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*Cell Injury, Repair, Aging and Apoptosis*

# Severely Impaired Insulin Signaling in Chronic Wounds of Diabetic *ob/ob* Mice

## *A Potential Role of Tumor Necrosis Factor-α*

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**Wound-healing disorders are major complications of diabetes mellitus. Here, we investigated insulin-mediated signaling in nonwounded skin and in cutaneous tissue regeneration of healthy C57BL/6 and diabetes-impaired leptin-deficient *obese/obese* (*ob/ob*) mice. The insulin receptor (InsR) was abundantly expressed in wound margins and granulation tissue during acute healing in healthy mice. Remarkably, active signaling from the InsR, as assessed by phosphorylation of downstream targets such as protein tyrosine phosphatase-1B, glycogen synthase (GS), and GS kinase, was nearly absent in nonwounded and acutely healing skin from *ob/ob* mice. Systemic leptin administration to *ob/ob* mice reverted the diabetic phenotype and improved tissue regeneration as well as the impaired expression of InsR, insulin receptor substrate-1 and insulin receptor substrate-2, and downstream signaling (phosphorylation of GS kinase and GS) in late wounds and nonwounded skin of *ob/ob* mice. Importantly, tumor necrosis factor (TNF)-α was a mediator of insulin resistance in keratinocytes *in vitro* and in *ob/ob* wound tissue *in vivo*. Systemic administration of a monoclonal anti-TNF-α antibody (V1q) in wounded *ob/ob* mice attenuated wound inflammation, improved re-epithelialization, and restored InsR expression and signaling in wound tissue of *ob/ob* mice. These data suggest that InsR signaling in diabetes-impaired wounds is sensitive to inflammatory conditions and that anti-inflammatory approaches, such as anti-TNF-α strategies, improve diabetic wound healing. *(Am J Pathol 2006, 168:765–777; DOI: 10.2353/ajpath.2006.050293)***

The functional connection between diabetes and foot ulceration was first recognized by the surgeon T.D. Pryce in 1887.[1] For the first time, Pryce claimed in an article published in *Lancet* that "diabetes itself may play an active part in the causation of perforating ulcers."[1] Diabetic foot ulcers are skin lesions with a loss of epithelium that may extend into the dermis and may sometimes involve bone and muscle.[2,3] It is now well established that ulcerations and subsequent amputation events of lower extremities represent serious complications of both types of diabetes mellitus and are associated with significant mortality.[3,4] Thus, diabetic ulcers characterize an increasing clinical problem. The annual incidence of foot ulceration in the diabetic population is just over 2%,[5,6] resulting in a lifetime risk of 15% for any diabetic patient to develop such a complication.[7,8] Diabetic ulcers still have a poor prognosis, and the 3-year survival rates are between 50 and 59%, as assessed for Italy and Sweden, respectively.[9,10] By contrast, the efforts to identify novel pharmacological approaches to improve significantly severe diabetes-impaired healing conditions have failed. Only recombinant platelet-derived growth factor (becaplermin) is now available for treatment of foot ulcers.[11] Thus, Jeffcoate and Harding[7] focus the challenge for future research in their review article on diabetic foot ulceration by their demand that "investment is urgently needed for basic research into the pathophysiology of chronic wounds."

Here, we have used the *obese/obese* (*ob/ob*) mouse as a model system of diabetes-impaired wound healing. These mice are characterized by severe diabetes and obesity syndromes.[12] The diseased phenotype is mediated by a functional loss of the *ob* gene, which normally

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encodes a 16-kd cytokine named leptin.[13] Severely impaired wound-healing conditions in *ob/ob* mice were strongly improved by administration of leptin, where leptin mediated wound re-epithelialization in a direct manner but attenuated chronic wound inflammation in an indirect manner.[14,15] In addition, systemic application of leptin to *ob/ob* mice also blunts both hyperglycemia and hyperinsulinemia and resolves the diabetic phenotype of the animals.[14–16] It was reasonable to suggest that dysregulation and insensitivity of the insulin signaling machinery in resident skin cells might contribute to diabetes-impaired repair and that a leptin-driven adjustment of insulin sensitivity in skin tissue might be functionally connected to an improved healing in the animals. In line, skin keratinocytes have been shown to express the insulin receptor (InsR), which is functionally implicated in keratinocyte differentiation and glucose uptake.[17,18]

There is increasing evidence for a functional link between insulin resistance, obesity, and diabetes. Initial studies demonstrated an increase in adipocyte-derived tumor necrosis factor (TNF)-$\alpha$ in obese rodents that was functionally connected to insulin resistance.[19] Interestingly, plasma TNF-$\alpha$ levels were also dependent on adipose tissue mass in humans,[20,21] and clinical studies confirmed that the presence of inflammatory mediators predicts the development of type 2 diabetes mellitus.[22–24] These observations suggest that obesity-associated inflammatory mediators such as TNF-$\alpha$ might contribute to insulin resistance in skin tissue. Using the leptin-deficient *ob/ob* mouse model, we investigated the insulin sensitivity of nonwounded and injured skin tissue under normal and diabetes-impaired conditions. Here, we provide evidence that disturbed insulin signaling pathways are associated with impaired repair in *ob/ob* mice and that TNF-$\alpha$ functionally interferes with insulin signaling and tissue regeneration at the wound site.

## Materials and Methods

### Animals

Female C57BL/6J (wild-type) and C57BL/6J-*ob/ob* mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and maintained under a 12-hour light/12-hour dark cycle at 22°C until they were 8 weeks of age. At this time, they were caged individually, monitored for body weight, and wounded as described below.

### Treatment of Mice

Murine recombinant leptin (2 $\mu$g/g body weight) (Calbiochem, Bad Soden, Germany) and purified monoclonal anti-TNF-$\alpha$ antibody V1q[25] (1 $\mu$g/g body weight) (Abcam Ltd., Cambridge, UK) were injected intraperitoneally in 0.5 ml of phosphate-buffered saline (PBS) for the indicated time periods. For local treatment, wounds of mice were covered with 1 $\mu$g of leptin in 20 $\mu$l of PBS twice a day (8:00 a.m. and 8:00 p.m.). Control mice were treated with PBS or an unspecific IgG (Santa Cruz, Heidelberg, Germany), respectively.

### Wounding of Mice

Wounding of mice was performed as described previously.[26,27] Briefly, mice were anesthetized with a single intraperitoneal injection of ketamine (80 mg/kg body weight)/xylazine (10 mg/kg body weight). The hair on the back of each mouse was cut, and the back was subsequently wiped with 70% ethanol. Six full-thickness wounds (5 mm in diameter, 3 to 4 mm apart) were made on the back of each mouse by excising the skin and the underlying panniculus carnosus. The wounds were allowed to form a scab. Skin biopsy specimens were obtained from the animals 1, 3, 5, 7, and 13 days after injury. At each time point, an area that included the scab, the complete epithelial and dermal compartments of the wound margins, the granulation tissue, and parts of the adjacent muscle and subcutaneous fat tissue was excised from each individual wound. As a control, a similar amount of skin was taken from the backs of nonwounded mice. For each experimental time point, tissue from four wounds each from four animals ($n$ = 16 wounds, RNA analysis) and from two wounds each from four animals ($n$ = 8 wounds, protein analysis) were combined and used for RNA and protein preparation. Nonwounded back skin from four animals served as a control. All animal experiments were performed according to the guidelines and approval of the local Ethics Animal Review Board.

### RNA Isolation and RNase Protection Analysis

RNA isolation and RNase protection assays were performed as described previously.[27,28] The cDNA probes were cloned using reverse transcriptase-polymerase chain reaction. The probes corresponded to nucleotides 3718 to 4080 (for InsR, NM010568.1), nucleotides 1173 to 1391 (for protein tyrosine phosphatase [PTP]-1B, BC010191.1), nucleotides 4261 to 4621 (for InsR substrate [IRS]-1, NM010570.2), nucleotides 1969 to 2201 (for IRS-2, AF090738), nucleotides 1294 to 1592 (for glucose transporter [Glut]-4, BC014282.1), nucleotides 796 to 1063 (for cyclooxygenase [COX]-2, M64291), nucleotides 541 to 814 (for TNF-$\alpha$, NM013693), nucleotides 1405 to 1649 (for TNF-$\alpha$ R1, p55, NM001065.2), nucleotides 1192 to 1475 (for TNF-$\alpha$ R2, p75, NM001066.2), and nucleotides 163 to 317 (for GAPDH, NM002046).

