Insulin is degraded extracellularly in wounds by insulin-degrading enzyme (EC 3.4.24.56)

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Insulin is degraded extracellularly in wounds by insulin-degrading enzyme (EC 3.4.24.56). Am. J. Physiol. 273 (Endocrinol. Metab. 36): E657–E664, 1997.—The exact mechanism by which insulin reverses impaired wound healing is unknown. Previous investigators have shown that insulin is degraded in experimental wounds, suggesting that the action of insulin may be locally modified. The following study corroborates these findings and identifies the major proteinase responsible for insulin degradation in wound fluid (WF). Adult male Fisher rats were wounded by subcutaneous implantation of polyvinyl alcohol sponges while under pentobarbital sodium anesthesia. WF and serum were collected on 1, 5, 10, and 14 days postinjury. Decreased insulin concentrations in late WF correlated with an increased insulin-degrading activity. Multiple proteinases appear to participate in the overall degradation of insulin in WF. However, the primary enzyme responsible for insulin degradation in WF was characterized by immunoprecipitation and immunoblotting and identified as the neutral thiol-dependent metalloprotease, insulin-degrading enzyme (EC 3.4.24.56). Exogenous steroid administration caused a decrease in WF insulin-degrading activity. Glucagon and adrenocorticotrophin degradation was also observed, whereas minimal degradation of insulin-like growth factors I and II and epidermal growth factor was detected in WF. The ability to extracellularly degrade insulin may represent a unique mechanism for the regulation of this hormone’s role in healing wounds.

Scar-breaking strength was significantly decreased by exogenous steroid administration (27) and increased collagenase and protease activity (15). Systemic insulin treatment is only effective if started before injury or during the first week postinjury (4). The importance of insulin degradation in wounds must also be considered. In this regard, Hunt (16) has shown that insulin concentrations are lower in wound fluid (WF) than in serum and that insulin is degraded when injected into wounds. The present study corroborates these findings and demonstrates extracellular degradation of insulin in the wound environment. Although multiple enzymes may participate in the extracellular degradation of insulin in the wound, the primary proteinase responsible for insulin degradation in the wound is characterized and identified as insulin-degrading enzyme (IDE, EC 3.4.24.56). Exogenous steroid administration markedly reduced insulin-degrading activity in WF. Possible roles for insulin degradation in wound healing include local modulation of insulin bioactivity and of insulin-growth factor interactions.

METHODS

Polyvinyl alcohol sponge wound model. Male Fisher rats (170–200 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). The lower back was shaved and the skin cleansed with a 70% solution of isopropanol. Polyvinyl alcohol sponges were inserted under sterile conditions. A longitudinal incision was made along the dorsal midline, extending 7 cm cephalad from the area just above the tail. Blunt dissection was used to create pockets in the connective tissue beneath the skin, and five sponges were implanted on each side of the midline. The incision was closed with autoclips. At designated times after implantation of the sponges, animals were killed by CO2 asphyxiation and the connective tissue capsule that surrounded them was harvested. Autoclips were removed from the wound, and sharp dissection was used to free the sponges from the connective tissue capsule that surrounded them.

Steroid-impaired wound healing. Corticosterone (200 mg) pellets were implanted subnuchally into male Sprague-Dawley rats (300–325 g), as described by Meyer et al. (20), under pentobarbital sodium anesthesia. Control rats for this treatment received implantation of a cholesterol pellet in the same manner. Four animals were used in each treatment group. Two days later, the rats were wounded as described above. After 14 days, animals were killed by CO2 asphyxiation and the sponges were harvested. The breaking strength of the scar produced by sponge implantation was determined using a tensiometer (Instron Model 1011, Canton, MA). Scar-breaking strength was significantly decreased by exogenous steroid administration compared with controls (420 ± 25 vs. 981 ± 109 g, respectively; P < 0.05).
Collection of WF. WF was obtained by placing sponges in a 10-ml syringe body that was suspended in a 50-ml sterile conical tube in ice. Sponges were centrifuged for 10 min at 500 g at 4°C. Cells released from the sponges formed a pellet at the bottom of the tube. The cell-free supernatants were stored at −80°C. Care was taken to ensure that sponges and WF were kept cold throughout the collection process.

