The Effects of Acute Hyperinsulinemia on Bone Metabolism

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Abstract

Aims: Insulin signaling in bone-forming osteoblasts stimulates bone formation and promotes the release of osteocalcin in mice. Only few studies have assessed the direct effect of insulin on bone metabolism in humans. Here, we studied markers of bone metabolism in response to acute hyperinsulinemia in men and women. Methods: 33 subjects from three separate cohorts (N=8, N=12 and N=13) participated in a euglycaemic hyperinsulinemic clamp study. Blood samples were collected before and at the end of infusions to determine the markers of bone formation (PINP, total osteocalcin, uncarboxylated osteocalcin) and resorption (CTX, TRAcP5b). Results: During 4-hour insulin infusion (40 mU/m²/min, low insulin), CTX level decreased by 11% (p<0.05). High insulin infusion rate (72 mU/m²/min) for 4 h resulted in more pronounced decrease (-32%, p<0.01) whereas shorter insulin exposure (40 mU/m²/min for 2h) had no effect (p=0.61). Markers of osteoblast activity remained unchanged during 4-hour insulin, but the ratio of uncarboxylated-to-total osteocalcin decreased in response to insulin (p<0.05 and p<0.01 for low and high insulin for 4h, respectively). During 2-hour low insulin infusion, both total and uncarboxylated osteocalcin decreased significantly (p<0.01 for both). Conclusions: Insulin decreases bone resorption and circulating levels of total and uncarboxylated osteocalcin. Insulin has direct effects on bone metabolism in humans, and changes in the circulating levels of bone markers can be seen within a few hours after administration of insulin.
Introduction

Bone is increasingly recognized as an effector in energy metabolism via its interactions with other tissues (1, 2). The role of bone-derived osteocalcin (OC) as a novel endocrine regulator of glucose and lipid metabolism was originally identified in mice, in which osteocalcin was shown to act as a hormone to increase proliferation of pancreatic β cells, insulin secretion and insulin sensitivity (3, 4). Osteocalcin is specifically expressed in osteoblasts and osteocytes, and it is secreted into the circulation during bone formation (5). The structure of osteocalcin is characterized by vitamin-K-dependent γ-carboxylation which gives the protein high affinity to hydroxyapatite in bone matrix (6). Only the uncarboxylated form of osteocalcin (ucOC) appears to induce the expression of insulin and adiponectin in β cells and adipocytes, respectively (4), putatively via the activation of GPCR6A (7, 8).

Bone is a potential target for insulin, which may exert positive effects on bone density and strength (9, 10). Clinical observations in patients with type 1 and type 2 diabetes mellitus suggest that insulin may act as an anabolic agent in bone and preserve bone mass in humans (11, 12). Insulin receptors have been detected in osteosarcoma cell lines and rodent osteoblasts (10, 13-15), osteoclast-like cells (16) and in primary human osteoblasts differentiated from bone marrow-derived mesenchymal stem cells (17). In vitro, insulin signaling promotes osteoblast differentiation and osteocalcin expression in mouse calvarial osteoblasts (18). The role of insulin receptor signaling in vivo was demonstrated by deleting insulin receptors specifically in the mouse osteoblasts (19, 20). Insulin signaling in osteoblasts stimulated bone formation and increased peak bone mass during development (19). In addition, the studies suggested that insulin downregulates the expression of osteoprotegerin, thereby indirectly up-regulating bone resorption and the release of matrix-bound osteocalcin, particularly in its uncarboxylated form (20). These data proposed a two-directional regulatory pathway, where bone protein (osteocalcin) regulates β-cells and insulin release, while
insulin participates in the release of osteocalcin from the skeleton (21). Whether a similar regulatory system is present in humans is controversial (22, 23). Several studies in humans have reported a relationship between osteocalcin and various measures of glucose metabolism, but the results have been inconsistent and mostly assessed total osteocalcin levels (22-27). The discrepancies between different study designs, populations, and comorbidities create uncertainty when evaluating the role of osteocalcin in glucose metabolism in human physiology. Studies are further complicated by the difficulties in accurately measuring circulating levels on ucOC and the controversy regarding the optimal assay design for ucOC (28). It is also unclear whether changes in osteoblast function and bone metabolism can be observed in response to hyperinsulinemia in humans in vivo.

