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No Effect of Aprotinin (Trasylol™) on Degradation of Exogenous and Endogenous Glucagon in Human, Mouse and Rat Plasma

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Abstract

Traditionally, aprotinin (Trasylol™) has been added to plasma samples prior to glucagon analysis. However, the evidence for the need of aprotinin is sparse and based on results obtained when radioimmunoassay (RIA) techniques were still in their infancy. Using RIAs directed against both the C-terminus and a mid-region of glucagon, we challenged the classical view that aprotinin is necessary. Glucagon concentrations in pools of human, mouse and rat plasma (n=30, 25 and 16 of each, respectively) with and without addition of increasing amounts of exogenous glucagon (5, 10, 20, 40 and 60 pM) were similar irrespective of whether or not aprotinin had been added. To investigate whether individual variation occurs in human samples, we measured plasma from 20 patients with gastrointestinal diseases and 20 healthy subjects with or without addition of aprotinin. Again, measured amounts of glucagon, endogenous or added, were not affected by the presence of aprotinin. The effect of aprotinin, present at blood sampling or added later (30 and 60 minutes), on endogenous glucagon values was investigated in T2DM patients (n=5), before and after insulin-induced hypoglycemia. There were no differences between the four treatments. In conclusion, we found no support for use of aprotinin for prevention of glucagon degradation.

Keywords: Trasylol™; Aprotinin; Glucagon assay; Radioimmunoassay (RIA); Peptide degradation

Introduction

Glucagon is the counter-regulatory hormone to insulin regarding their actions on the liver. Glucagon is released from the pancreatic α-cells in response to hypoglycemia and stimulates hepatic glucose output. It is composed of 29 amino acids and is liberated from the precursor molecule, proglucagon, by tissue-specific posttranslational cleavage by prohormone convertase (PC2) in the pancreatic α-cells [1,2]. Neutral endopeptidase 24.11 (NEP) and plasmin activities have been linked to the short half-life (around 5 min) of glucagon in vivo, but evidence points to the kidney as the main site of metabolic clearance [3,4].

With the development of the radioimmunoassay (RIA) technique in 1956 [5], it became possible to measure endogenous glucagon concentrations [6]. At that time, it was speculated that enzymatic degradation of glucagon might occur during storage and handling of the plasma samples, and the addition of the protease inhibitor, aprotinin, to blood samples has since become standard procedure before glucagon measurements [7]. The first glucagon RIAs were developed using the isotope I^{131}, which has a short half-life of 8 days, to label the tracer, and Eisentraut et al. observed that addition of aprotinin was essential for prevention of the damaging effect of human plasma on the glucagon tracer (but was not necessary for the protection of unlabeled, endogenous glucagon [8]). Heding [9] and Nonaka and Foa [10], however, both described a significant loss of endogenous glucagon levels if samples were not treated with aprotinin. Other proteolytic inhibitors (such as benzamidine) have also been investigated and recommended for reducing glucagon degradation in plasma [11]. In contrast to these early assays, tracers used today are prepared using I^{125}, which has a half-life of 60 days, but it has never been systematically examined whether aprotinin is still required in modern-day glucagon assays.

The aim of this study was, therefore, to investigate whether aprotinin (Trasylol™) prevents glucagon degradation. We studied possible degradation of exogenous glucagon in rat, mouse and human plasma, as well as endogenous glucagon in blood samples from T2DM patients obtained during fasting and hypoglycemia (stimulating secretion).

Materials and Methods

Peptides and aprotinin (Trasylol™)

Synthetic glucagon 1-29 (No: H-6790) was obtained from Bachem, Switzerland, and dissolved in phosphate buffer. The concentration of glucagon in prepared solutions was confirmed

by RIA using antibody code 4305 [2] (recovery 95%), and by Quantitative Amino Acid Analysis (recovery 92%) at Department of Systems Biology, Enzyme and Protein Chemistry, Soltofts Plads, DTU, building 224, DK-2800 Kgs. Lyngby, Denmark. Aprotinin, Trasylol™, 10000KIE/ml was from Bayer Health Care AG 51368 Leverkusen, Germany. Aprotinin was added in amounts to give a final plasma concentration of 500KIE/ml.

