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Factors leading to the degradation/loss of insulin in postmortem blood samples

Cora Wunder *, Gerold F. Kauert, Stefan W. Toennes

Institute of Legal Medicine, Forensic Toxicology Department, Kennedyallee 104, 60596 Frankfurt am Main, Hessen, Germany

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ABSTRACT

Since lethal insulin injection has been used in murder and suicide cases, its non-ambiguous detection in postmortem, mostly hemolytic blood samples is still a problem. In the present study the stability of insulin and reasons for its loss in those blood samples were examined. When incubated with buffer, serum or with intact blood cell suspensions insulin concentrations were found to remain stable over time, but a significant loss of insulin was observed in hemolyzed blood samples. This was not due to an enzymatic cleavage, but predominantly to the presence of hemoglobin. Incubation of insulin with a hemoglobin solution containing the same hemoglobin content as hemolyzed blood caused a dramatic decrease of the insulin concentration. Degradation of insulin reached its maximum after 23 h of incubation. The charge state of the ferric ion of hemoglobin could not be held accountable for the insulin-loss, but rather the protein part of hemoglobin. Alkylation experiments using iodoacetamide suggested that the thiol groups of the globin molecule are involved in the insulin loss preventing degradation at least partially. The same was observed by lowering the pH to 2.7 in the incubation mixture. Two degradation products of insulin were identified by mass spectrometry such as modified insulin A and B chains with 4 (A chain) and 2 Da (B chain) lower masses. These results suggest that thiol groups of hemoglobin cause splitting of the disulfide bonds of insulin which immediately leads to the formation of new intramolecular disulfide bridges, a reaction which occurs in hemolytic blood and may explain the gradual loss of insulin in postmortem blood samples.

1. Introduction

Insulin is a peptide hormone consisting of two polypeptide chains (A and B) linked by two disulfide bridges. Another intramolecular disulfide bond is located in the A chain. Insulin is an essential hormone regulating the blood glucose level.

In-vivo, insulin has a half-life of about 5 min and is degraded after receptor-binding and internalization of the insulin–receptor complex. The degradation process is initiated by the insulin degrading enzyme (IDE) [1–3]. Insulin hydrolysis commences in the B chain followed by the reduction of the disulfide bonds by the protein disulfide isomerase (PDI) [4]. The resulting fragments are further cleaved, probably by multiple proteolytic enzymes [5]. Duckworth et al. found that IDE is present in multiple tissues such as liver, kidney and muscles [5] as well as in erythrocytes [6]. Ca^{2+} ions are enhancing the activity of IDE whereas 1,10-phenanthroline, bacitracin and *N*-ethylmaleimide inhibit the enzymes activity [5,7,8].

Forensic investigations have shown that insulin has been used in suicide and murder [9]. Today, detection and quantitative estimation of insulin is still based on immunoassays which have not been validated for postmortem samples which is usually hemolytic blood. Evidence for decreased insulin stability has been obtained by incubation experiments [10] and an enzymatic cleavage of insulin by IDE has been assumed. Steinke et al. [11] hypothesized that hemolytic blood was triggering insulin inactivation in erythroblastosis fetalis disease which leads to hyperplasia of the pancreas followed by increasing insulin production. It has been suggested that insulin is inactivated by SH-containing compounds like glutathione or through reversible binding to hemoglobin [11].

The stability of insulin in blood is obviously depending on storage conditions which is still not sufficiently investigated. According to Lindquist and Rammer [12] and Schuck et al. [13] insulin seems to be stable in frozen postmortem blood or serum during several days. During storage at room temperature Reimers et al. [14] observed a significant loss of 20% of insulin in serum after 2 days and of 72% after 8 days. Hemolysis significantly accelerated the disappearance of insulin.

* Corresponding author. Tel.: +49 69 6301 7561; fax: +49 69 6301 7531.
E-mail addresses: wunder@med.uni-frankfurt.de, cora.wunder@gmx.de (C. Wunder).

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In the present study insulin stability in blood samples was investigated to identify potential factors responsible for its postmortem degradation.

2. Materials and methods

2.1. Chemicals

Insulin (Insuman Rapid®) was purchased from Sanofi Aventis (Frankfurt, Germany). Bovine insulin, human hemoglobin (pure substance), 1,10-phenanthroline, protease inhibitor cocktail (aprotinine, bestatine hydrochloride, E-64-[*N*-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamid], leupeptide hemisulfate, pepstatin A) were obtained from Sigma–Aldrich (Munich, Germany) and 4-dimethylaminophenol (4-DMAP, 250 mg/5 ml) from Franz Köhler Chemie (Alsbach Hähnlein, Germany). All other chemicals and solvents were purchased from Merck (Darmstadt, Germany) and were of analytical or HPLC grade. Human blood samples were obtained from a healthy volunteer.