Here, we employed euglycemic hyperinsulinemic clamp technique (29) to study whether insulin affects markers of bone remodeling, particularly osteocalcin, in humans. A few studies have applied this technique to assess acute insulin-driven changes in bone metabolism (30-32) with the focus on physiological variations in circulating levels of insulin. We analyzed in parallel three cohorts, in which insulin infusion rate (40 or 72 mU/m$^2$/min) and duration of the clamp (2 or 4 hours) varied to achieve physiological or supraphysiological insulin concentrations in the presence of normoglycemia. This experimental set-up should isolate the direct effects of insulin on bone.

**Subjects and methods**

*Study subjects*

Three separate cohorts were recruited (Table 1). Eight healthy volunteers without any chronic diseases or regular medications participated in cohort 1 (five men and three women) with median age of 54.4 yrs and body mass index (BMI) of 32.7 kg/m$^2$ (33). Cohort 2 consisted of twelve healthy young men with median age of 23.5 yrs and BMI of 21.4 kg/m$^2$ (34). Cohort 3 consisted of
thirteen healthy elderly women (median age 73.7 yrs) with median BMI of 26.1 kg/m². All subjects were normoglycemic.

Study design

All cohorts were studied after an overnight fast using euglycaemic hyperinsulinemic clamp. Cohort 1 had a hyperinsulinemic (40 mU/m²/min, termed “low insulin” hereinafter) euglycemic (5 mmol/L) clamp for four hours. Cohort 2 also had a four-hour euglycemic clamp but with supraphysiological insulin infusion rate (72 mU/m²/min, termed “high insulin” hereinafter). The elderly women in cohort 3 had a shorter protocol of hyperinsulinemic (40 mU/m²/min) euglycemic clamp for two hours. All studies were approved by the Ethical Committees of Helsinki University Central Hospital (cohorts 1 and 2) or Turku University Hospital (cohort 3) and a written informed consent was given by all study participants. The studies were carried out according to the principles of Declaration of Helsinki.

The clamp study was initiated after an overnight fast at 9 a.m. and blood samples were collected at the beginning of the study before infusions (0 min) and at the end of infusions (120 or 240 min, depending on the experimental protocol, Figure 1). Serum was stored in aliquots at -70 °C until analysis and all samples were analyzed in duplicate in a blinded fashion. Whole body insulin sensitivity was calculated as glucose disposal rate (GDR, or M value; µmol/kg/min) during the last hour of the clamp [22].

Serum total and uncarboxylated osteocalcin

Serum total osteocalcin (total OC) was determined with two-site immunoassay using previously described protocols (35, 36). Two-site immunoassay for total OC is based on monoclonal antibodies (Mabs) 2H9 and 6F9 and detects the N-terminal midsegment of the OC molecule (37). In brief, 200 ng of biotinylated 2H9 and 100 ng of europium-labelled 6F9 per well was used. Synthetic peptide
of human OC amino acids 1-49 (Advanced Chemtech, USA) was used as a calibrator. Streptavidin-coated microwell plates were from Kaivogen (Turku, Finland) and other immunoassay reagents (Delfia® Assay Buffer, Wash Solution and Enhancement solution) from Perkin Elmer Life Sciences (Turku, Finland). Time-resolved fluorescence was measured with Victor2 Multilabel Counter (PerkinElmer Life Sciences). Intra- and inter-assay variations for the assay are reported to be <5% and <8%, respectively (35). Uncarboxylated osteocalcin (ucOC) was determined with in-house assay protocol based on hydroxyapatite (HA) binding, as previously used in mouse studies (3). The amount of osteocalcin bound to HA is dependent on carboxylation status and the amount of HA used (38), and we optimized the amount of HA best suitable for serum samples. In the optimized assay protocol, 100 µL of diluted sample was mixed with 1 mg hydroxyapatite slurry (Type I, buffered aqueous suspension, Sigma) and incubated for 15 min on ice. Supernatant was collected by centrifugation (8000g, 5min) and OC content in the unbound fraction (containing ucOC) was measured with the total OC immunoassay procedure based on antibodies 2H9 and 6F9 described above, allowing us to compare total OC and ucOC values. Serum samples were diluted 1:1 prior to analysis (7.5% bovine serum albumin in Tris-HCl pH 7.8). Protocol was validated with synthetic peptides of carboxylated human OC (with γ-carboxyglutamic acid at positions 17, 21 and 24, from Advanced Chemtech, USA) and uncarboxylated human OC (with glutamic acid at positions 17, 21 and 24, from Peptide 2.0, USA).

