values in the patients were significantly higher than those in the healthy subjects. This difference in basal bone lead concentration might be the result of previous long-term calcium supplementation in the older patient population. To address this issue, we reviewed the 14 patients’ charts and determined that three patients disclosed prior calcium supplementation. Two men took calcium carbonate, one at 800 mg/day for 12 years and the other at 2 g/day for 2 years. The remaining woman took calcium citrate at 200 mg/day for 2 years. The mean bone lead content in these three patients (2.8 ± 1.0 ng/mg bone ash) was not significantly different and actually less than that for the remaining 11 patients (3.4 ± 2.0, \( P = 0.646 \)). The value was not significantly different from the four younger subjects (\( P = 0.227 \)). This suggests that prior calcium supplementation may not totally explain the differences in basal bone lead concentration between the young controls and older patients. However, because the number of patients for which this comparison was made is small, this contention cannot be dismissed. Alternatively, a longer lifetime exposure to environmental lead in the older patient population may also explain this difference. In addition to the above findings, there was no correlation between the duration of calcium administration and the change in bone lead concentrations (Fig. 1). This is not surprising as the lead concentration in the Citracal tablet was 100 ng/tablet as determined by inductively coupled plasma mass spectrometry (personal communication C. Wabner, Mission Pharmacal). Thus, at 800 mg of calcium per day (4 tablets), a total lead ingestion of 400 ng/day from calcium tablets (orally) would be expected. Over 5 years, this would represent 22% of the total bone lead increase observed during the treatment period in the present study, assuming that all ingested lead was absorbed and deposited in the skeleton of a 70-kg person with a total bone mineral phase of 3500 g.

Another consideration is the effect that a skeletally active drug such as estrogen, diphosphonate, or sodium fluoride might have on bone lead concentration when taken concurrently with a calcium supplement. Indeed, it would be expected that such drugs, which increase bone mass, would raise bone lead concentration as the newly formed bone becomes mineralized with calcium from the oral calcium supplement. In the current patient population, 11 patients took Neosten® (sustained-release sodium fluoride, 25 mg twice a day) concurrently with Citracal. When compared with the three patients who took Citracal alone, there was no significant difference between the change in bone lead content between the two groups (0.7 ± 2.7 for fluoride-treated vs 1.6 ± 2.4 ng/mg bone ash for calcium alone, \( P = 0.639 \)). At present, we do not know if concurrent use of estrogen or bisphosphonates with calcium supplementation would yield similar findings. Thus, on the basis of this small number of patients, we conclude that calcium supplementation in the form of calcium citrate (Citracal) does not present a potential risk for lead intoxication.

References

Effect of Hemolysis on the Concentration of Insulin in Serum Determined by RIA and IRMA, Didier Chevenne,1,2*, Annick Letailleur,1 François Trivin,2 and Dominique Porquet1 [1] Hôp. Robert Debré, Lab. de Biochim.-Hormonol., 48 blvd. Séjurier, 75019-Paris, France; 2 Hôp. Saint-Joseph, Lab. de Biochim., 185 rue Raymond Losserand, 75674-Paris Cédez 14, France; *author for correspondence: fax (33) 1 40 03 47 90

Insulin-degrading enzyme (IDE; EC 3.4.99.45) was first described 40 years ago [1]. It is widely distributed in various tissues, including red blood cells (RBC) [2–4]. IDE may not play a key role in insulin metabolism, and fundamental questions on the biological role of IDE remain [2, 3]. Recently, IDE was characterized as a peroxisomal protease [3]. The specificity of IDE is selective: Only insulin and transforming growth factor-α (\( K_m \neq 0.1 \mu mol/L \)) are good substrates; insulin-like growth factor 1 and proinsulin are poor substrates [2–4]. On denaturing

Fig. 1. Correlation between the change in bone lead concentration and the duration of therapy with calcium citrate in osteoporotic subjects (○).

○, young healthy subjects who did not receive any calcium supplementation during the 3-month interval between pre- and post-bone biopsies.
polyacrylamide gels, IDE appears as a single polypeptide of 110 kDa [4], but in nonreducing conditions, IDE has an Mr of 300 000, suggesting that the enzyme exists in polymer form [4]. The cleavage sites indicate that IDE recognizes the tertiary structure rather than a particular amino acid sequence [2]. Inhibitors of IDE include p-hydroxymercuribenzoate (0.1 mmol/L), p-chloromercuriphenylsulfonic acid (pcMPS, 0.1 mmol/L), bacitracin (1 g/L), N-ethylmaleimide (1 mmol/L), 1,10-phenanthroline (1 mmol/L), EDTA (5 mmol/L), and diamide (5 mmol/L) [4–6]. The degradation of insulin by IDE is not inhibited by lysosomal enzyme inhibitors like aprotinin (500 000 kU/L) or leupeptin (0.1 g/L) [4, 6].

Although most insulin immunoassay kits indicate that hemolyzed samples should not be analyzed, few extensive studies have been done on the degree of insulin degradation by RBC IDE or how to prevent it [5–9]. To our knowledge, the interference of hemolysis with insulin values has been studied with RIAs [5–9] but not with specific IRMAs involving monoclonal antibodies. In RIAs, the mechanism of the reduction in insulin concentrations involves IDE-mediated degradation of plasma insulin and 125I-labeled insulin (used as tracer). Hemolysis is partly dependent on the material used for venipuncture [10] and cannot always be eliminated. We therefore determined the precise influence of hemolysis on human insulin RIA results (Phadesep Insulin, Pharmacia) using polyclonal antibodies, and those of IRMA (Bi-Inulin IRMA, Sanofi-Pasteur) using monoclonal antibodies without cross-reactivity with intact and des (31,32) proinsulins. We also investigated ways of overcoming the problem.