Measurement of insulin. A rat insulin radioimmunoassay kit (Linco, St. Louis, MO) was used to measure insulin in WF and serum samples. Measurements were made on sample duplicates according to the standard kit protocol, except all steps were conducted at 4°C. The intra-assay coefficient of variation was <10%.

Degradation assay. Insulin degradation was determined using a trichloroacetic acid (TCA) precipitation assay as described by Saric et al. (24). In addition, glucagon, adrenocorticotropic hormone (ACTH), insulin-like growth factor (IGF)-I, IGF-II, and EGF degradation were determined. Assays were conducted by incubating 20 fmol of 125I-labeled peptide in 50 µl of 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.5) containing 1.5 mM MgCl2, 5 mM KCl, 0.5% bovine serum albumin, and 45 µg of WF protein for 30 min at 25°C. The reaction was stopped by the addition of 50 µl of ice-cold 15% TCA. The percentage of peptide degradation was calculated by subtracting the mean percentage of TCA-soluble radioactivity in three blanks, which contained no WF, from the mean percentage of TCA-soluble radioactivity in samples of WF from different rats. The number of rats used for each experiment varied, as listed in the legends to Figs. 1–7.

A profile of the sensitivity of insulin-degrading activity in WF to specific protease inhibitors was performed to identify the class of protease responsible for insulin degradation in WF. Inhibitors were added to the reaction mixture of the TCA precipitation assay in the following concentrations: 1 mg/ml bacitracin, 1 mM N-ethylmaleimide (NEM), 100 µg/ml p-chloromercuriphenylsulfonic acid (pCMPS), 10 mM EDTA, 5 mM 1,10-phenanthroline, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mg/ml leupeptin, and 20 mg/ml pepstatin A. These chemicals were purchased from Sigma Chemical, St. Louis, MO.

Size-exclusion chromatography. To determine the approximate size of the proteinases responsible for degradation of insulin, glucagon, and ACTH, day 10 WF was fractionated by size-exclusion chromatography. A Bio-Sil SEC 400–5 high-performance liquid chromatography column (Bio-Rad, Hercules, CA) with a molecular weight range of 20,000 to 1,000,000 was used. A 100-µl sample of WF was injected onto the column and eluted with phosphate-buffered saline (PBS, pH 7) at a flow rate of 0.5 ml/min. After 5 min, fractions were collected in 1-min intervals for 20 min. Degrading activity of each fraction was determined using the TCA-precipitation assay.

Immunoprecipitation of insulin-degrading activity. To determine if the enzyme responsible for insulin degradation in WF was the previously identified protease, IDE (EC 3.4.24.56), immunoprecipitation was performed using an anti-IDE monoclonal antibody (9B12) that binds to rat IDE (9). For the experiment, various amounts of 9B12 anti-body were added to 5 µl of day 15 WF. The total volume was raised to 25 µl by the addition of PBS. Mouse immunoglobulin G (IgG) controls were set up in the same manner. After overnight incubation at 4°C on a rotating mixer, immobilized protein G (Pierce, Rockford, IL) was added to each tube. The tubes were incubated for an additional 2 h at room temperature before centrifugation. The supernatant was removed and assayed for insulin-degrading activity.

RESULTS

Temperature dependence of WF insulin recovery. The accuracy of the measurement of insulin concentration in WF was determined by measuring the recovery of insulin added to WF. Rat insulin (5 ng/ml) was added to day 10 WF or serum, and the samples were incubated for 15 min at 4 or 25°C. The measurement of serum insulin concentrations was unaffected by incubation temperature, and 100% of the added insulin was recovered. Recovery of insulin from WF was also 100% when the assay was conducted at 4°C but was reduced to ~20% in assays performed at 25°C. On the basis of this experiment, subsequent insulin assays were performed at 4°C. These results suggested the presence of a potent insulin-degrading protease in WF.

Insulin concentrations and insulin-degrading activity in day 1, 5, 10, and 14 WF and serum. The insulin concentrations of serum and WF were determined in samples collected at various times after injury (Fig. 1A). There were no changes in serum insulin concentrations over time. However, WF insulin concentrations decreased over time and were lower than serum concentrations by day 10 and 14.

Insulin degradation was measured by TCA precipitation in serum and WF collected at various times after injury (Fig. 1B). Insulin-degrading activity increased in the late WF. Insulin degradation was not detectable in serum (data not shown).