**Serum CTX-I, TRAcP5b and PINP**

Bone resorption and osteoclast activity was assessed by measuring serum levels of C-terminal crosslinked telopeptides of type I collagen (CTX) and tartrate-resistant acid phosphatase 5b (TRAcP5b) using serum IDS-iSYS CTX-I (CrossLaps®) ELISA and BoneTRAP® (TRAcP 5b) ELISA (both from IDS Ltd, UK), respectively. Bone formation was assessed by measuring serum intact N-terminal propeptide of type I collagen (PINP) using IDS-iSYS Intact PINP assay (IDS Ltd, UK). According to the manufacturer, intra- and inter-assay variations for the assays are for βCTX-I
<6% and <10%, for TRAcP5b <6% and <6% and for PINP <4% and <6%, respectively. Measurements were performed by ValiRx Finland Ltd. In addition, the plasma levels of 25-hydroxy vitamin D (25(OH)D), the predominant circulating metabolite of vitamin D, were measured with automated electrochemiluminescence binding assay (Cobas 8000, Roche Diagnostics) in cohort 3.

Statistical analysis

The data are given as medians with range, or interquartile range (IQR). Bone markers were non-normally distributed (Shapiro-Wilk test <0.95) and the statistical differences between values before and after infusion were calculated using Wilcoxon’s signed rank test. Spearman’s test was used to study the correlations between variables. For statistical analysis we used SPSS, version 22 (SPSS Inc) and the level of significance was set at p < 0.05.

Results

Glycemic control during studies

Low insulin infusion rate increased serum insulin levels 6-fold (p=0.012) in cohort 1 and 8-fold (p=0.003) in cohort 3. High insulin infusion rate (cohort 2) resulted in a 30-fold increase in serum insulin (p=0.003) and 67% higher insulin level during clamp as compared to cohort 1 and cohort 2. Plasma glucose levels remained at 5 mmol/L during insulin infusions in all cohorts (data not shown). Whole body glucose uptake had variation in all cohorts. In cohort 1 (overweight men and women), the median GDR value was 30.5 µmol/kg/min (range 11.9 – 46.5). The median GDR value in cohort 2 (young men with normal bodyweight) was 54.7 µmol/kg/min (range 46.3 - 81.5) and in cohort 3 (elderly women), the median GDR was 26.7 µmol/kg/min (range 8.7 – 53.3). In cohort 3, the median plasma level of 25(OH)D was 75.2 nmol/L, and only two subjects (15%) had insufficient levels of circulating 25(OH)D (i.e. below 50 nmol/L by the Finnish National Guidelines).
Bone resorption in response to euglycemic hyperinsulinemia

After 4-hour insulin infusion, there was a small but significant decrease in bone resorption marker CTX (median -11%, p=0.035, Fig.2A). High-dose insulin exposure resulted in a similar, but more pronounced suppression of bone resorption measured by CTX (median -32%, p=0.008, Fig.2B). In contrast, shorter 2-hour insulin infusion did not alter serum CTX (median +1.0%, p=0.61, Fig.2C). Levels of osteoclast-derived TRAcP5b, a marker of osteoclast number, varied and no consistent pattern in response to insulin were observed. A small, but significant decrease of 4.6% (p=0.01) was observed in 2-hour insulin exposure and a decreasing trend was observed also after the longer 4-hour exposure (median -13%, p=0.05). However, no decrease was detected in TRAcP5b in cohort 2 (p=0.27). The changes in bone markers in response to insulin infusion are summarized in Figure 2 and Supplementary Figure 1.

