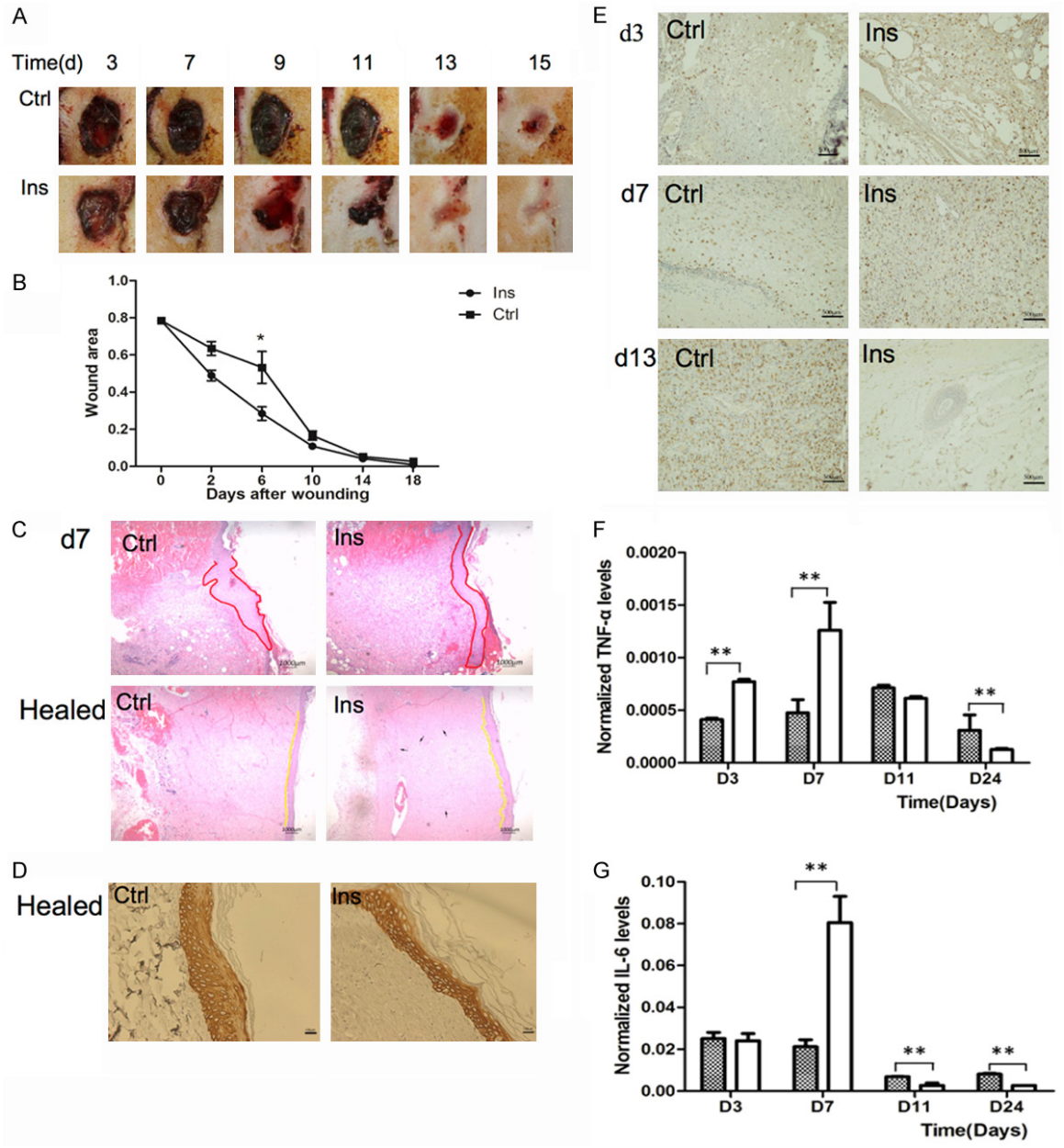

Figure 2. Topical insulin applications improved diabetic wound healing by regulating wound inflammatory cells and repairing cell function. Diabetic wounds were treated with vehicle (20 μL saline solution) or 0.2 U insulin/20 μL saline solution every day until healing was achieved and (A) representative images of the healing process were monitored at day 3, 7, 9, 11, 13, 15 after injury. (B) Wound areas were quantified every other day and expressed as the percentage of wound healing (n=6); statistics are shown as comparisons between the insulin and saline groups. *$P < 0.05$; **$P < 0.01$ versus control. (C) Representative H&E stained sections show shorter migrating tongue, fewer vessels in saline wounds at day 7 after wounding, and less mature epidermis after wound healing. Scale bar =1,000 μm. The migration tongues are outlined in red and the reticular edge zone is highlighted in yellow. Blood vessels are indicated by arrows. (D) Immunolabeling for Keratin14, a marker for keratinocyte differentiation, showed that the basal layers of the epidermis of healed wounds are better organized and differentiated when insulin was applied to the wounds. Scales bar =100 μm. (E) Macrophage recruitment on day 3 and 7 after wounding and the day the wound healed is represented by images of immunohistochemical staining for CD68, a specific marker of macrophage. Scale bars=500 μm. Real-time PCR analyzes mRNA expression by comparing fold change of (F) TNF-α and (G) IL-6 mRNA expression at insulin- and saline-treated wounds on day 3 and day 7 after wounding and on the day wound healing was achieved. Data were obtained from 6 wounds, and are shown as mean ± SD. **$P < 0.01$.