### Immunohistochemistry

Mice were wounded as described above. Animals were sacrificed at day 5 after injury. Complete wounds were isolated from the back, bisected, and frozen in tissue-freezing medium. Six-micrometer frozen sections were subsequently analyzed using immunohistochemistry as described previously.[26] Additionally, wounds were fixed in formalin and embedded in paraffin. Paraffin-fixed sections were stained with hematoxylin and eosin. Antiserum against the $\beta$-subunit of InsR (Santa Cruz) was used for immunodetection.

### *Immunoblot Analysis*

Wound, muscle, and liver tissue and cell culture lysates were prepared as described previously.[26,29] Fifty micrograms of total protein lysate was separated using sodium dodecyl sulfate (SDS)-gel electrophoresis, and specific proteins were detected using antisera directed against InsR$\beta$, Glut-4 (Santa Cruz), PTP-1B, IRS-1, IRS-2 (Biomol, Hamburg, Germany), phospho-glycogen synthase kinase (GSK)-3$\alpha/\beta$, phospho-glycogen synthase (GS), phospho-tyrosine (Cell Signaling, Frankfurt, Germany), or actin (Sigma, Deisenhofen, Germany).

### *Determination of Phospho-InsR$\beta$ by Immunoprecipitation*

Five hundred micrograms of total wound protein lysate was incubated overnight with 2.5 $\mu$g of a monoclonal, biotinylated anti-phospho-tyrosine (4G10) antibody (Biomol). Next, 150 $\mu$g of streptavidin-coupled magnetic beads (MyOne; Dynal, Hamburg, Germany) was added for 2 hours. Magnetic beads were isolated and washed with PBS, and protein was eluted using Laemmli buffer.

### *Determination of Glucose Uptake*

Quiescent, confluent human HaCaT keratinocytes were incubated in 35-mm wells with 1 ml of Krebs-Ringer solution (Sigma, Deisenhofen, Germany) in the presence or absence of insulin (0.1 and 1 $\mu$g/ml) for 20 minutes. Subsequently, 0.5 $\mu$Ci of D-[2-H$^3$]glucose (Amersham, Freiburg, Germany) was added per 35-mm well. Cells were harvested using 10% (w/v) SDS after different time points of incubation.

### *Enzyme-Linked Immunosorbent Assay*

Total wound lysate was analyzed for the presence of immunoreactive TNF-$\alpha$ by enzyme-linked immunosorbent assay (ELISA) using the Quantikine murine ELISA kit (R&D Systems, Wiesbaden, Germany).

### *Determination of Blood Glucose, Insulin, and Leptin Levels*

Blood glucose levels were determined using the Accutrend sensor (Roche Biochemicals, Mannheim, Germany). Serum insulin and leptin were analyzed by ELISA (Crystal Chemicals, Chicago, IL) as described by the manufacturer.

### *Determination of Cell Viability*

Viability of cultured keratinocytes was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay following a published protocol[30] and the lactate dehydrogenase assay. The lactate dehydrogenase assay was performed according to the instructions of the manufacturer (Roche Biochemicals, Mannheim, Germany).

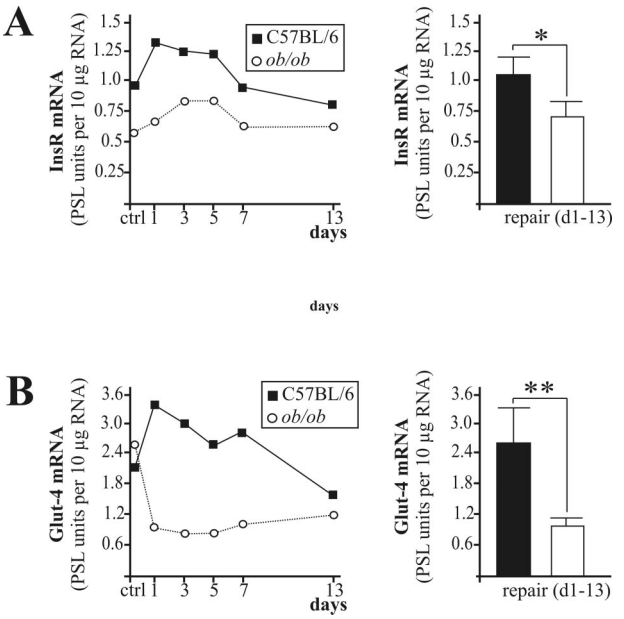



**Figure 1.** Expression of insulin signaling molecules in skin repair. Regulation of InsR (**A**) and Glut-4 (**B**) mRNA expression in nonwounded back skin (*ctrl*) and wound tissue isolated from wild-type (*C57BL/6*) and *ob/ob* mice. The time after injury is indicated. Each single experimental time point represents 16 wounds ($n = 16$) isolated from four individual mice of two experimental series. Mean expression levels during repair are shown in the **right panels**. **, $P < 0.01$; *, $P < 0.05$. Bars indicate the mean ± SD over the healing period (days 1 to 13) from 80 wounds from 20 animals.