The possibility that the increased insulin degradation observed in WF was an inadvertent consequence of...
cell damage during WF collection was also investigated. For this determination, WF was collected from 14-day sponges in situ by making a small incision through the fibrous capsule surrounding each sponge and withdrawing 25 µl/sponge with a pipettor. The WF was centrifuged for 5 min at 500 g at 4°C. The sponges were then removed from the animal, and the WF remaining in the sponges was processed as described in METHODS. WF was collected from sponges in this manner from three rats. Analysis of insulin-degrading activity in WFs collected by these two techniques showed no difference.

Characterization of insulin-degrading activity in WF. Maximal insulin-degrading activity in WF occurred at neutral pH (Fig. 2A). The apparent Michaelis-Menten constant (K_m) for insulin degradation in WF was estimated at 229 nM (Fig. 2B). Addition of excess nonradio-labeled insulin masked the detection of insulin degradation by specifically competing with radiolabeled insulin as a substrate in this assay (Fig. 2C). Insulin degradation measured in the presence of various protease inhibitors, bacitracin, the sulfhydryl-blocking reagents, NEM and pCMPS, and metal chelators, EDTA and 1,10-phenanthroline, completely inhibited insulin degradation in WF. PMSF partially inhibited insulin degradation, whereas leupeptin and pepstatin A produced only slight inhibition (Fig. 2C). A major portion of insulin degradation in WF appears to be dependent on a neutral thiol-dependent metalloproteinase. However,
the inhibitory profile suggests that other proteinases also participate in insulin degradation in WF.

Identification of IDE as insulin-degrading enzyme in WF. Varied concentrations of 9B12 antibody were tested to assess the possible role of IDE in insulin degradation in WF. Each concentration of 9B12 antibody tested immunoprecipitated ~90% of the insulin-degrading activity in the WF. In contrast, nonimmune mouse IgG control did not significantly reduce this activity (Fig. 3A). Likewise, degradation of glucagon was reduced by ~62% in WF immunoprecipitated with 1 µg of the 9B12 antibody (Fig. 3, inset). These results strongly suggest that a significant portion of the insulin-degrading activity in WF can be accounted for by IDE.

The immunoblot for IDE using 9B12 antibody (Fig. 3B) showed a single band of specific immunoreactivity in day 15 WF immunoprecipitated with 9B12. The size of the band corresponds to the known molecular mass of IDE (110 kDa).

Degradation of peptide hormones and growth factors in WF. The degradation of other peptide hormones and growth factors of similar size and composition to insulin was measured to evaluate the specificity of WF for insulin degradation (Fig. 4). In addition to insulin, substantial degradation of glucagon was detected; ACTH also was degraded but to a lesser extent. In contrast, degradation of the peptide growth factors IGF-I, IGF-II, and EGF by WF was minimal.

Size-exclusion chromatography of hormone-degrading activity in WF. The approximate molecular weight of hormone-degrading activity in WF was determined by size-exclusion chromatography under nondenaturing conditions (Fig. 5). Insulin-degrading activity was...
detected in fractions 11–14, which correspond to a molecular mass of 150–300 kDa. Glucagon-degrading activity was present in a similar molecular mass fraction; however, ACTH-degrading activity was present in a high molecular mass fraction.

Comparison of insulin, IGF-I, and IGF-II degradation in WF. Because IGF-I and IGF-II have previously been reported to be degraded by IDE (7), a more extensive comparison of insulin, IGF-I, and IGF-II degradation in WF was performed. Insulin was degraded to a much greater extent than either IGF-I or IGF-II (Fig. 6A). Similar rates of degradation to those shown in Fig. 6A, using 0.4 nM IGFs, were observed in assays containing up to 200 nM of IGF-I or IGF-II. The ability of the IGFs to interfere with insulin degradation also was determined. Insulin degradation was inhibited by both IGF-I and IGF-II in a dose-dependent manner, with IGF-II being a more potent inhibitor than IGF-I (Fig. 6B).