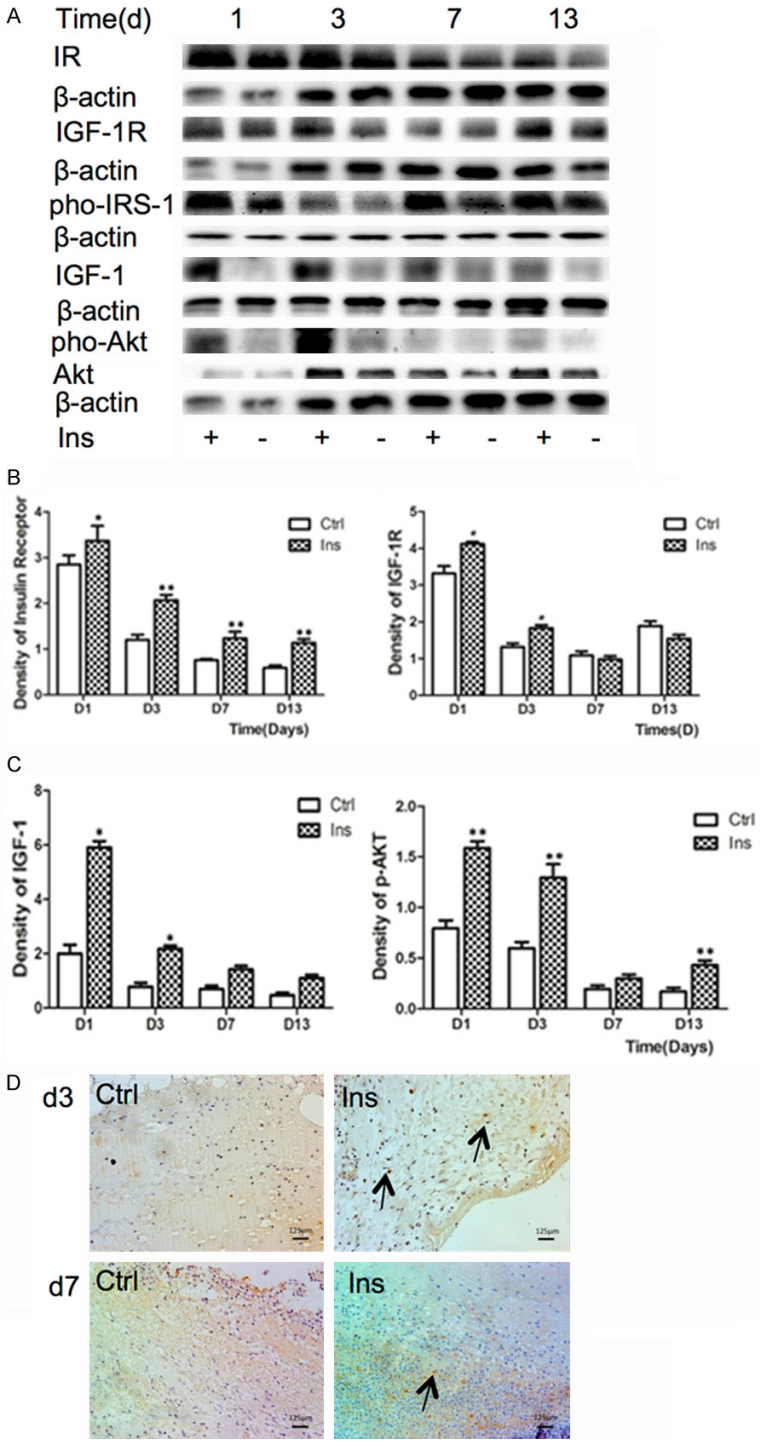

Figure 3. Topical insulin application regulated expression of insulin signaling related proteins on wound area. Diabetic wound were treated with vehicle (20 μL saline solution) or 0.2 U insulin/20 μL saline solution. Wound samples were collected on day 1, 3, 7, and 13 after injury, homogenized, and analyzed. (A) Western blot analysis was performed to detect protein expression of insulin receptor, IGF-1 receptor, phos-IRS-1, IGF-1 and phos-AKT. The antibody dilution ratio is 1:1000. β-actin was detected as a loading control. (B, C) The quantified expression of the proteins were determined using Image J. Data are shown as the mean value ± SD (n=6, $*p < 0.05$; $**p < 0.01$ versus control). Significant differences between means were determined by analysis of variance followed by unpaired t-test. (D) Representative immunohistochemical sections showed an increased number of phos-AKT- expressing cells in insulin-treated wounds at day 3 and 7. Scale bar =125 μm.

we also observed significant enhanced phos-IRS-1 (**Figure 3A**) and phos-AKT (**Figure 3A**, **3D**) expression under insulin treatment in wounds.

Topical insulin application regulated expression of Glut1

To prove that insulin plays a role in regulation of glucose transport during healing, a time course analysis of protein expression was performed to monitor Glut1 protein level, which was altered upon insulin treatment. Immunoblotting showed aberrantly decreased Glut1 expression in diabetic wounds (**Figure 1A**), and persistent increase of Glut1 expression during the diabetic wound healing process, with the maximal expression of Glut1 on day 1 after wounding (**Figure 4A**). IHC staining showed that Glut1 could be found in the epidermal layer and the dermis right under the epidermis (data not shown). In the presence of insulin, increased Glut1 expression in the plasma membranes of the basal cells were observed at day 3 and 7 after wounding (**Figure 4B**), which suggested insulin might increase transportation of glucose that provides the metabolic energy necessary for cell migration and proliferation.

Discussion

Insulin resistance is an essential feature of type II diabetes, which is most evident in clas-