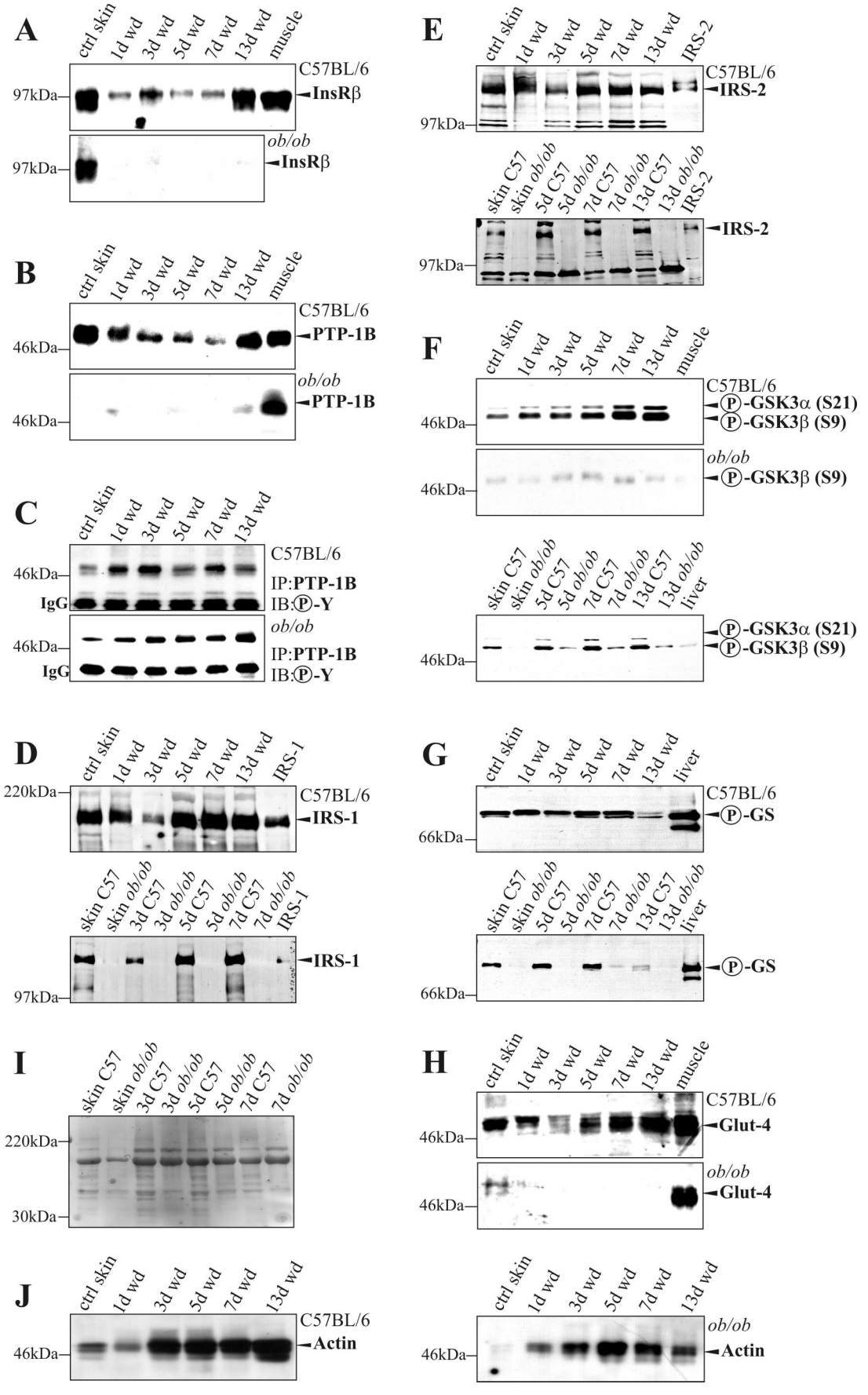
### *Statistical Analysis*

Data are shown as means ± SD. Data analysis was performed using the unpaired Student's *t*-test with raw data. Statistical comparison between more than two groups was performed by analysis of variance (Dunnett's method).

## *Results*

### *Insulin Signaling Molecules in Skin and Wound Tissue of Healthy and Diabetic Mice*

In this study, we investigated the availability of insulin signaling pathways in skin of normal and diabetes-impaired wound healing. The *ob/ob* mouse is characterized by a severe type 2 diabetes mellitus[12] and suffers from disturbed wound-healing conditions.[14,15,31] We found that expression of InsR (Figure 1A) and Glut-4 (Figure 1B) mRNA was significantly reduced in impaired wounds of diabetic *ob/ob* mice when compared with acutely healing wounds of C57BL/6 mice. By contrast, mRNA expression levels did not markedly change during healing in both groups for PTP-1B, IRS-1, and IRS-2 (data not shown). However, protein expression revealed marked differences in the availability and activation of insulin-sensitive components in skin tissue of control and diseased mice. In clear contrast to healthy mice, the constitutively expressed InsR completely diminished on injury in diabetes-impaired wound tissue (Figure 2A). Notably, InsR and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Ⓟ-GSK3β (S9)
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liver
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66kDa
Ⓟ-GS
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Ⓟ-GS
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Actin

PTP-1B expression during normal healing paralleled a reduced amount of protein during the acute repair process (Figure 2, A and B, top panels, 1 day wound to 7 days wound). However, PTP-1B was barely detectable in *ob/ob* mice (Figure 2B, bottom panel). PTP-1B represents a central negative regulator of insulin action[32] that is inactivated by tyrosine phosphorylation.[33] Interestingly, we observed an increase of tyrosine phosphorylated and thus inactive PTP-1B (Figure 2C, top panel, 1 day wd to 7 days wd) that paralleled InsR down-regulation (Figure 2A) during acute healing in control mice. Nevertheless, the small amounts of PTP-1B in wounds of *ob/ob* mice were most likely inactive, because tyrosine phosphorylation of PTP-1B increased during repair (Figure 2C, bottom panel). We also found the expression of InsR adaptor molecules IRS-1 (Figure 2D) and IRS-2 (Figure 2E) to be markedly reduced in skin and wounds of diabetic *ob/ob* mice. Finally, we investigated possible consequences of altered InsR, PTP-1B, or IRS-1 and -2 availability during diabetes-impaired repair. To this end, we determined activation of the key down-stream molecules GSK3$\alpha$ and -$\beta$ and GS in normal and wounded skin. Insulin causes inactivation of GSK3 as a result of serine phosphorylation of the kinase, finally leading to serine dephosphorylation and functional activation of GS.[34] We observed increasing amounts of phosphorylated GSK3$\alpha$ (Ser21) and GSK3$\beta$ (Ser9) during normal repair, which were nearly completely absent in *ob/ob* mice (Figure 2F). However, although wound GSK3$\alpha/\beta$ activity was most likely blunted by serine phosphorylation, we recognized a marked phosphorylation (Ser641) of GS during the complete healing period in healthy mice, which did not persist in diseased animals (Figure 2G). Moreover, Glut-4 was constitutively expressed during normal repair but was strongly down-regulated in diabetes-impaired wound conditions (Figure 2H).

## *Localization of Insulin-Responsive Cells in Normal Wound Tissue*

Immunohistochemistry revealed the InsR to be expressed in proliferating keratinocytes located at the wound margins and within the granulation tissue in 5-day wound tissue of healthy mice (Figure 3A), indicating that both epidermal and mesenchymal wound cells were potentially sensitive toward insulin. Notably, we could barely detect InsR-specific signals in wound sections isolated from diabetic *ob/ob* mice (Figure 3B), which were characterized by clearly reduced hyperproliferative epithelia located at the margins of the wound.[14]

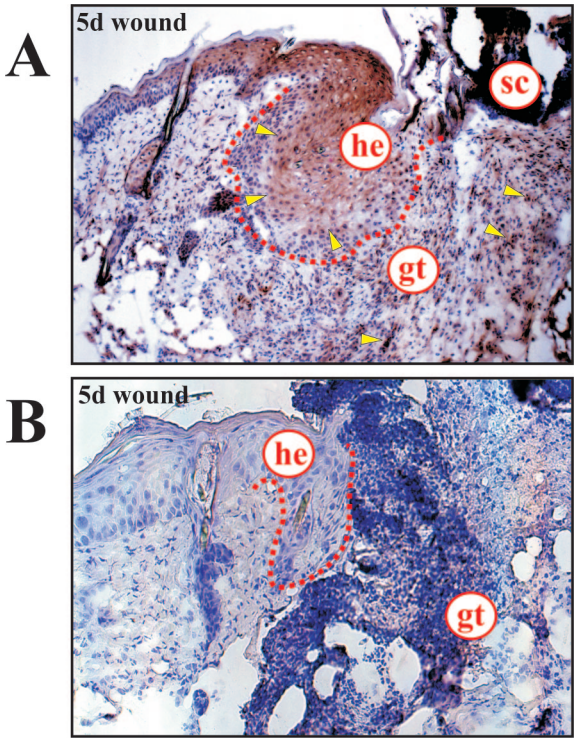


**Figure 3.** Induction and localization of the InsR at the wound site. Immunohistochemical localization of InsR protein in 5-day wounds of healthy C57BL/6 (**A**) and diabetic *ob/ob* mice (**B**). Particularly strong signals for InsR expression are indicated by **arrows**. he, hyperproliferative epithelium; gt, granulation tissue; sc, scab.

## *Systemic Leptin Administration Resolves the Diabetic Phenotype, Improves Skin Repair, and Adjusts Disturbed Wound Insulin Signaling Pathways in* ob/ob *Mice*

It is well established that systemic treatment of leptin-deficient *ob/ob* mice with recombinant leptin results in resolution of both the diabetic and impaired healing phenotype.[14,16] Thus, *ob/ob* mice were injected intraperitoneally with recombinant leptin (2 $\mu$g/g body weight, once a day) for 13 days. As shown in Figure 4A, we found high serum leptin levels 3 hours after injection. The diabetic phenotype of leptin-injected *ob/ob* mice was resolved, because hyperinsulinemia and blood glucose were rapidly adjusted to normal. Moreover, after 13 days of leptin treatment, mice revealed a significant loss of body weight (Figure 4A) and an improved healing, as assessed by the loss of scabs after wound re-epithelialization and reduction of wound areas (Figure 4, B and C). Histological analysis of 13-day wound tissue convincingly demonstrated the potency of systemically administered leptin to

**Figure 2.** Key proteins of the insulin signaling cascade during normal and diabetes-impaired wound (wd) healing. Immunoblots showing InsR (**A**), PTP-1B (**B**), tyrosine-phosphorylated (*Y-P*) PTP-1B (**C**), IRS-1 (**D**), IRS-2 (**E**), phosphorylated (S21, S9) GSK3$\alpha/\beta$ (**F**), phosphorylated (S641) GS (**G**), and Glut-4 (**H**) in nonwounded (ctrl skin) and wounded skin in *C57BL/6* and *ob/ob* mice as indicated. A control for equal loading (Ponceau S staining) is shown in **I**. **J:** Integrity of protein lysates is again controlled by immunodetection of actin. The time after injury is indicated. Each time point depicts eight wounds ($n = 8$) from four individual mice ($n = 4$). Liver and muscle tissue was used as control tissue to prove specificity of antibodies.