2.2. Analysis of insulin

The analysis of insulin and of its degradation products was performed using an Agilent (Waldbronn, Germany) 1100 series liquid chromatograph interfaced to an Agilent 1100 series oa-TOF system operated in positive electrospray ionization mode (ESI). ESI source parameters were set according to the recommendations of the supplier for a flow rate of 0.4 ml/min (nebulizer 45 psig, capillary voltage 4000 V, drying gas flow 10 l/min at 350 °C). The fragmentor voltage was set to 225 V according to preliminary optimization studies. Data acquisition was performed in a mass range from m/z 120–3500 Da with simultaneous internal mass calibration in each recorded spectrum (system reference mixture supplied by the Agilent dual-sprayer interface) providing mass accuracies in the range of ±5 ppm. Chromatographic separation was achieved on a 100 × 2.0 mm Polaris C18-Ether column with 3 μm particle size (Varian, Darmstadt, Germany) at 60 °C using a mobile phase gradient consisting of 0.1% formic acid (solvent A) and acetonitrile (solvent B) (0–0.5 min 20% B, 2.0 min 30% B, 9.0 min 32% B, 10 min 36% B, 10.1 min 37% B, 18 min 39% B, 24–28 min 100% B). The injection volume was 10 μl. Identification of analytes, internal standard (bovine insulin) and integration were performed automatically using Agilent's MassHunter quantitative (version B.03.02.) and qualitative (version B.03.01.) software. For evaluation the relative area of insulin vs. the internal standard was used.

2.3. Incubation of insulin and sampling procedure

The incubation medium (see below) was preincubated for 30 min at 37 °C and 1 ml of human insulin (0.5 mg/ml water) was added. At specified time intervals 200 μl samples were transferred into Eppendorf tubes containing 10 μl of bovine insulin (0.1 mg/ml water) as internal standard. Acetonitrile (200 μl) was added dropwise to the samples while vortexing. After centrifugation for 3 min at 16,000 × g, 100 μl of the supernatant were used for analysis. For evaluation, "relative insulin concentrations" were determined as percentage of the insulin area ratio at the beginning of the incubation.

2.4. Study on insulin stability in serum, red blood cell suspension and hemolyzed red blood cells

Fresh human blood was centrifuged for 10 min at 3500 × g to separate serum and blood cells. The blood cell fraction was divided into two parts; one part was washed three times with saline to yield red blood cell suspension. The second part was hemolyzed by adding twice the volume of distilled water and was incubated for 30 min at room temperature. 1 ml of serum, intact and hemolyzed red blood cell suspension was diluted with 3 ml of Tris buffer (0.1 M, pH 7.4) and was incubated for five hours with insulin as described above.

2.5. Studies on insulin degradation

To 1 ml of hemolyzed whole blood 1 ml of 1 mM 1,10-phenanthroline in ethanol or 50 μl of a protease inhibitor cocktail were added and the mixture diluted with Tris buffer (0.1 M, pH 7.4) to a total volume of 4 ml which was incubated for 24 h with insulin as described above.

The hemoglobin content of hemolyzed blood was determined in duplicate (OSM3 Hemoximeter from Radiometer Copenhagen, Denmark) yielding 264 mg/ml. 1 ml of hemolyzed blood or 1 ml of a 264 mg/ml solution of human hemoglobin in water were diluted with Tris buffer (0.1 M, pH 7.4) to a total volume of 4 ml and both were incubated for 5 h with insulin as described above (n = 5 each). Various amounts of hemoglobin (0, 10, 40, 80, 160, 250 mg) were dissolved in Tris buffer (0.1 M, pH 7.4) to a total volume of 4 ml and were incubated for 24 h with insulin as described above.

Globin was separated from the heme entity according to the method of Tayot [15]. Hemoglobin reference substance (50 mg) was dissolved in 1 ml of water and 1 ml of 0.1 N HCl was added dropwise at 4 °C. Acetone (20 ml, containing 200 μl 0.1 N HCl) was added, the solution was heavily shaken and was allowed to stand for 1 h at room temperature during which the heme group remains solved while the globin chains precipitate. The suspension was centrifuged for 10 min at 3500 × g, the supernatant discarded and the residue washed three times with the acidic acetone solution. This resulting globin preparation was lyophilized and from the weight a yield of over 90% was assumed.

For incubation, 20 mg globin preparation was dissolved in 4 ml Tris buffer (0.1 M, pH 7.4) and incubated for 48 h with insulin as described above.

1 ml of hemoglobin solution (40 mg/ml, 0.1 M Tris buffer pH 8.4) was mixed with 1 ml of iodoacetamide solution (0.5 mg/ml in water). The mixture was diluted with Tris buffer to a total volume of 4 ml and preincubated at 37 °C for 3 days according to preliminary optimization experiments. Afterwards, 1 ml insulin was added and incubated for 48 h as described above (n = 3). Hemoglobin was also dissolved in 1 ml Tris buffer (0.1 M, pH 7.4) or 0.1% formic acid (pH 2.7). Each mixture was diluted in their corresponding solvents to a total volume of 4 ml and was incubated with insulin for 72 h as described above (n = 2).

3. Results

To examine whether insulin is stable in postmortem or hemolyzed blood, it was incubated in buffer solution at physiological pH, serum and a red blood cell suspension. Fig. 1 shows that the insulin concentration in these incubation mixtures was constant over the incubation period of 5 h. However, the insulin concentration significantly decreased in hemolyzed blood by 84% of the initial insulin concentration.

Hemolysis leads to the release of hemoglobin and of enzymes. Assuming that insulin loss in hemolyzed blood is due to enzymes like IDE or other proteases, 1,10-phenanthroline and a protease inhibitor cocktail was added to samples of insulin in hemolyzed blood. Fig. 2 demonstrates that the insulin concentration decreased in a similar manner as in hemolyzed blood despite the addition of enzyme inhibitors suggesting that the loss of insulin is a protease-independent process.

To determine whether hemoglobin might be responsible for the insulin loss, insulin was incubated with hemolyzed blood and with a solution of human hemoglobin, both with the same hemoglobin