Spiked human, mouse and rat plasma

Frozen plasma aliquots from 30 healthy subjects, 25 mice and 16 rats were thawed on ice, pooled into three species-specific pools of plasma, and centrifuged at 4°C. Each pool was divided in 6 portions which were spiked with 60pM, 40pM, 20pM, 10pM, 5pM or 0pM glucagon, respectively. Each spiked plasma sample was then divided into four portions: 1st portion was extracted immediately; aprotinin (final concentration, 500 KIU/ml) was added immediately to the 2nd portion which was then extracted; the 3rd and 4th portions were kept on ice, and aprotinin was added after 30 and 60 minutes, respectively, followed by extraction. After extraction, samples were measured as indicated below.

To investigate whether individual variation occurs in human plasma, the effect of aprotinin on glucagon degradation was investigated in samples from 20 healthy subject as well 20 patients with various gut related diseases: Crohn's disease, colitis ulcerosa and diverticulitis. Glucagon concentrations (endogenous and after spiking with 40pM exogenous glucagon) were analyzed in samples without and with the addition of aprotinin at the time of thawing for analysis.

Patient samples

The clinical part of the protocol was approved by the Scientific-Ethical Committee of the Capital Region of Denmark (registration number: H-D-2009-0078), registered with ClinicalTrials.gov (registration number: NCT-0143-6734) and took place at Gentofte Hospital, Denmark. The study was performed in accordance with the Helsinki Declaration II, and written informed consent was obtained from all participants before inclusion. Five male patients with T2DM (age (mean±standard error of the mean (SEM)): 63±2 years; body mass index (BMI): 29±1 kg/m^2; fasting plasma glucose (FPG): 7.8±0.4; HbA_{1c}: 6.1±0.2%; duration of diabetes: 28±8 months) were studied. The induction of hypoglycemia by insulin was performed as previously described [12]. Blood samples were collected in the fasting state and after induction of hypoglycaemia (see legend to figure 3) in EDTA tubes containing either aprotinin or distilled water (to maintain same dilution as the other portions). Both tubes were mixed gently and immediately centrifuged (3500G for 15 minutes at 4°C), after which the plasma was removed and stored on ice until analysis. The plasma without aprotinin was divided into three portions and aprotinin (to a final plasma concentration of 500 KIU/ml) was added to the second and third portion after 30 and 60 minutes, respectively.

Radioimmunoassay (RIA)

All samples were extracted with ethanol (final concentration 70%). Samples spiked with exogenous glucagon were measured with both a mid-region specific glucagon antiserum (code no 4304 [1]) and a C-terminally directed antiserum which requires the intact fully processed C-terminus of glucagon (code no 4305 [2]). Endogenous glucagon samples were measured with antiserum 4305.

Results analysis

For the analysis of aprotinin effect on glucagon degradation, we compared slopes (α-values of the linear regression curves) for the different treatment groups. For statistical analysis, we used repeated measurements one-way ANOVA followed by Tukey post test when applicable, using GraphPad Prism 5, USA (CA).

Results

Plasma pools

Figure 1 shows concentrations of glucagon in the plasma pools with added glucagon. Concentrations were not adjusted from inherent losses in the extraction process. For each individual recovery series (0, 5, 10, 20 40 and 60 pmol/l added to each aliquot) a regression analysis was carried out, and the slopes (n = 6 for each aprotinin treatment) were then compared by ANOVA. In general, there was good correlation between added and measured glucagon in all samples (see figure legends (Figures 1A-1F) for slopes and r-values). The mean recovery (mean of slopes) of glucagon was 0.69±0.03 without aprotinin and 0.70±0.02 with aprotinin in human plasma. There were no significant differences between the slopes obtained with or without aprotinin obtained with the four plasma treatments for human, mouse and rat plasma, whether using mid-region specific antisera or C-terminal specific antisera. Aprotinin treated spiked rat plasma (2nd portion) measured with mid-region specific antisera did though show lower recoveries and r-values in one case (Figure 1C), however the two other aprotinin treated groups (3rd and 4rd portion) was not significantly different from the portion treated without aprotinin (1st portion), perhaps indicating this as an outlier. The concentration results for samples without glucagon addition were also unaffected by aprotinin addition.