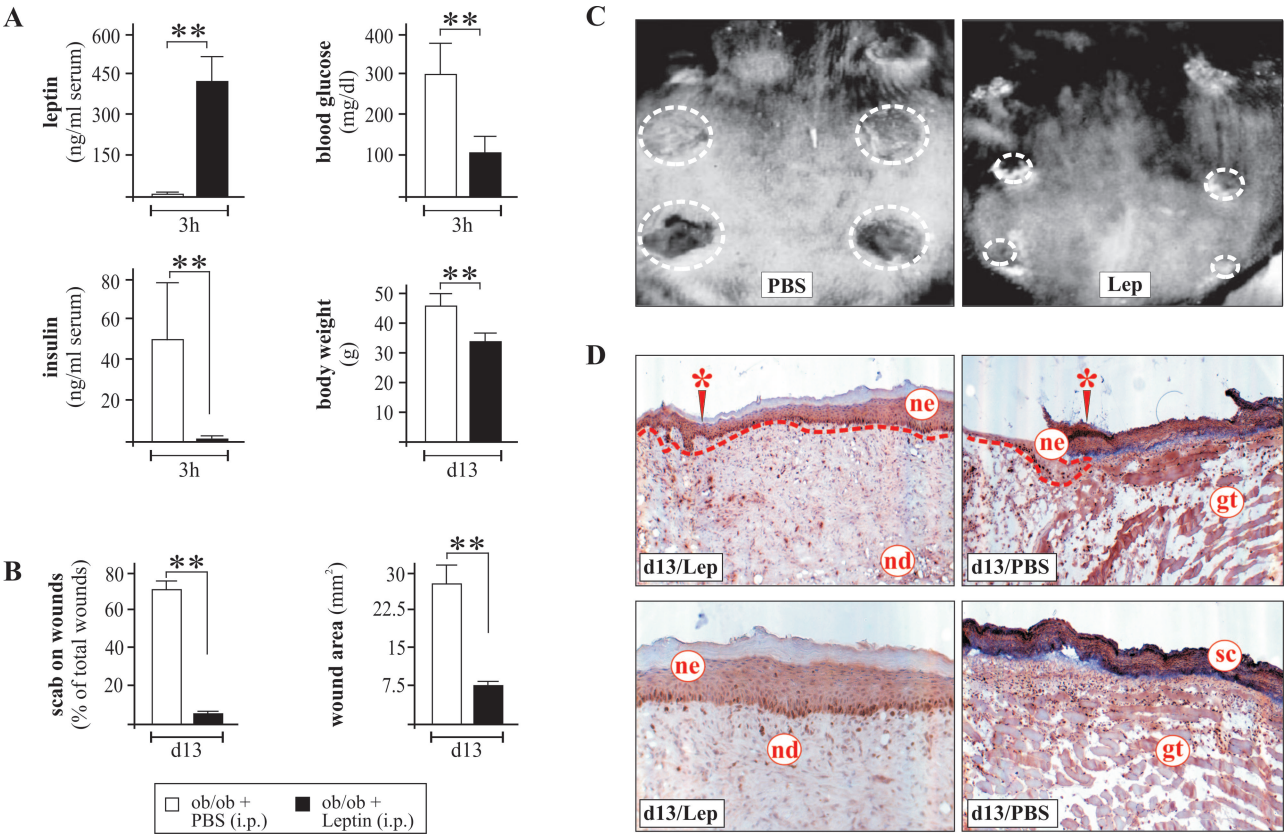


**Figure 4.** Administered leptin is biologically active. **A:** Blood leptin, insulin, and glucose levels 3 hours after systemic application of recombinant leptin. The *ob/ob* mice were treated with leptin for 13 days, after which the body weight of the animals was monitored. **B:** Presence of scab-covered wounds and wound area after 13-day treatment with PBS or leptin. **, $P < 0.01$ as compared with PBS-treated animals. Bars indicate the means ± SD from nine individual animals ($n = 9$). **C:** Photographs of 13-day wounds in PBS- or leptin-treated *ob/ob* mice. **D:** Representative histological analysis of a 13-day wound tissue of a leptin-treated (**left panels**) or PBS-treated (**right panels**) *ob/ob* mouse. **Top panels** show the wound margin area (site of initial injury is marked by an **asterisk**); **bottom panels** show the middle of the wound. gt, granulation tissue; nd, neo-dermis; ne, neo-epidermis; sc, scab.

improve wound re-epithelialization in *ob/ob* mice (Figure 4D). Late wound areas from leptin-treated mice were characterized by a robust formation of a multilayered and organized neo-epidermis and neo-dermis, which completely covered the site of injury (Figure 4D, left panels). By contrast, wounds of PBS-treated mice revealed only small and reduced neo-epithelia at the wound margins and completely failed to cover the site of injury with a well-developed granulation tissue and neo-epithelium. At this stage of impaired healing, wound coverage was represented not by cells but by a robust scab (Figure 4D, right panels).

Next, we investigated the presence and activation of key insulin signaling molecules in late chronic and improved wounds (day 13 after wounding) isolated from PBS- or leptin-treated *ob/ob* mice. We could not detect significant changes in total InsR, PTP-1B, IRS-1, and Glut-4 mRNA expression in improved and diabetes-impaired wounds, but IRS-2 mRNA levels were significantly increased in impaired wound tissue (data not shown). However, chronic diabetes-impaired wounds in PBS-injected *ob/ob* mice were characterized by low protein expression levels of InsR and its negative regulator PTP-1B[32] (Figure 5A). Interestingly, PTP-1B was highly phosphorylated in chronic wounds from PBS-treated mice and thus most likely is in an inactive state[33] (Figure 5A, right panels). This observation was functionally supported by a strong phosphorylation of the InsR, which was only detectable in the presence of phosphorylated PTP-1B in impaired wound tissue of PBS-treated mice (Figure 5A, left panel). Moreover, the InsR adaptor molecules IRS-1 and -2 were expressed in wounds after leptin administration but were absent in chronic healing conditions (Figure 5B). Additionally, we found GSK3$\beta$ (Ser9) (Figure 5C, left panel) and the GS (Figure 5C, right panel) to be phosphorylated after leptin treatment. Finally, Glut-4 protein was expressed in both normal and impaired repair; however, the protein isolated from leptin-treated improved wounds appeared to be slightly different in terms of its migratory behavior in SDS gels (Figure 5D). It is noteworthy that the effects of leptin on the insulin signaling cascade were restricted to its systemic properties. We tested the potency of topically applied leptin to amend the disturbed insulin cascade in wounds of *ob/ob* mice. In the absence of systemic short-term (blood insulin and glucose levels) and long-term (body weight) effects of topically applied leptin (Figure 6A), we could not detect any re-increase in InsR expression in wounds of topically treated animals (Figure 6B).