Effects of exogenous steroid administration on wound insulin-degrading activity. Exogenous steroid adminis-
treatment reduces inflammatory cell infiltration of wounds, decreases angiogenesis, and markedly impairs wound healing (19). A marked decrease in insulin-degrading activity in WF from steroid-treated rats was observed (Fig. 7). Control WF immunoprecipitated with 9B12 antibody had higher insulin-degrading activity than WF from steroid-treated rats, suggesting that both IDE and non-IDE insulin-degrading activity was reduced by steroid treatment. No further reduction of insulin-degrading activity of WF from steroid-treated rats could be detected after immunoprecipitation with 9B12 antibody. This was true even for assays allowed to proceed for up to 2 h to increase sensitivity. Thus steroid treatment appeared to deplete IDE in the wound.

DISCUSSION

Our results demonstrate that insulin is degraded extracellularly in WF at least in part by the activity of the neutral thiol-dependent metalloproteinase, IDE (EC 3.4.24.56). Table 1 compares the characteristics of insulin-degrading activity in WF with purified preparations of IDE (5, 6, 8). WF insulin-degrading activity and IDE have a similar pH optimum, and the $K_m$ of WF insulin-degrading activity is in the range reported for unpurified preparations of IDE. In addition, both are inhibited by metal chelators, sulfhydryl blocking reagents, and bacitracin. A wide range of molecular masses has been reported for IDEs (9). The size-exclusion chromatography method used in this study gives a crude estimation of WF insulin-degrading activity molecular mass that falls within the ranges previously reported (Table 1). The most convincing evidence for the presence of IDE in WF is that specific anti-IDE antibody (9B12) immunoprecipitates a significant portion of WF insulin-degrading activity and detects a band of appropriate size using Western analysis.

However, the results also indicate the participation of other proteinases present in WF in insulin degradation. For example, WF insulin-degrading activity is partially inhibited by serine-aspartic proteinase inhibitors, whereas IDE is not, and not all of the insulin-degrading activity is immunoprecipitated by anti-IDE antibody. The TCA precipitation assay also suggests the participation of multiple proteinases. Detection of degradation by this assay is dependent on the size of the degradation product. Typically, the maximum amount of insulin degradation detected with this assay using purified IDE is 20% (6). The maximal amount of degradation detected using crude WF ranges from 60 to 70%, suggesting that the degradation of insulin in crude WF is considerably more extensive than that observed for purified IDE. The identity of fragments produced by purified insulin has been well characterized (8). Several of these fragments are bioactive but bind to the insulin receptor with reduced potency (8). The non-IDE insulin-degrading activity in WF may further modify the bioactivity of the insulin degradation products. The possibility that these fragments elicit bioactivity in wounds through a mechanism not mediated by the insulin receptor has not been investigated.

Glucagon is known to be degraded by IDE (6, 8), and glucagon degradation occurs in molecular weight fractions similar to insulin and is reduced in WF treated with anti-IDE antibody. Like insulin, glucagon degradation is not completely eliminated by immunoprecipitation of IDE, suggesting the participation of other proteinases. Additionally, glucagon degradation has a different temperature sensitivity than insulin (unpublished observation). Also, the presence of additional

Table 1. Comparison of characteristics of insulin-degrading enzyme to wound fluid insulin-degrading activity

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>IDE</th>
<th>WF IDA</th>
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</thead>
<tbody>
<tr>
<td>pH optimum</td>
<td>7.0–7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>$K_m$, nM</td>
<td>29–130</td>
<td>229</td>
</tr>
<tr>
<td>Chelators (EDTA, 1,10-phenanthroline)</td>
<td>Inhibit</td>
<td>Inhibit</td>
</tr>
<tr>
<td>Serine and aspartic protease inhibitors (PMSF, leupeptin, pepstatin A)</td>
<td>Do not inhibit</td>
<td>Partially inhibit</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>Inhibits</td>
<td>Inhibits</td>
</tr>
<tr>
<td>Molecular mass, kDa</td>
<td>130–300</td>
<td>~150–300</td>
</tr>
<tr>
<td>Gel filtration (nondenaturing)</td>
<td>110</td>
<td>~110</td>
</tr>
<tr>
<td>SDS-PAGE (reducing denaturing)</td>
<td></td>
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IDE, insulin-degrading enzyme; WF IDA, wound fluid insulin-degrading activity; $K_m$, Michaelis constant; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.
specific glucagon-degrading enzymes (5) in WF cannot be excluded. Because ACTH-degrading activity is present in a markedly different molecular weight fraction of WF, a proteinase separate from IDE appears to be responsible for ACTH degradation.