Individual variation

Figure 2 shows glucagon concentrations in individual plasma samples taken without and with addition of 40 pmol/L exogenous glucagon and with and without addition of aprotinin. Linear regression analysis showed a slope value of 0.97 and a coefficient of correlation of R^2: 0.97, indicating almost identical values for plasma levels of endogenous as well as exogenous glucagon whether aprotinin had been added or not.

Patient samples

Fasting plasma glucagon concentrations in samples from the patients were similar irrespective of whether or not aprotinin had been added to the plasma (Figures 3A and 3B). As expected, induction of hypoglycemia by insulin significantly increased glucagon levels, but again, there were no differences in measured concentrations in samples with or without aprotinin (Figures 3C and 3D).

Discussion and Conclusion

Our study shows that neither recovery of exogenous nor

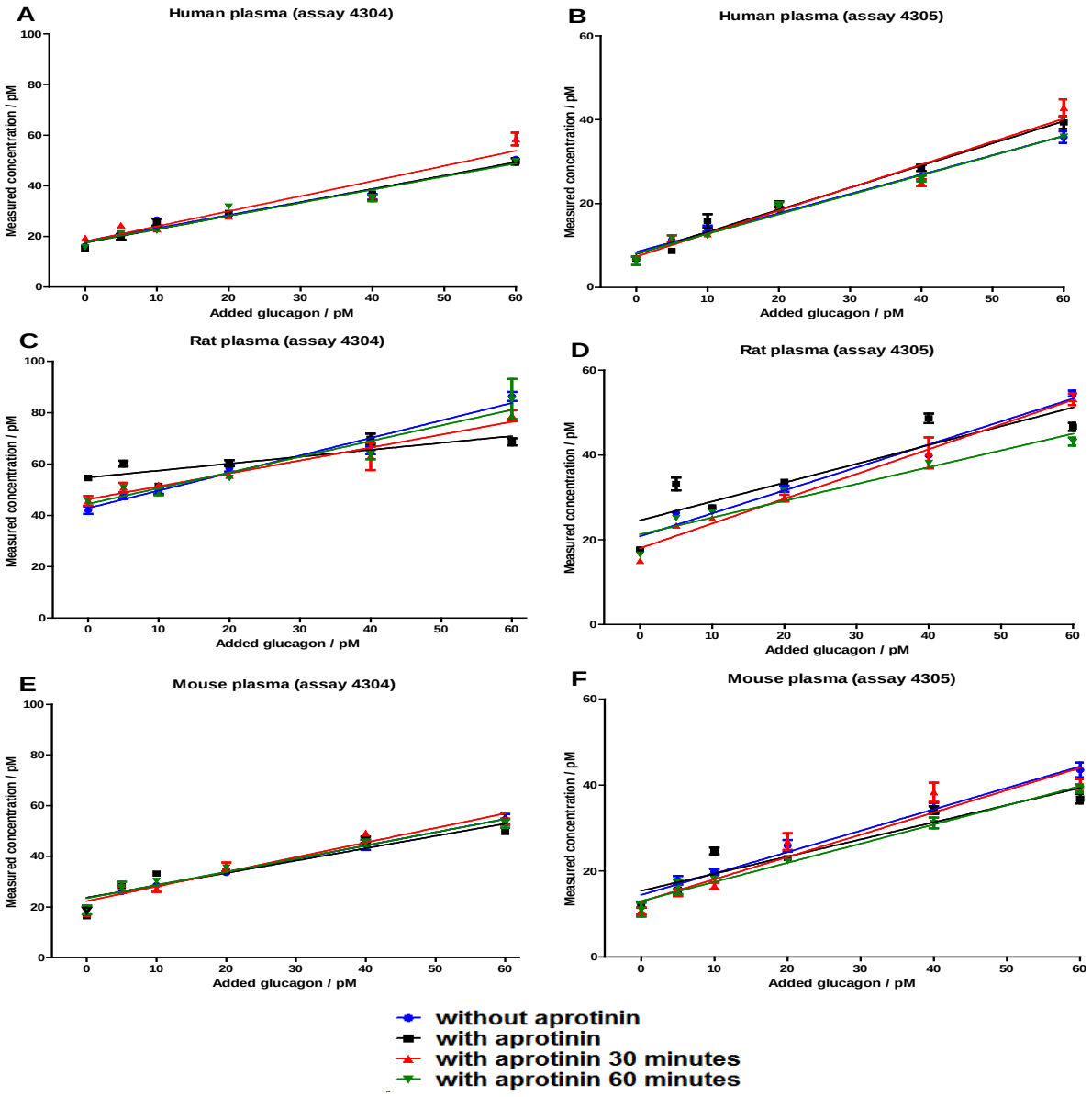

Figure 1: A): Human spiked plasma measured with RIA assay 4304; without aprotinin, slope = 0.67±0.02, r^2=0.93±0.02; with aprotinin, slope = 0.68±0.03, r^2=0.93±0.04; with aprotinin added 30 minutes later, slope = 0.70±0.05, r^2=0.87±0.03; with aprotinin added 60 minutes later, slope = 0.68±0.04, r^2=0.92±0.03. Slopes were not significantly different (ANOVA for repeated measurements).

B): Human spiked plasma measured on RIA assay 4305; without aprotinin, slope = 0.69±0.02, r^2=0.96±0.03; with aprotinin, slope = 0.72±0.02, r^2=0.93±0.02; with aprotinin added 30 minutes later, slope = 0.71, r^2=0.92±0.03; with aprotinin added 60 minutes later, slope = 0.68±0.02, r^2=0.96±0.04. Slopes were not significantly different (ANOVA for repeated measurements).