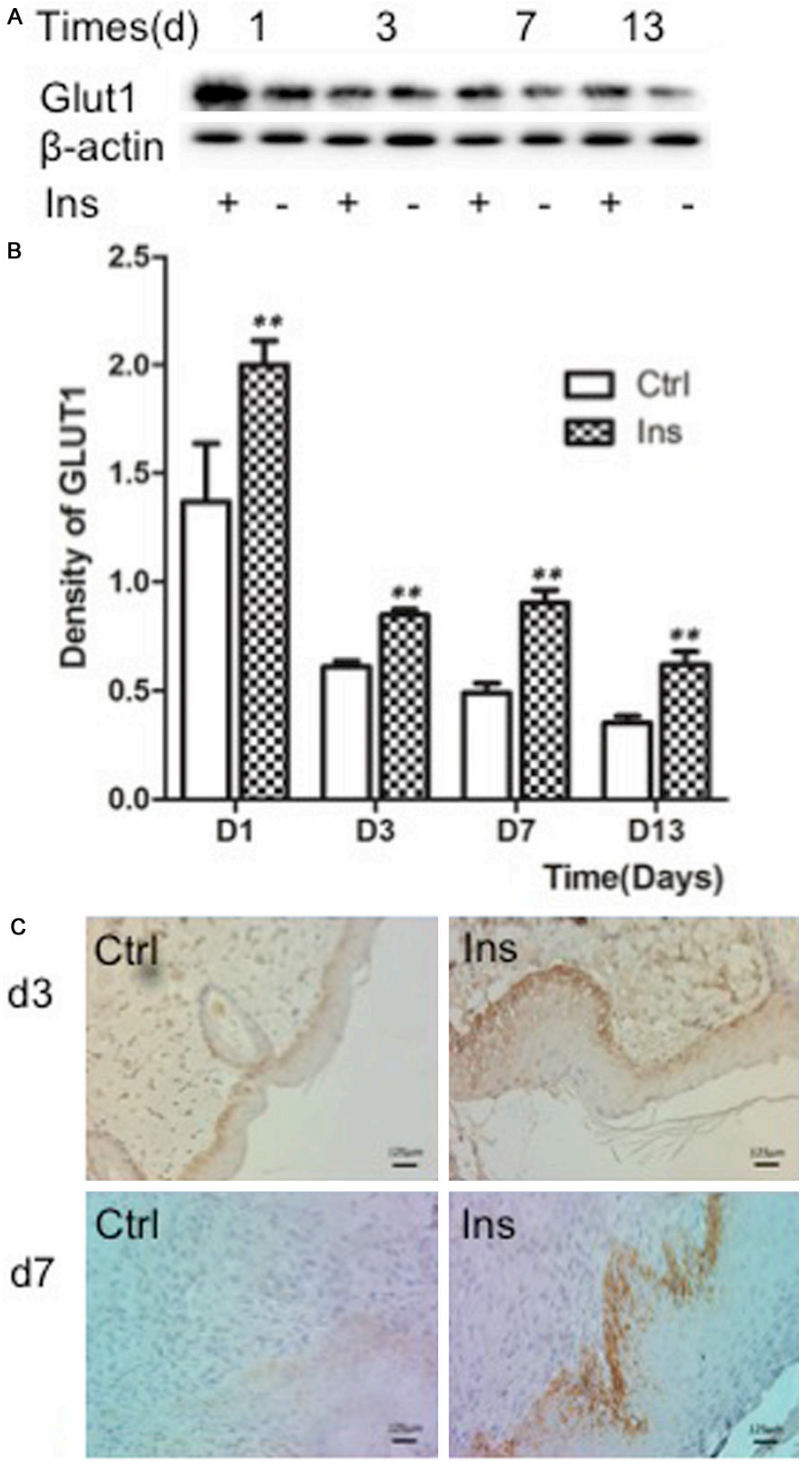

Figure 4. Topical insulin application regulated expression of Glut1. Diabetic wound samples treated with vehicle (20 μL saline solution) or 0.2 U insulin/20 μL saline solution were collected on day 1, 3, 7, and 13 after injury, homogenized, and analyzed. (A) Glut1 levels were detected by western blot analysis. The antibody dilution ratio is 1:1000. β-actin was detected as a loading control. (B) The quantified expression was determined using Image J. Data are shown as the mean ± SD (n=6, $**p < 0.01$). Statistics are shown as comparisons between the treatment and control groups. (C) Images immunostained with antibodies specific to Glut1 are presented. Insulin treatment promotes Glut1's translocation to the plasma membrane, which was found to be increased 30% on day 3 and 60% on day 7 compared with controls.

sic insulin target organs, adipose tissue, liver, and muscle. It is not clear whether insulin resistance is also present on diabetic skin tissue and how it prevents insulin from organizing an efficient healing process. Here, we introduced a type II diabetic rat model and studied the characteristics of insulin resistance on skin tissue by detecting insulin signaling related proteins, including IR, IGF-1, IGF-1R, phos-AKT, and IRS-1 expression, as well as the responsiveness of skin tissue to insulin by monitoring the change of insulin signaling related proteins during the insulin-induced healing process. Our experiments showed that: (1) although impaired insulin sensitivity was noticed on type II diabetic rat skin, vigorous responsiveness of skin tissue but not liver after insulin challenge suggested a significant difference of insulin resistance on skin tissue and classic insulin target tissue; (2) topical insulin application significantly changes the expression of insulin signaling related proteins and expression of Glut1 on wound areas; and (3) topical insulin applications improved diabetic wound healing by regulating wound inflammatory cells and repairing cell function.