Bone formation in response to euglycemic hyperinsulinemia

PINP, a marker of bone formation, remained unchanged during both high and low as well as long and short insulin infusions (p>0.05 for all). The circulating levels of total OC remained unchanged in response to insulin in 4-hour hyperinsulinemic clamps (p>0.05 in both cohorts). In contrast, the level of ucOC was reduced by a median of 27% in response to high insulin infusion rate (p=0.010, Fig.2B) and a similar, although not significant, decrease was observed with low insulin infusion rate (median -25%, p=0.069, Fig.2A). The ratio of uncarboxylated-to-total OC was decreased in response to insulin exposure both with low (p=0.012) and high (p=0.005) insulin infusion. Shorter 2-hour insulin exposure resulted in decrease in both total osteocalcin (-22%, p=0.002) and ucOC (-35%, p=0.009, Fig.2C). The ratio of uncarboxylated-to-total OC varied and was not significantly altered (p=0.53). We also performed all analyses by excluding the two obese individuals (BMI >30) in the 2-hour insulin exposure experiment. The main findings were the same after the
exclusions, with a decrease of 22% in totalOC (p=0.005) and 38% in ucOC (p=0.019) and no change in uncarboxylated-to-total OC ratio (p=0.51).

Associations between bone markers and insulin sensitivity

We also explored the association between glucose disposal rate, a measure of insulin sensitivity, and baseline levels of bone markers. Insulin sensitivity was positively associated with baseline bone resorption measured by CTX (r=0.91, p=0.002) in cohort 1. Correlations with other bone markers were not significant, although a similar trend of positive association was seen for PINP (r=0.62, p=0.102), total OC (r=0.67, p=0.071), and ucOC (0.62, p=0.102). Insulin sensitivity of elderly women in cohort 3 also tended to associate with several bone markers, although the associations were not statistically significant (CTX r=0.48, p=0.12; PINP r=0.48, p=0.12; total OC r=0.37, p=0.22; ucOC r=0.44, p=0.14). In cohort 2 consisting of young individuals with low BMI and high baseline bone turnover, no associations between insulin sensitivity and baseline bone markers were found (data not shown). This may be due to their higher insulin sensitivity but it is also possible that the relationship between insulin and bone metabolism depends on physical condition, age and/or gender (39).

Discussion

Our data indicate that acute pharmacological changes in circulating insulin levels have immediate effects on bone metabolism in humans. Insulin suppresses bone resorption (CTX) and decreases the levels of total and uncarboxylated osteocalcin in dose- and time-dependent manner.

Bone resorption marker was decreased while no change was seen in bone formation markers four hours after insulin exposure. This suggests that insulin may act as an anabolic agent for bone metabolism, by shifting the balance in the favor of bone formation. However, no direct effect of
insulin on osteoblasts can be confirmed on the basis of the current study, since we observed no change in the circulating levels of PINP or total osteocalcin after 4 hours. This is in conflict with the mouse studies reporting increased bone formation and osteocalcin secretion upon activation of insulin receptor signaling (19, 20). Furthermore, resorption marker CTX was decreased in response to 4-hour insulin infusion, opposite of increased bone resorption predicted by the mouse models. By measuring bone markers two or four hours after insulin exposure we were thus unable to confirm the direct stimulatory effect of insulin on bone observed in mice. Species-specific differences in osteocalcin between mice and humans, such as the differences in the regulatory elements of osteocalcin gene and the relatively low sequence conservation, may complicate the extrapolation of the findings of mouse models to humans (22, 23, 40).