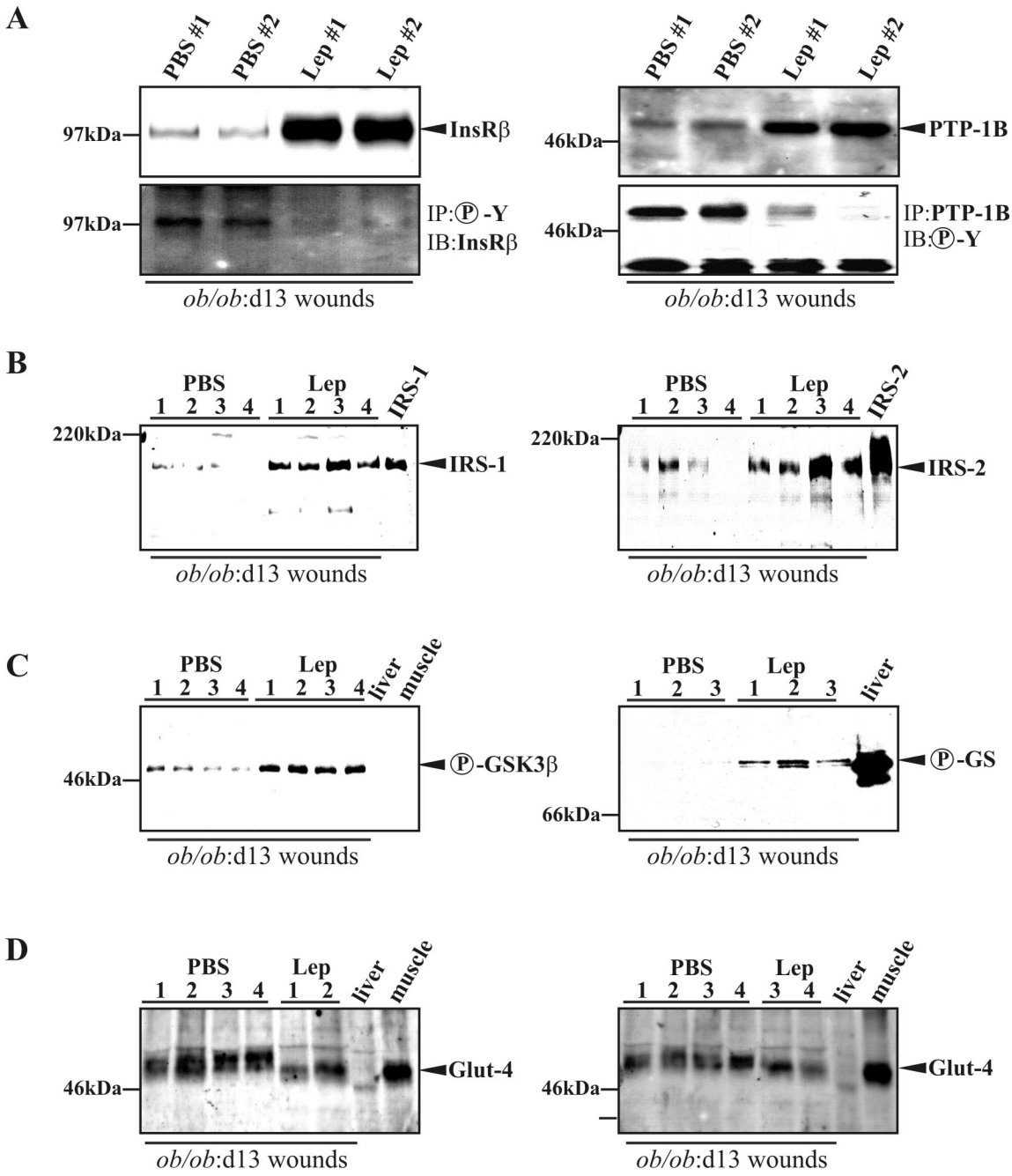


**Figure 5.** Disturbed key components of insulin signaling are adjusted by systemic leptin administration in wounds of diabetic *ob/ob* mice. Immunoblots showing InsR, phospho-InsR, PTP-1B, and phospho-PTP-1B (**A**); IRS-1 and IRS-2 (**B**); phospho-GSK3$\alpha/\beta$ and phospho-GS (**C**); and Glut-4 (**D**) in 13-day wounds of PBS- and leptin-treated *ob/ob* mice as indicated. Numbers indicate individual mice. Each time point depicts eight wounds ($n = 8$) from four individual mice ($n = 4$). Liver and muscle tissue and lysates from IRS-1- and IRS-2-overexpressing cells were used as controls to demonstrate specificity of antibodies.

### *Leptin Improves Sensitivity of the Insulin Signaling Cascade in Nonwounded Skin of Diabetic* ob/ob *Mice*

As a next step, we determined expression and activation of key molecules of the insulin pathway in nonwounded skin of PBS- and leptin-treated *ob/ob* mice. We did so to clarify whether the above-mentioned alterations in insulin sensitivity were restricted to a disturbed healing or a common disturbance in skin tissue of these diabetic mice. Protein expression of the InsR (Figure 7A), IRS-1 (Figure 7B), PTP-1B (Figure 7C), and Glut-4 (Figure 7F) and phosphorylation of GSK3$\alpha$ (Figure 7D) and the GS (Figure 7E) were increased in nonwounded skin tissue on leptin treatment. These data clearly indicate disturbed insulin sensitivity in skin tissue before wounding, which is most likely transferred into the impaired healing process.

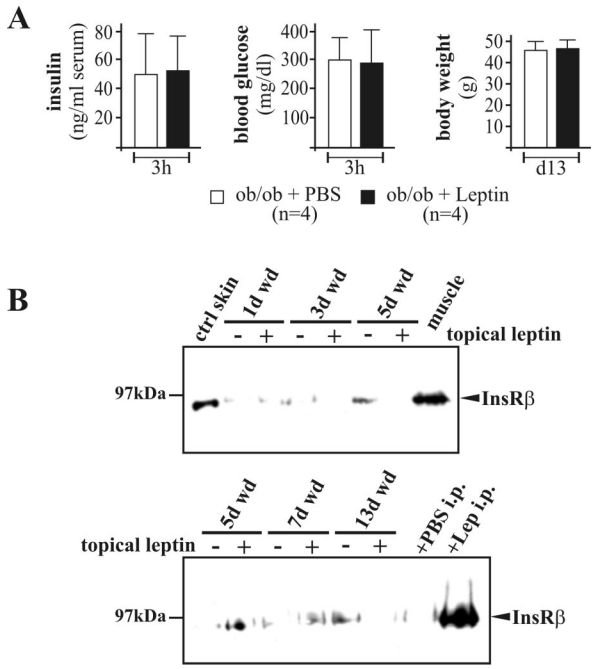


**Figure 6.** Topical treatment of wounds (wd) with leptin did not improve InsR expression. **A:** Blood insulin and glucose levels 3 hours after topical application of recombinant leptin (1 $\mu$g/wound). After 13 days of leptin treatment, the body weight of the animals was monitored. Bars indicate the means ± SD from four individual animals ($n = 4$). **B:** Immunoblots showing InsR expression in nonwounded skin (*ctrl skin*) and wounds of topically treated *ob/ob* mice as indicated. Each time point depicts eight wounds ($n = 8$) from four individual mice ($n = 4$). Thirteen-day wound tissue from leptin-injected mice and muscle tissue served as positive controls.

## *Chronic Insulin and TNF-α Exposure Might Contribute to Impaired Insulin Sensitivity in Keratinocytes*

Finally, we aimed to identify possible mediators that might be responsible for the systemic effects of leptin on insulin sensitivity in diabetic wound tissue. Because *ob/ob* mice suffer from hyperinsulinemia (Refs. [12] and [16]; this study), we hypothesized that chronic exposure of resident skin cells to insulin might contribute to insulin insensitivity. Pretreatment (24 hours) of human HaCaT keratinocytes with low amounts of insulin (50 ng/ml) completely abolished the observed dose-dependent glucose uptake into the cells after acute insulin stimulation (0.1 and 1.0 $\mu$g/ml) (Figure 8A).