Insulin resistance, in the setting of clinical state, results from a combination of altered function of insulin target cells, effect of circulating antagonists of insulin, and the accumulation of macrophages that secrete proinflammatory mediators [21]. At the mo-

lecular level, reduced insulin receptor on target cells along with impaired insulin-dependent PI3K activation and downstream signaling play crucial roles in pathological progression of insulin resistance [22].

Over 100 known pathologic factors could contribute to deficiencies in wound healing [23]. Disruption of insulin signaling pathway, one of the most distinctive pathological changes of type II diabetes [24], might be one of these factors. In addition to the effect on glucose disposal, insulin plays a role in stimulating DNA synthesis and cell proliferation. Dysfunction of insulin is frequently associated with proliferative tissue abnormalities of the skin (acanthosis nigricans), ovary, and heart [25]. Insulin resistance is normally known as inability of metabolic tissues, including adipose tissue, liver, and skeletal muscle to respond to insulin [26]. Whether insulin resistance is present on type II diabetic skin tissue and how it affects diabetic wound healing is still uncertain. We observed a decreased number of IR and IGF-1 and less activated AKT (phos-AKT), but increased TNF-α expression and activated ERK expression in diabetic rat skin, compared with normal skin. PI3K/AKT and MAPK are regarded as a key switch of insulin sensitivity. Hyperinsulinemia might impair the balance between PI3K/AKT and MAPK [27]. The lower expression of phos-AKT and the higher expression of phos-ERK in diabetic skin imply the presence of insulin resistance in diabetic skin. In addition, the deficiency of growth factors such as IGF-1 is also an indicator of chronic wounds *in vivo* [28].

In contrast to the high insulin level in cava, the insulin level is significantly low in skin of diabetic patients. The diminished levels of insulin or IGF-1 in skin are partially the result of the increased insulin-degrading enzyme activity observed in experimental wounds of animals [29]; they also cause the “invisible damage” of diabetic skin, as well as impaired healing of diabetic wounds. Dysfunction of glucose transporters in internal organs of diabetic patients is one of the characteristics of diabetes [30]. Impaired Glut4 signaling shown by decreased Glut4 expression upon insulin challenges has been observed in the literature [31]. However, the major glucose transporter Glut1 in skin is not disrupted in our diabetic model. It is reported that in response to insulin the PI3K/PKB/mTOR/4E-BP 1 pathway is activated to increase Glut1 mRNA expression and enhance the regulative effect of Glut1 on tissue growth [32]. Glut1 in myocardia of diabetic patients reportedly takes major responsibility in response to insulin, via the PI3K-mediated signaling pathway [33]. Topical insulin application-regulated Glut1 function might be one of the cellular mechanisms of insulin-induced wound healing.

Utilizing the experimental diabetic wound model, we found that insulin significantly increased the healing rate through modulation of inflammatory response and repairing cell function. An appropriate inflammatory response, including proper infiltration and resolution of inflammatory cells, is essential to a concerted wound healing response. An abnormal inflammatory response is a noticeable characteristic of impaired diabetic wound healing [34]. Proper healing requires timely macrophage influx followed by infiltration of neutrophils into wounds. Macrophages phagocytose and digest tissue debris and neutrophils in wounds, and also secrete anti-inflammatory cytokines and growth factors that promote cell proliferation and migration as well as tissue repair [35]. Dysfunction in macrophage infiltration could inhibit the timely removal of bacterial and necrotic tissue from wounds, and could lead to delayed inflammatory cells resolution [36, 37]. The infiltration and resolution of macrophages in the diabetic wounds in our study were significantly delayed in saline-treated rather than insulin-treated wounds: massive macrophages existed in the wound areas at day 16 after wounding, whereas relatively low amounts of macrophages were found in insulin-treated wounds at the same time point, which suggested that insulin could induce diabetic wound healing through regulation of wound inflammatory response.