The role of IDE in intracellular processing of insulin has been studied extensively. It appears that the function of IDE is not simply to degrade and inactivate insulin but that it may regulate action of insulin via intracellular peptide-signaling pathways (2, 6). In addition to its role in intracellular processing, a number of other roles have been proposed for IDE. In addition to insulin and glucagon, IDE degrades other cellular proteins, including atrial natriuretic factor, transforming growth factor-α, and oxidatively damaged hemoglobin (6). Other functions involving IDE include processing of insulin by antigen-presenting cells, regulation of growth factor concentrations, propeptide processing, and an involvement in myoblast differentiation (5, 6).

Expression of IDE is developmentally regulated, and its sequence identity is evolutionarily conserved (17, 18). Together these findings suggest general physiological importance for IDE.

The finding of decreased IDE in WF of animals treated with high levels of corticosterone provides a useful tool for identifying the source of IDE in the wound. In addition to impaired wound healing, a primary effect of chronic steroid treatment includes a reduction of the inflammatory infiltrate in the wound, as well as decreased angiogenesis, fibroplasia, and collagen accumulation (19). An accumulation of extracellular lactate dehydrogenase and arginase over time in WF collected from the standard wound model (3) raises the possibility that IDE is derived from cell lysis. Decreased inflammatory cell infiltration of the wound and perhaps decreased cell lysis (as indicated by decreased WF arginase activity (26)) in steroid-impaired wounds support this possibility. Erythrocytes also are enriched with IDE (8, 9), and hemolysis of erythrocytes leaking into the extravascular space from newly formed vessels in the wound may also participate in delivery of IDE to the wound. The angiostatic effects of steroids that reduce vascularization of the polyvinyl alcohol sponge wounds would decrease IDE delivery to the wound by this mechanism. However, the exact mechanism for the deposition of IDE in WF remains to be determined. Experiments using immunohistochemistry and in situ hybridization techniques will be helpful in determining the location(s) and source(s) of IDE in the wound.

The possible preferential degradation of insulin compared with IGF-I and IGF-II in WF may begin to explain the “biological priority” of healing wounds. This concept, described by Moore (21), stated that 75% of wounds or surgical incisions healed to the point of tensile integrity during a period of negative energy and nitrogen balance. In healthy individuals, despite injury-induced abnormalities in circulating hormones known to affect wound healing, most wounds heal after injury (21). The ability of the wound to heal in the face of varying hormone and substrate supply suggests that the wound has a biological priority over the host and further suggests that the metabolic and functional determinants of normal wound repair are inherent within the local wound environment. In support of this hypothesis, we know that the insulin concentration in WF decreases over time (Fig. 1A), whereas IGF-I and IGF-II accumulate in WF (10, 25). A possible explanation for this dichotomy may involve differences in the rate of degradation in the wound environment. Although the TCA precipitation assay underestimates the degradation of IGF-I and IGF-II, a similar difference in the rate of degradation between insulin and the IGF’s has been demonstrated with the use of a receptor binding assay (7). This difference in degradation can be explained by the higher affinity of insulin for IDE compared with IGF-I and IGF-II (1, 7). Furthermore, the concentrations of IGF-I and IGF-II in WF (~100 and 50 nM, respectively) are far below their K_m for IDE and below the concentration needed to produce inhibition of insulin degradation by IDE (Fig. 6B). The potential production of IGF-I and IGF-II by wound cells (22) also favors local modulation of regulatory factors within the wound environment. Despite their similarities, insulin and IGF-I and IGF-II mediate distinct biological effects (22), and the importance of local insulin degradation to wound healing remains to be determined. Studies designed to inhibit IDE activity in vivo will provide direct information regarding the impact of IDE on wound healing.

In summary, insulin is rapidly degraded in WF. The principle enzyme responsible for insulin degradation in WF is a previously identified neutral thiol-dependent metalloprotease, IDE. The ability to extracellularly degrade insulin may represent a unique mechanism for the regulation of this hormone’s action in healing wounds. Several possible roles for regulated degradation of insulin in the wound include 1) differential degradation of insulin and growth factors, allowing local control of wound healing; 2) degradation of insulin, allowing healing to stop by removal of this important growth factor; and 3) production of degradation products of insulin that produce biological effects different from those of intact insulin.

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