Additionally, chronic release of TNF-$\alpha$ from adipose tissue has been shown to functionally contribute to systemic insulin resistance in obese rodents.[19] Although an acute stimulation of HaCaT keratinocytes by TNF-$\alpha$ strongly increased glucose uptake into the cells, we observed a complete inhibition of insulin-mediated glucose uptake into keratinocytes after chronic TNF-$\alpha$ pretreatment (25 ng/ml, 72 hours) of the cells (Figure 8B). Here, it is important to note that chronic exposure of keratinocytes to TNF-$\alpha$ did not reduce the viability of cells (Figure 8, C and D). Moreover, exposure to TNF-$\alpha$ inhibited insulin-stimulated serine phosphorylation of the GSK3$\beta$ isoform in the cells (Figure 8E). Interestingly, HaCaT keratinocytes expressed both p55 and p75 TNF-$\alpha$ receptors

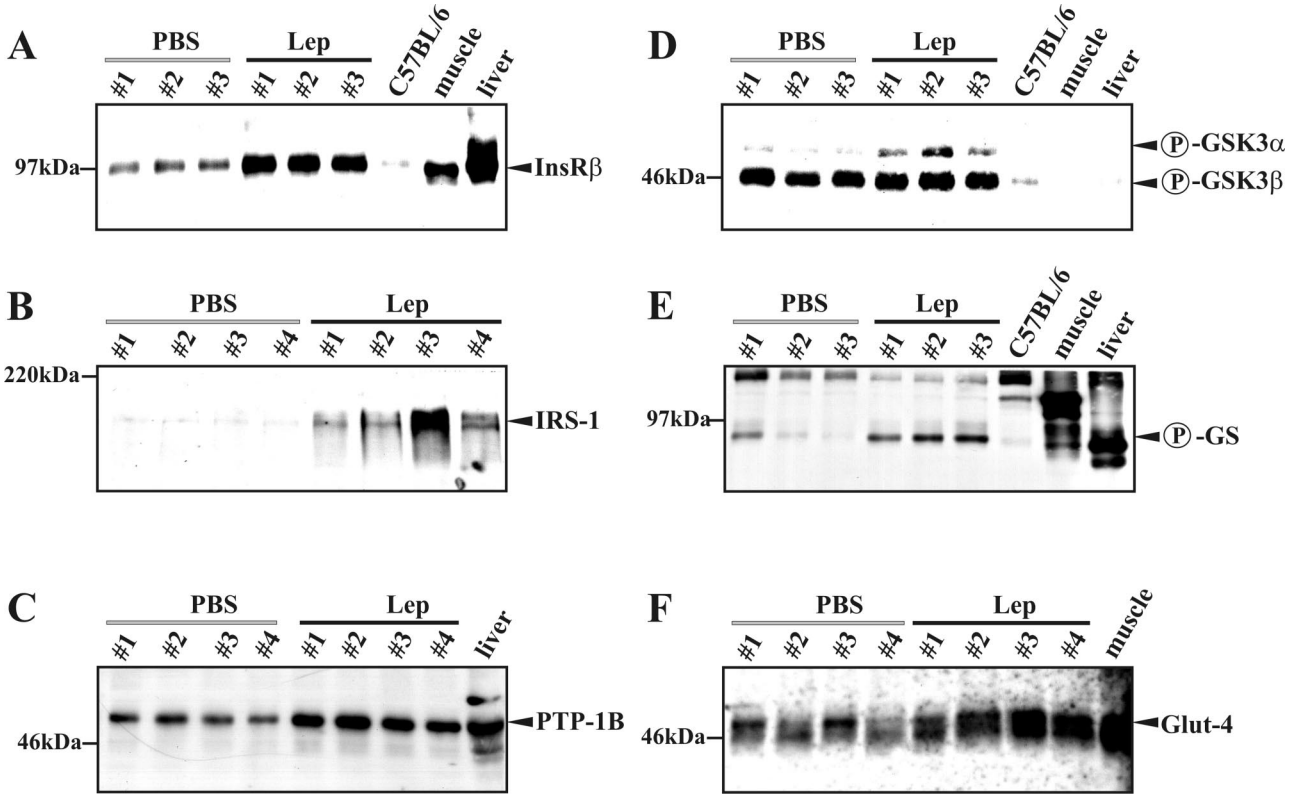


**Figure 7.** Leptin adjusts key molecules of insulin signaling in nonwounded diabetic skin tissue. *ob/ob* mice were systemically treated with PBS or leptin (Lep) for 7 days. Immunoblots showing InsR (**A**), IRS-1 (**B**), PTP-1B (**C**), phospho-GSK3$\alpha/\beta$ (**D**), phospho-GS (**E**), and Glut-4 (**F**) in nonwounded skin of PBS- and leptin-treated *ob/ob* mice as indicated. Numbers indicate individual mice. Skin of C57BL/6 mice, or liver and muscle tissue were used as controls.