Significant suppression in bone metabolism is observed after ingestion of glucose and the effect of postprandial insulin release on bone metabolism is usually of greater magnitude than the effects observed in our study (36, 41). Part of the postprandial effect may be mediated by insulin but also by the incretins, such as GIP (42) or GLP-2 (43), or even glucose itself may directly affect the bone cells (44). The objective of our study was to test the effects of insulin on bone metabolism with a steady glucose level. The suppression of bone metabolism observed after hyperinsulinemic euglycemic clamp suggests that insulin also has direct effects in the bone microenvironment. The differential responses in CTX and osteocalcin after 2-hour and 4-hour insulin infusion suggest that bone markers and total and uncarboxylated forms of osteocalcin have different kinetics in response to insulin. We cannot, however, exclude that insulin also regulates other aspects of bone metabolism over a different time period, or that the skeletal effects may be different in acute and chronic metabolic challenge in humans.

Small clamp studies isolating the effect of insulin on bone markers have suggested that acute changes in insulin levels do not regulate bone metabolism in humans (30, 31). Basu and co-workers
used a 2-3 hour stepped insulin infusions accompanied by hyperglycemic (9.3 mmol/L) clamp and failed to observe changes in bone markers, including CTX and uncarboxylated OC (31). We used high insulin infusion, longer exposure time and glucose was clamped at normal physiological concentration (5 mmol/L). Longer exposure to higher insulin may have uncovered some effects which were not observed previously. Clowes et al. used a 2-hour hyperinsulinemic euglycemic clamp with high insulin infusion rate (80 mU/m²/min) and reported no significant change in bone formation and resorption markers (30), which is in line with our 2-hour hyperinsulinemic euglycemic clamp, except for osteocalcin. In our study, the association between insulin sensitivity and bone turnover was weak, if any. Insulin sensitivity was positively, although not significantly, associated to bone turnover in two of the cohorts, but not in the cohort consisting of young men with normal body weight. Levinger and co-workers observed a positive association between whole body insulin sensitivity and osteocalcin in middle-aged obese men (32) and another study reported a borderline association between total osteocalcin and insulin resistance which appeared to be mediated by BMI (45). Based on these findings, the proposed link between insulin sensitivity and bone metabolism remains uncertain in humans and further studies are required.

The strengths of our study include the use of two different insulin infusion rates and durations. Our main finding of a decrease in circulating CTX levels and to lesser extent also uncarboxylated-to-total OC ratio by insulin is corroborated by similar data from three independent cohorts. The three different cohorts cannot, however, be directly compared to each other due to differences in baseline characteristics. We assessed the carboxylation of osteocalcin with in-house HA binding method. The assay performance was greatly dependent on the diluent and the amount of HA, as reported (38), and a detailed characterization of assays based on HA binding is warranted. The limitations of our study include the small number of subjects, due to the highly laborious and invasive study protocol. Due to the small sample size, it was not possible to statistically adjust for confounding factors or to take into account the gender differences (39). Differences in baseline characteristics of
the cohorts, such as age and BMI, may explain some of the differences observed in the magnitude
of responses. Further limitation is the unavailability of PTH measurements. Previous studies have
reported a transient reduction in PTH in response to insulin (30) and it remains to be evaluated
whether the decrease in PTH mediates the changes in bone metabolism.

In summary, pharmacologically elevated insulin levels decrease bone resorption and the levels of
total and uncarboxylated osteocalcin within a few hours in euglycemic conditions. Acute insulin
exposure does not directly regulate bone formation in humans, as assessed by the circulating marker
of bone formation (PINP).

Declaration of interest
The authors have no conflict of interest.

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Author contributions
KI: study conception, acquisition of data, analysis and interpretation of data, drafting and revising
the article; MH, PE, MB, VH, PN: study design, acquisition of data, revising the article; KV: study
design, revising the article; HK, study design, acquisition of data, interpretation of data, revising the
article. All authors approved the final version of the article.
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Figure legends

Figure 1. Experimental design. Glucose was clamped at 5 mM and insulin infusion rates were 40 or 72 mU/m²/min. Timepoints for sample collection are indicated with triangles. Samples were analysed for PINP, CTX, TRAcP5b, TotalOC and ucOC.