C): Rat spiked plasma measured with RIA assay 4304; without aprotinin, slope = 0.68±0.02, r^2=0.92±0.03; with aprotinin, slope = 0.60±0.04, r^2=0.55±0.05; with aprotinin added 30 minutes later, slope = 0.67±0.03, r^2=0.71±0.03; with aprotinin added 60 minutes later, slope = 0.69±0.04, r^2=0.67±0.04. Slopes were not significantly different (ANOVA for repeated measurements).

D): Rat spiked plasma measured on RIA assay 4305; without aprotinin, slope = 0.70±0.02, r^2=0.92±0.03; with aprotinin, slope = 0.66±0.04, r^2=0.83±0.04; with aprotinin added 30 minutes later, slope = 0.61±0.03, r^2=0.82±0.04; with aprotinin added 60 minutes later, slope = 0.61±0.03, r^2=0.93±0.05. Slopes were not significantly different (ANOVA for repeated measurements).

E): Mouse spiked plasma measured with RIA assay 4304; without aprotinin, slope = 0.59±0.02, r^2=0.92±0.03; with aprotinin, slope = 0.61±0.03, r^2=0.89±0.02; with aprotinin added 30 minutes later, slope = 0.57±0.03, r^2=0.89±0.05; with aprotinin added 60 minutes later, slope = 0.63±0.04, r^2=0.89±0.02. Slopes were not significantly different.

F): Mouse spiked plasma measured on RIA assay 4305; without aprotinin, slope = 0.66±0.03, r^2=0.96±0.04; with aprotinin, slope = 0.59±0.04, r^2=0.72±0.04; with aprotinin added 30 minutes later, slope = 0.67±0.02 r^2=0.88±0.03; with aprotinin added 60 minutes later, slope = 0.59±0.04, r^2=0.93±0.03. Slopes were not significantly different (ANOVA for repeated measurements).

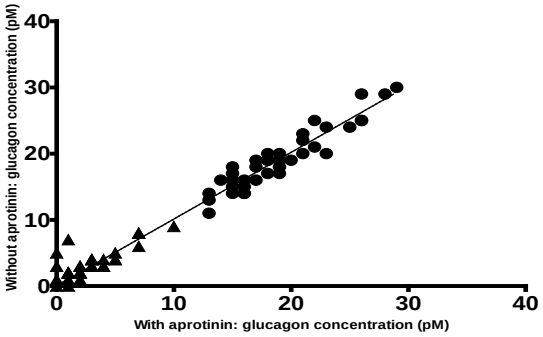

Figure 2: Human spiked plasma measured using RIA assay 4305 without aprotinin and with aprotinin. N=40. Linear regression analysis: slope: 0.97, R: 0.97. Triangles illustrate endogenous glucagon levels and circles (endogenous + exogenous) glucagon.