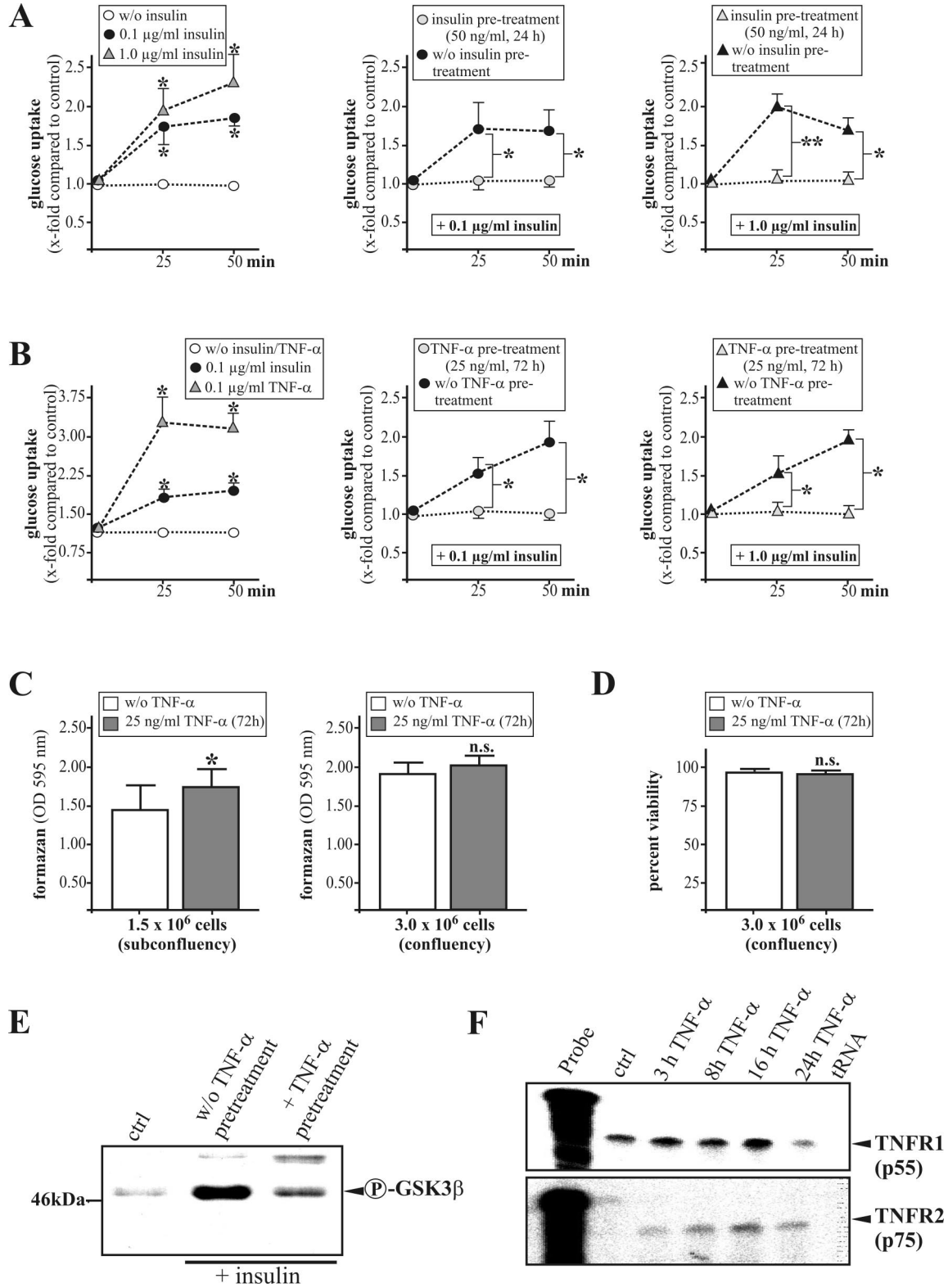


**Figure 8.** The role of chronic insulin and TNF-α exposure for insulin actions in cultured keratinocytes. Serum-starved HaCaT keratinocytes were stimulated for glucose uptake (x-fold compared with control) in the presence or absence of chronic insulin (**A**) or TNF-α (**B**) pretreatment as indicated. **, $P < 0.01$; *, $P < 0.05$ as compared with controls. Bars indicate the means ± SD obtained from four independent cell culture experiments ($n = 4$). Keratinocyte viability as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (**C**) or lactate dehydrogenase (**D**) assay in the presence or absence of a 72-hour TNF-α stimulation as indicated. **E:** Immunoblot demonstrating phospho-GSK3β on insulin stimulation in the presence or absence of TNF-α pretreatment. **F:** RNase protection assay demonstrating mRNA expression of TNF-α receptor 1 (TNFR1, p55) and TNF-α receptor 2 (TNFR2, p75) in starved HaCaT keratinocytes on TNF-α (2 nmol/L) stimulation as indicated. Hybridization against tRNA was used as a negative control.

(TNFRs), although only the p75 receptor was induced by its own ligand (Figure 8F).

### *Systemic Neutralization of TNF-α Attenuated Wound Inflammation and Strongly Improved Wound Morphology in Diabetic* ob/ob *Mice*

Our *in vitro* observations provided strong evidence that TNF-α might also represent an inhibitor of insulin action in skin cells, because *ob/ob* mice were characterized by increased levels of circulating TNF-α.[19] To confirm a possible functional connection between elevated TNF-α levels, wound insulin resistance and diabetes-disturbed wound morphology in *ob/ob* mice *in vivo*, we treated the animals at days 9 and 12 after wounding with a systemic application of the monoclonal anti-TNF-α antibody V1q.[25] Remarkably, the above-mentioned leptin-mediated improvement of insulin signaling was clearly associated with a strong attenuation of TNF-α expression at the wound site (Figure 9A). More importantly, only two systemic applications of the anti-TNF-α antibody V1q into *ob/ob* mice resulted in a significant attenuation of wound inflammation as assessed by the expression of COX-2 mRNA at the wound site (Figure 9B). It is important to note that even a short-term systemic neutralization of TNF-α by V1q in *ob/ob* mice restored the expression of the InsR and phosphorylation of GSK3α at the wound site *in vivo* to levels comparable with leptin treatment (Figure 9C). Moreover, V1q treatment improved diabetes-disturbed wound healing in *ob/ob* mice as shown by scab-free wound tissue (Figure 9, D and E, left panel) and re-epithelialized wounds associated with reduced wound areas (IgG, $26.8 \pm 5.5\ mm^2$, versus V1q, $19.6 \pm 3.9\ mm^2$, $P < 0.001$; $n = 18$ wounds) (Figure 9, D and E). Moreover, wounds from V1q-administered *ob/ob* mice were characterized by the strong formation of granulation tissues covered by organized and robust neo-epithelia (Figure 9F). Here, it is noteworthy that untreated control animals (IgG) completely failed to form an epithelial tongue at this stage of healing (Figure 9F; see also PBS-treated control mice in Figure 4D).

## *Discussion*

Diabetes mellitus currently develops into one of the main threats of human health with an explosive increase in the number of people diagnosed with diabetes worldwide.[35] Visceral obesity is now well established to represent one of the predominant risk factors in the development of an insulin resistance.[35] One major complication of the diabetic disease is the ulceration of the foot.[2,3] Although diabetic ulcerations are of clinical importance, nearly all promising applications of recombinant factors, with the exception of platelet-derived growth factor-BB,[11] failed to be transferred into the human system. Thus, it is reasonable to emphasize that we need a fundamental understanding of the pathophysiology of diabetic wounds to specifically identify future therapeutic strategies.

A novel approach to expand our understanding of pathomechanisms underlying diabetic ulcerations might come from important findings that functionally connect insulin resistance in liver and muscle tissue to elevated serum levels of adipocyte-derived TNF-α in obese rodents. Even more important, systemic neutralization of TNF-α improved peripheral uptake of glucose in response to insulin in these obese animals.[19] Here, it is worthy to note that human adipose tissue has also been shown to constitutively express TNF-α,[20] and that there is strong evidence for a positive correlation between body mass index and circulating TNF-α levels in humans[21] and the development of type 2 diabetes dependent on inflammatory parameters.[22–24] Thus, it was now reasonable to hypothesize that diabetes-impaired wound healing in obese mice might be characterized by the failure of resident cells at the wound site to respond properly to an insulin stimulus. Moreover, we postulated that a potential insulin resistance of injured skin tissue, and thus comparable with systemic insulin insensitivity, might be functionally connected to increased obesity-mediated TNF-α production.[19]

In line with this hypothesis, we first recognized that regulation of key molecules of the insulin signaling cascade were disturbed in nonwounded and injured skin of diabetic mice. Interestingly, marked changes occurred at the protein level on injury, because we found a robust constitutive mRNA expression for central proteins (eg, InsR, PTP-1B, IRS-1, and IRS-2), indicating that expressional regulation most likely occurred from stably transcribed mRNA pools. Additionally, because wound tissue is characterized by a dynamic infiltration of cells, there is evidence that cell influx also contributes to differential changes in total wound mRNA and protein expression. The diabetes-associated expressional down-regulation of InsR, as the primary sensor for insulin at the wound site, appeared especially to represent one major event in decreasing peripheral insulin sensitivity at sites of ongoing wound inflammation in these animals.[15,36] The loss of a functional InsR might counterregulate glucose utilization by resident skin cells to break the vicious circle of energy production by fermentation that acidifies the chronic wound microenvironment under prolonged inflammatory conditions. In good accordance with this hypothesis, we found that chronic exposure of TNF-α was able to inhibit insulin-stimulated glucose uptake into keratinocytes, thus contributing to the above-mentioned effect by a desensitization of cells toward an insulin signal.

It became obvious that impaired wounds in *ob/ob* mice were characterized by the nearly complete absence of InsR expression, which was normally expressed at the wound margins and within the granulation tissue in healthy animals. Interestingly, the residual InsR protein in chronic wound tissue was strongly phosphorylated at tyrosine residues. The overall low abundance of the InsR in diabetes-impaired wounds was in close functional connection to reduced amounts of PTP-1B, representing a negative regulator of InsR activation *in vivo*.[33] Phosphorylation of PTP-1B leads to its enzymatic inactivation.[33] As