Figure 2. % changes in bone markers in response to A) 4-hour euglycemic hyperinsulinemic (40 mU/m²/min) clamp (cohort 1), B) 4-hour euglycemic hyperinsulinemic clamp with high (72 mU/m²/min) insulin infusion rate (cohort 2) or C) 2-hour euglycemic hyperinsulinemic (40 mU/m²/min) clamp (cohort 3). Results are shown as % change from the baseline (0’) value. The lines inside the boxes represent the 50th percentile; the limits of the boxes represent the 25th and 75th percentiles, and the whiskers the 10th and 90th percentiles. Individual samples are shown with crosses and the mean value with a square. P values for the changes (from baseline to the endpoint, Wilcoxon signed ranks test) are shown below the x-axis (**p<0.01, *p<0.05).

Supplementary Figure 1. Levels of bone markers A) PINP, B) CTX, C) TRACP5b, D) TotalOC and E) ucOC before and after euglycemic hyperinsulinemic clamp in cohort 1 (40 mU/m²/min, 240min), cohort 2 (72 mU/m²/min, 240min) and cohort 3 (40 mU/m²/min, 120min). Results are shown for the median values (solid diamond) and for each individual (open squares and circles for males and females, respectively).
Table 1. Characteristics of the three cohorts analyzed using euglycaemic hyperinsulinemic clamp technique. Plasma glucose was clamped at 5 mmol/L in all experimental cohorts.

<table>
<thead>
<tr>
<th></th>
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<th>Cohort 2 (N=12)</th>
<th>Cohort 3 (N=13)</th>
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<tr>
<td></td>
<td>median range</td>
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<tr>
<td>Insulin infusion rate (mU/m²/min)</td>
<td>40 -</td>
<td>72 -</td>
<td>40 -</td>
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<tr>
<td>Clamp duration (min)</td>
<td>240 -</td>
<td>240 -</td>
<td>120 -</td>
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<td>0 / 12 -</td>
<td>13 / 0 -</td>
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<td>179.2 173 - 190</td>
<td>161.8 150 - 168</td>
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<tr>
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<td>21.4 18.3 - 28.7</td>
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<td>Waist-to-hip ratio</td>
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<td>0.86 0.76 - 0.91</td>
<td>0.89 0.79 – 0.96</td>
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<td>Fasting plasma glucose (mmol/L)</td>
<td>5.4 4.7 - 6.6</td>
<td>5.4 4.9 - 5.9</td>
<td>5.8 5.3 – 6.4</td>
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<tr>
<td>Fasting serum insulin (mU/L)</td>
<td>10.0 4 - 16</td>
<td>3.0 2 - 6</td>
<td>8.0 3 – 19</td>
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<td>Serum insulin at the end of insulin infusion (mU/L)</td>
<td>63.0 34 - 75</td>
<td>105.0 69 – 114</td>
<td>76.0 51 - 99</td>
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<td>Whole body glucose uptake (µmol/kg/min)</td>
<td>30.5 11.9 - 46.5</td>
<td>54.7 46.3 – 81.5</td>
<td>26.7 8.7 – 53.3</td>
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Hyperinsulinemic euglycemic (5mM) clamp

- Cohort 1 (N=8) Ins 40 mU
- Cohort 2 (N=12) Ins 72 mU
- Cohort 3 (N=13) Ins 40 mU

= sample for bone marker analysis
A  
Cohort 1  
Insulin 40 mU/m²/min, 240 min  

Change from baseline (%)  

<table>
<thead>
<tr>
<th>PINP</th>
<th>CTX</th>
<th>TRACP5b</th>
<th>TotalOC</th>
<th>ucOC</th>
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<td>-100</td>
<td>80</td>
<td>-80</td>
<td>-60</td>
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B  
Cohort 2  
Insulin 72 mU/m²/min, 240 min  

Change from baseline (%)  

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<th>TotalOC</th>
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C  
Cohort 3  
Insulin 40 mU/m²/min, 120 min  

Change from baseline (%)  

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