In addition to its well-known effect on promoting glucose metabolism (glucose, amino acid transport), insulin, at different concentrations, regulates protein and DNA synthesis by a variety of cellular mechanisms, thus influencing cell growth and differentiation [38]. The insulin signaling related protein in nondiabetic skin has already been investigated [39]. Here, we investigated these proteins in diabetic skin and diabetic wound healing. Topical insulin application is implemented by placing insulin solution directly onto open-excision wounds to minimize the interference of subcutaneous injection dur-

ing the healing process. Our results show that in both insulin- and saline-treated wounds, the expression of insulin signaling related proteins gradually decreased along with the progress of healing. Topical insulin application rapidly induced upregulation of insulin signaling related proteins on wound areas after injury. IRS-1 is a central player of insulin effect, whose functions include regulation of glucose transportation [40]. Increased phosphorylation/activation of IRS-1 tyrosine kinase was observed since day 3 after wounding. IRS-1 binds PI3-kinase, one of the SH2 proteins, through its multiple tyrosine phosphorylated sites [41, 42] (Kahn 1994; Lavan and Lienhard 1993). Being a downstream effector of PI3-kinase, AKT phosphorylation was also found increasingly after insulin treatment. We also demonstrate the presence of phos-AKT localized in the dermal layer of skin. Although insulin stimulation rapidly increased IR expression, insulin has no evident effect on IGF-1 receptor; no significant upregulation of IGF-1 receptor could be seen after insulin treatment since day 3 after wounding. This is in accordance with a recent report that claimed in proliferating keratinocytes both insulin and IGF-1 induced phosphorylation of both receptors [43], whereas insulin stimulation of terminally differentiated cells resulted in phosphorylation of the IR alone. Generally speaking, insulin-stimulated growth promotion at supraphysiologic concentrations is mediated through IGF-I receptor and insulin-stimulated growth promotion at physiologic concentrations is mediated by insulin receptor [44]. With less IGF-1R activated by insulin, there will be less overgrowth effect.

Proliferation and migration of cells during wound healing is a metabolically demanding process; for example, migrating cells require high levels of glucose to provide a substrate for glycolysis [45-47]. Insulin regulates both Glut4- and Glut1-mediated glucose uptake, although Glut1-mediated glucose uptake is usually considered to be responsible for non-insulin-dependent glucose transportation [48]. In fact, insulin-mediated-AKT activation is responsible for glucose uptake in muscle and liver through Glut4 and tissue repair through Glut1. It has been proven that glucose transporters are present in keratinocytes and can sensitively respond to hormones as do insulin and IGF-1 [49, 50]. We found insulin increased the total amounts of Glut1 and its expression in the plasma membrane of the basal cells in skin. This is consistent with the observation that at the early and middle stages when the animals do not have complete wound closure, keratinocytes will continue to migrate to close the wound. It is suggested that insulin induces growth promotion by increasing expression of Glut1 in the plasma membrane via the activation of PI3K/AKT /mTOR pathway [32], which can be one biological factor that contributes to the effectiveness of insulin in diabetic skin.

To our knowledge, this is the first comparison of insulin-induced AKT phosphorylation between skin tissue with liver tissue. The effectiveness of insulin in diabetic skin may also be associated with the ability to regulate Glut1. Moreover, the inflammatory environment in skin may be different from that in other organs *in vivo*. A lower insulin resistance in skin is likely to explain the fact that a low dose of topical insulin is sufficient to accelerate healing. Hyperinsulinemia induces cardiovascular disease, vascular endothelial injury, and primary hypertension [51, 52]. There is also evidence of systemic insulin application in diabetic mice leading to increased prevalence of biofilms in the diabetic wound area [53]. Topical administration of insulin might be a promising method because it is capable of activating insulin signaling and inducing accelerated wound healing without the deleterious effects associated with systemic administration.

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Disclosure of conflict of interest

None.

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