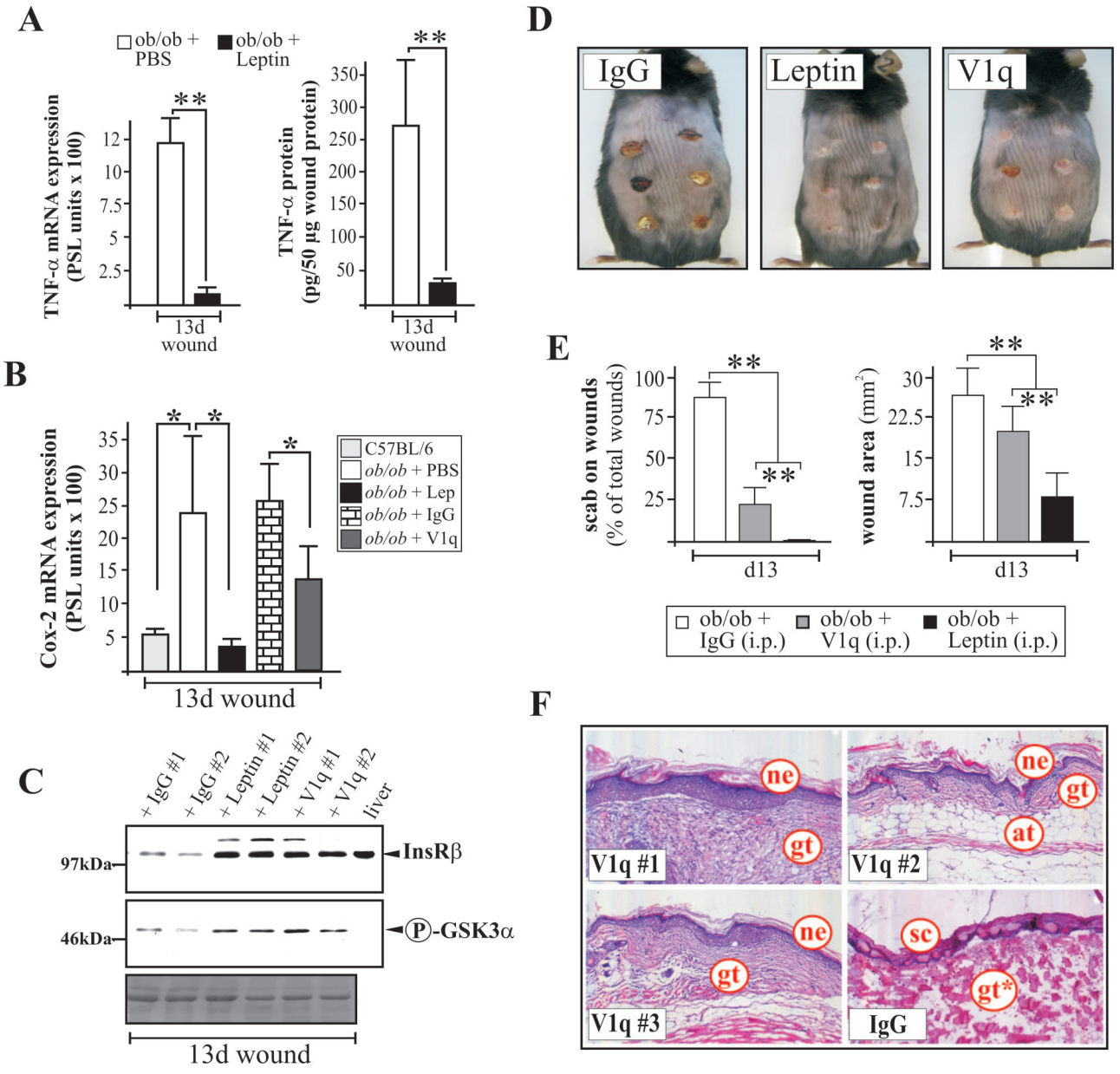


**Figure 9.** Systemic neutralization of TNF-α strongly improves diabetes-impaired wound healing. **A:** Quantification of TNF-α mRNA (RNase protection assay; **left panel**) and protein (ELISA; **right panel**) expression in 13-day wounds from PBS- and leptin (Lep)-treated *ob/ob* mice. **, $P < 0.01$ as indicated by the brackets. Bars indicate the means ± SD obtained from 18 wounds ($n = 18$) isolated from nine animals ($n = 9$). **B:** COX-2 mRNA expression in 13-day wound tissue from *ob/ob* mice that have been treated with PBS, leptin, unspecific control antibody (IgG), or a monoclonal anti-TNF-α antibody (V1q) as indicated. *, $P < 0.05$ as indicated by brackets. Bars indicate the means ± SD obtained from wounds ($n = 16$) isolated from four different animals ($n = 4$). **C:** Immunoblot showing the expression of InsR and phospho-GSK3α in 13-day wounds from IgG-, leptin-, or anti-TNF-α antibody (V1q)-injected *ob/ob* mice as indicated. Ponceau S staining of the blot is shown as a loading control. **D:** Photographs of 13-day wounds in IgG-, leptin-, and anti-TNF-α (V1q)-treated *ob/ob* mice. **E:** Presence of scab-covered wounds and wound area of 13-day wounds in IgG-, leptin-, and anti-TNF-α (V1q)-treated *ob/ob* mice. **, $P < 0.01$ as indicated by the brackets. Bars indicate the means ± SD from 18 wounds ($n = 18$) from three individual animals ($n = 3$). **F:** Histological analyses of 13-day wound tissue isolated from three ($n = 3$) individual anti-TNF-α (V1q)- or IgG-treated *ob/ob* mice as indicated. Formalin-fixed, paraffin-embedded 6-µm sections were counterstained using hematoxylin and eosin. at, adipose tissue; gt, granulation tissue; gt*, atrophied gt; ne, neo-epidermis, sc, scab.

a consequence, we found highly phosphorylated InsR molecules in diabetes-impaired wound tissues. This observation might reflect a compensatory mechanism of disturbed wound tissue to allow residual signaling from markedly reduced InsR molecules. However, the remaining InsR appeared to be functionally inactive, because effective signaling from the phosphorylated InsR remained severely disturbed. The consequences of these findings include the presence of resident cells in non-wounded skin and chronic wounds of diabetic mice that are most likely disturbed in terms of sensitivity toward external signals. This observation might explain, at least partially, that topical application of growth factors into chronic wound tissue has failed to improve wound healing to date. Obviously, resident cells could not respond properly to insulin because their signaling machinery is severely disturbed, an effect that might also hold true for other factors. Keratinocyte behavior is especially influ-

enced by the presence of an undisturbed insulin signaling. Lack of InsR in keratinocytes led to an abrogation of glucose uptake and an impairment of differentiation.[17,18] Additionally, these findings clearly impact nonwounded diabetic skin that was characterized by a markedly reduced expression of InsR.

Here, we could show that systemic leptin administration to *ob/ob* mice resolved the diabetic phenotype, attenuated wound inflammation and TNF-$\alpha$ expression (Goren et al[15]; this study), and improved skin repair and insulin sensitivity. Notably, topical administration of small amounts of leptin (1 $\mu$g/wound) failed to influence systemic parameters such as serum insulin (this study) and glucose (Bing et al[31]; this study) as well as corticosterone levels.[31] Because we could not observe restored InsR expression after topical treatment, it is tempting to argue that leptin-mediated improvement of insulin sensitivity and healing of wounds pivotally involves systemic mechanisms.

Nevertheless, the central question still remained whether obesity and insulin resistance of resident wound cells might be functionally connected by TNF-$\alpha$. To finally determine a functional role for TNF-$\alpha$ in this process, we have treated animals with the monoclonal antibody V1q, which has been shown to efficiently neutralize TNF-$\alpha$ in mice *in vivo*.[25] Treatment was initiated from day 7 after wounding, because we had to avoid interference with physiologically elevated levels of TNF-$\alpha$ during acute wound inflammation.[15] It is important to note here that anti-TNF-$\alpha$ treatment strongly improved the disturbed wound-healing conditions in *ob/ob* mice. Wounds of anti-TNF-$\alpha$-treated *ob/ob* mice revealed an attenuated wound inflammation associated with improved wound contraction and granulation tissue formation as well as a complete re-epithelialization. Moreover, this process was paralleled by the strong re-appearance of InsR expression in wounds of *ob/ob* mice. Thus, our findings strongly suggest that systemic TNF-$\alpha$ levels were functionally connected to impaired healing conditions under diabetic and obese conditions in *ob/ob* mice. Additionally, our data provide strong evidence that anti-inflammatory pharmacological strategies to interfere with potentially elevated levels of inflammatory mediators such as TNF-$\alpha$ might restore insulin sensitivity in diabetes-impaired wounds and thus might represent a novel therapeutic approach to improve healing.

In summary, we have shown that key components of the insulin signaling machinery were dysregulated in nonwounded and injured skin of diabetic *ob/ob* mice. Systemic administration of leptin normalized these impaired conditions. However, leptin action on insulin signaling was indirect and partially related to an attenuation of chronic inflammatory conditions. Moreover, our data provide evidence that functional signaling from InsR participates in skin repair. InsR signaling in diabetes-impaired wounds appeared to be sensitive to inflammatory conditions, suggesting that anti-inflammatory approaches, such as anti-TNF-$\alpha$ strategies, could improve diabetic wound healing.

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