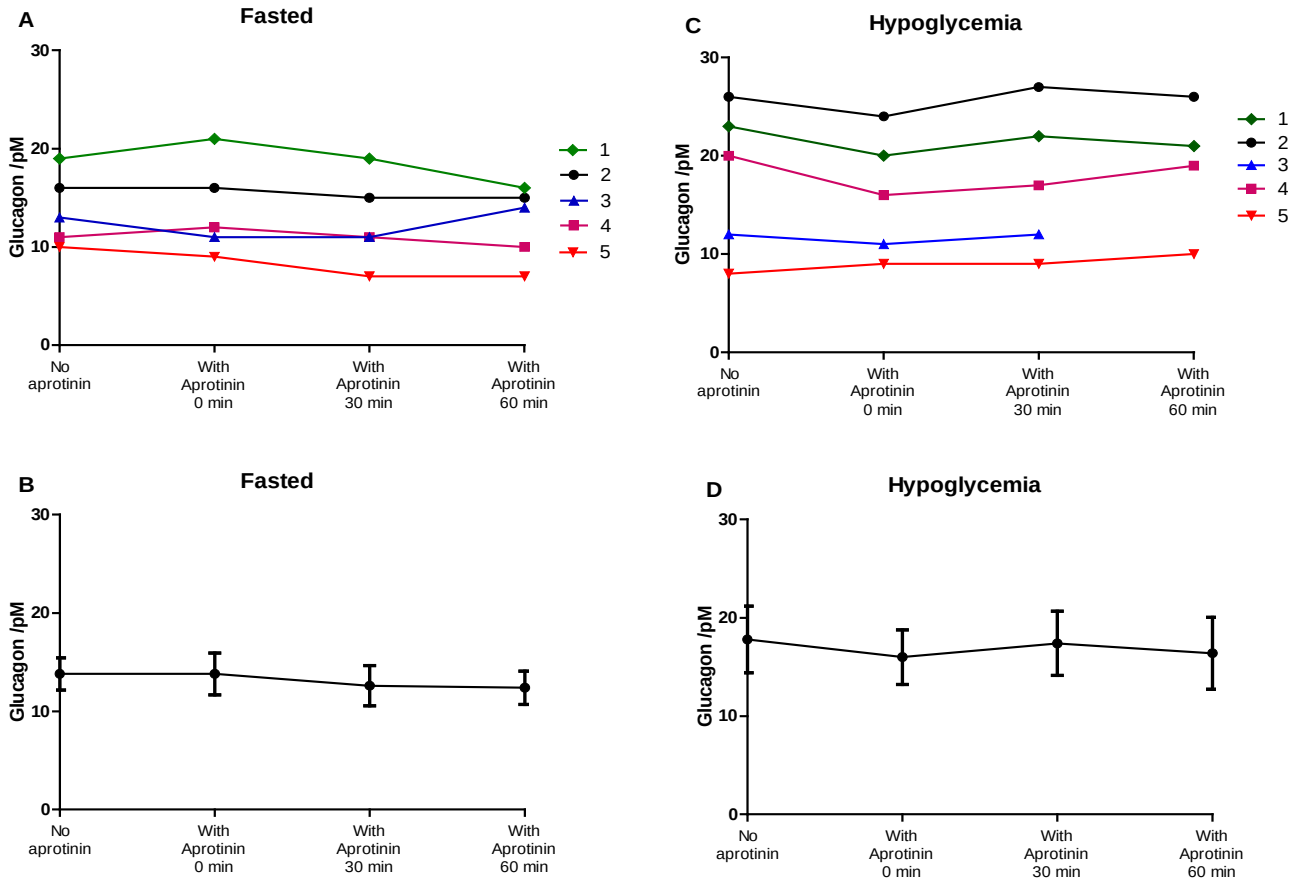

Figure 3: A-B: Fasting plasma glucagon levels, measured with RIA assay 4305, in patients with T2DM, shown individually and as mean (n=5). Glucose levels: patient 1 (7.4); patient 2 (6.5); patient 3 (9.3); patient 4 (7.9); patient 5 (7.9). Mean 7.8 mmol/L; SD 1.0; SEM 0.5.