We studied the effects of hemolysis on insulin degradation by adding lysed RBCs to serum. After centrifugation and removal of serum and white cells by aspiration, RBCs were washed three times in saline and lysed by freezing. Red cell debris was removed by centrifugation and the supernatant was added to serum to obtain hemoglobin concentrations of 0.5, 1, 2, 4, and 6 g/L. Inhibition of IDE was studied either by first adding pcMPS (0.4 mmol/L), diamide (5 mmol/L), 1.10-phenanthroline (1 mmol/L), or EDTA (5 mmol/L) (all products from Sigma) to serum, followed by the RBC hemolysate, or, in another experiment, by maintaining a constant temperature of 4 °C for 1 h after the addition of the RBC hemolysate to serum without inhibitor. To reproduce the usual conditions of blood sampling (temperature, time between sampling and analysis or storage, and the usual degree of hemolysis), we incubated serum for 1 h at 20 °C and 37 °C with hemolyzed RBC.

All insulin samples are measured in duplicate. Mean basal insulin concentration determined by IRMA was 53 mIU/L (range 17.3–101.2). Results are expressed as percentage insulin recovery (Table 1). Differences were analyzed with the nonparametric Wilcoxon rank test with StatView 4.1 software (Abacus Concepts). In all analyses P <0.05 was considered significant.

Although our study was not performed under the same conditions of hemolysis, time, temperature, and assay, the hemolysis-induced insulin loss determined with IRMA was similar to previous results [5, 7–9]. In particular, slight hemolysis (0.5 g/L) significantly reduced the observed insulin concentration at 20 °C, and massive hemolysis (6 g/L) degraded >90% of insulin after 1 h at 37 °C. Although percentage insulin recovery was lower in IRMA than in RIA, marked insulin loss was also observed with RIA. The difference between the two methods can be explained by the lack of specificity of polyclonal antibodies (which could cross-react with insulin fragments) and (or) degradation of 125I-labeled insulin used as a tracer in RIA.

Generally, IDE inhibitor activity has been determined in assays measuring insulin degradation by mixing purified IDE and 125I-labeled insulin at 37 °C, pH 7.4, the reaction is terminated by the addition of trichloroacetic acid (TCA), which precipitates nondegraded insulin [4]. With IRMA, the ion chelators EDTA (5 mmol/L) and 1,10-phenanthroline (1 mmol/L) had no influence on insulin degradation (data not shown); in contrast, in the TCA assay they completely inhibit the insulin-degrading activity of IDE [4]. As chelators inhibit the activity of IDE but not insulin binding to IDE, the insulin epitopes could be masked, thereby preventing the monoclonal antibodies from binding insulin [11]. Conversely, pcMPS (and bacitracin) inhibit insulin binding to IDE, which may explain

| Table 1. Effect of hemolysis on the apparent concentration of insulin. |
|-------------------|---|---|---|---|---|---|
| Hemoglobin conc., g/L | 0.5 | 1 | 2 | 4 | 6 |
| 20 °C RIA | 7 | ND | 83.1 ± 5.0b | 76.2 ± 5.3b,c | 59.6 ± 12b,c | 45.0 ± 11b,c |
| 20 °C IRMA | 20 | 92.5 ± 3.5a,b | 85.2 ± 7.2b | 72.6 ± 9.8b | 51.7 ± 16.6b | 33.1 ± 15.5b |
| 37 °C IRMA | 7 | 69.8 ± 9.5bc | 54.4 ± 12.9bc | 31.4 ± 14.4bc | 9.5 ± 7.9bc | 2.5 ± 2.3bc |
| 4 °C IRMA | 10 | ND | ND | 90.0 ± 6.2bc,b,c | 80.1 ± 9.8bc,b,c | 70.1 ± 9.5bc,b,c |
| 20 °C + pcMPS IRMA | 15 | ND | 97.0 ± 1.7bc,b,c | 95.2 ± 2.2bc,b,c | 90.8 ± 4.0bc,b,c | 84.3 ± 5.0bc,b,c |
| 20 °C + Diamide IRMA | 6 | ND | ND | 94.2 ± 3.5bc,b,c | 90.2 ± 3.8bc,b,c | 82.5 ± 4.8bc,b,c |

a n = 6
b Different from nonhemolyzed serum
c Different from 20 °C IRMA
ND, not determined.
the difference in activity between the different inhibitors. Although the insulin loss was not completely prevented in the conditions of our study, pcMPS or diamide markedly reduced insulin degradation by IDE. The mean hemolysis-induced insulin loss was <10% when the plasma hemoglobin concentration was <4 g/L. In most situations this insulin loss has little impact on the clinical interpretation of the results.

Our study clearly showed the effect of temperature on IDE activity (Table 1). Although IDE activity was reduced by maintaining hemolyzed samples at 4 °C, the inhibitory effect of low temperature was less effective than pcMPS or diamide (Table 1). Moreover, maintaining a constant low temperature from blood sampling to plasma/serum freezing is not easy. The effect of diamide on IRMA has been studied by adding diamide (at a final concentration of 5 mmol/L) in 32 nonhemolyzed serum (insulin concentration from 1.2 to 155 mIU/L). Diamide shows no influence on insulin measured by the Bi-Insulin IRMA kit (P = 0.46).

In summary, our study shows that even slight hemolysis degrades serum insulin immunoreactivity assayed by RIA and IRMA. Ion chelators like EDTA or 1,10-phenanthroline have no effect on insulin degradation; in contrast, when pcMPS (0.4 mmol/L) or diamide (5 mmol/L) are added first, the hemolysis-induced insulin loss is <5% with a serum hemoglobin concentration of 2 g/L and 10% at 4 g/L. Low temperature significantly reduces insulin losses but is less effective than diamide or pcMPS.

References