C-D: Plasma glucagon levels, measured with RIA assay 4305 in patients with T2DM after induction of hypoglycemia (t=90 min), shown individually and as mean (n=5). Glucose levels: patient 1 (3.1); patient 2 (3.5); patient 3 (3.5); patient 4 (3.3); patient 5 (3.0). Mean 3.3 mmol/L; SD 0.2; SEM 0.1.

levels of endogenous glucagon were affected by the addition of aprotinin (Trasylol™) to plasma from mice, rats and humans. This lack of effect in human plasma is in agreement with the findings by Eisentraut et al. [8], although they found aprotinin necessary for the subsequent assay procedure to protect the tracer from degradation. Heding, Nonaka and Foa, and Shima et al. [9,10,13] all reported a protective effect of aprotinin addition in human and dog plasma. Our results clearly differ from these older studies. One important difference, as already alluded to, is the use of I^{125}-labelled glucagon as tracer. Moreover, our tracer is mono-iodinated and highly purified, and may be less sensitive to enzymatic degradation (than di-iodinated tracer). Perhaps more importantly, our assay involves a prior extraction step using ethanol, whereby a large part of circulating proteins are

removed, undoubtedly including a large number of enzymes. The extraction step was introduced mainly to avoid unspecific protein interference, which often can lead to spuriously high results [9]. As indicated by the recoveries of 69-70 %, the ethanol extraction step is, as expected, associated with a loss of measurable glucagon, but again this was unaffected by aprotinin addition [9].

Glucagon is generally measured with radioimmunoassays employing C-terminally directed antibodies, since these do not react with proglucagon products from the gut, including glicentin and oxyntomodulin, both of which contain the glucagon sequence. A good C-terminal antibody (including the one we use) is sensitive to even minor modifications of the C-terminal structure, and neither reacts with molecular forms shorter than glucagon nor with extended forms. For these studies we also used an antibody directed against the 6-15 region of glucagon [14], and the results obtained were rather similar. We did not include measurements with N-terminal antibodies (which are considered "non specific" because they also react with oxyntomodulin), and we therefore cannot exclude that some degradation may have occurred at the N-terminus, which could have been prevented by aprotinin. N-terminal degradation would undoubtedly affect the bioactivity of glucagon (des-his glucagon is 100-fold less potent than native glucagon); however, estimations of the secretion of glucagon using C-terminal and mid region specific assays should not be affected by N-terminal degradation [15]. Glucagon has been reported to be affected by the plasma activity of the enzyme dipeptidyl-peptidase-4 (DPP-4), which removes an N-terminal dipeptide from susceptible substrates [16]. However, although this may occur with high concentrations of glucagon under in vitro conditions [17,18], DPP-4 inhibition in vivo clearly was of little physiological relevance [18]. Nevertheless, N-terminal degradation could seriously affect estimations made using N-terminal assays. Ideally, therefore, a sandwich assay employing a combination of N- and C-terminal antisera is required in order to determine plasma levels of intact, bioactive glucagon, but such assays are not presently available. Whether or not addition of aprotinin would be beneficial for such assays would require additional study, although it is likely that an ethanol extraction step, as employed in the present study, would also eliminate N-terminal degradation [9].

In conclusion, our results show that addition of aprotinin is not necessary for conventional C-terminal (specific assays) or midregion (unspecific, "total") glucagon assays in plasma from humans, mice and rats. We recommend EDTA plasma for best results, which may also help to minimize any degradation by reducing calcium-dependent proteolytic activity in plasma. Conventional vacutainers can, therefore, be used for blood sampling without having to break the closed system for addition of aprotinin. The lack of necessity to add aprotinin will reduce study costs and simplify sample handling in the future.

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