PTEN expression and its association with glucose control and calorie supplementation in critically ill patients

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Running head: PTEN, glucose control, and calories in ICU

Abstract

Backgroud and Aim: Phosphatase and tensin homologue (PTEN) reduces insulin sensitivity. Since critically ill patients present insulin resistance, we aimed at assessing the role of PTEN expression on glucose homeostasis and clinical outcome in patients admitted to an intensive care unit (ICU) and receiving artificial nutrition.

Methods: Observational, single-center study conducted in one ICU in Rome, Italy on adult patients hospitalized for trauma. Plasma glucose levels and its variability were recorded in patients receiving artificial nutrition. PTEN expression was measured by western blotting analysis and the associations between PTEN, plasma glucose levels and its variability, and calories administered were investigated. Parametric and non-parametric tests were used, as appropriate.

Results: Twenty consecutive patients (13 men and 7 women, mean age of 37.3 ± 12.7 years) were studied. No correlation between plasma glucose and PTEN was documented (r= -0.15, P= 0.55), neither between glycemic variability and PTEN expression (r= -0.00, P= 0.99). However, total kcal/day administered and PTEN expression significantly correlated (r= 0.56, P= 0.01). Also, patients with PTEN levels below the median received less kcal/day than those with PTEN above the median (P=0.048). This association was more pronounced when adjusted for body weight (P= 0.03) and for the average of insulin daily administered (P= 0.02).

Conclusions: PTEN expression might significantly contribute to glucose homeostasis and disposal in critically ill patients receiving artificial nutrition. Larger samples are necessary to confirm our observation.

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Key words: PTEN, ICU, glycemic control, trauma patients, artificial nutrition, calories

Introduction

Glycemia management is a key factor in the care of critically ill patients [1]. However, tight glucose control should be avoided in patients in those intensive care units (ICUs), where the staff is not sufficiently trained [2]. In contrast, maintaining a blood glucose target ≤180 mg/dL is associated with improved clinical outcome [3]. More importantly, low glycemic variability appears protective and associated to reduced ICU specific and in-hospital death [4].

Whether tight or permissive glycemic control is the optimal approach to ICU patients has been since long time matter of debate. Similarly, contrasting evidence support the use of either underfeeding or early parenteral supplementation. Focusing on interventions aimed at improve glucose homeostasis, glutamine-induced improved glucose disposal [5, 6] is associated with increased expression of heat shock protein 70 and reduced risk of infections [7-9]. Also, calorie restriction, including a significant reduction of glucose intake, lengthens the lifespan of many animal species, and likely of humans [10]. However, recent experimental data show that the protective effects of calorie restriction are not observed in presence of a constitutive activation of the intracellular signal chain induced by insulin [11]. A crucial role for the constitutive activation of insulin signaling is played by mutations of intracellular enzyme phosphatidylinositol-3 kinase (PI3K), whose expression and activity affect different physiological responses [12]. The hyperactivation of signal transduction pathways can also be obtained by the inactivation or defective expression of the phosphatase and tensin homologue (PTEN), a physiological inhibitor of PI3K [12]. Recent data demonstrate that PTEN plays a key role in peripheral insulin resistance [13]. Also, during sepsis PTEN expression tends to increase [14].

We previously suggested that stress-induced differential expression of PTEN [15] may contribute to altered insulin intracellular signaling and glucose homeostasis, which in turn impact on clinical outcome. Confirming the potential of insulin signaling in modulating

biochemical responses, calorie restriction modulates oxidative damage only when insulin signaling is preserved [16]. These potentially positive effects might disappear if a constitutively activated intracellular signal chain is present. In this light, reduced expression of PTEN may condition calorie supplementation in ICU patients.

Therefore, the aim of this study was to evaluate the role of PTEN expression on the control of plasma glucose levels, calorie intake and clinical outcome in ICU patients.

Materials and Methods

Patients and clinical parameters

This was an observational, single-center study performed on patients in ICU. The study was registered on www.clinicaltrials.gov (NCT01796847). All procedures were in accordance with the ethical standards of the Helsinki Declaration issued in 1975, and later amendments. After approval of the local Ethics Committee and after obtaining written informed consent from patients or, in case of impossibility, from the closest family member, consecutive adult trauma patients hospitalized in the ICU of Policlinico Umberto I, Sapienza University of Rome, Italy, were considered. Exclusion criteria were the presence of diabetes mellitus or impaired fasting glucose, cancer, chronic kidney disease. Patients receiving any therapy interfering with glucose metabolism (i.e., corticosteroids) were also excluded.

Patient demographic characteristics (age, gender, weight, height, body mass index – BMI) were recorded. Acute physiology and chronic health evaluation (APACHE) score was calculated at the admission to ICU.

Length of ICU hospitalization, complications (i.e. infections, cardiovascular events) and outcome(s) of ICU hospitalization were recorded. The number of days of mechanical ventilation, antibiotic therapy and its duration were collected.

Artificial nutrition and glycemic control

Nutritional care is provided based on the European Society for Clinical Nutrition and Metabolism (ESPEN) Guidelines for the use of artificial nutrition in critically ill patients [17, 18]. In particular, patients not meeting at least 70-75% of their nutritional needs received nutritional support by means of enteral nutrition [17, 18]. The calorie goal was set at 25 Kcal/Kg body weight (BW)/day and the nitrogen goal at 1.2-1.5 g amino acid/kg BW/day. In case of intolerance to enteral nutrition (i.e. diarrhea, vomiting, etc.), the calorie and protein gap was supplied by parenteral nutrition.

The expected glycemic range in all patients was 110-180 mg/dL (6.1-10 mmol/l). The adjustment of the insulin dose was performed according to whole-blood glucose levels, measured at 1- to 4-hour intervals in arterial blood or, when an arterial catheter was not available, in capillary blood using a point-of-care glucometer (GEM Premier 4000, Instrumentation Laboratory). A continuous infusion of insulin (50 IU insulin Humulin R, Lilly in 50 ml of 0.9% sodium chloride solution) through a time pump (Perfusor Space, Braun) was started when the blood glucose level exceeded the value of 150 mg/dL (10 mmol/l) to maintain a blood glucose level between the expected range. When the glycemic levels were lower than 110 mg/dL (6.1 mmol/l), the insulin infusion was reduced or stopped. The insulin infusion adjustments were performed by the nurse in charge of the patient.

Blood sample collection for PTEN expression analysis

Blood samples were obtained from patients at the moment of admission in the ICU and collected in ethylenediaminetetraacetic acid tubes. Each plasma sample was washed with phosphate buffered saline (PBS) to reach a final volume of 40 mL and added with 10 ml of Ficoll to separate the white blood cells. Samples were centrifuged with swinging-bucket rotor without brake (45 min at 1800 rpm at room temperature), lymphocytes were recovered

(arranged in a ring) and brought to a final volume of 30 ml adding with PBS. After additional centrifugation with brake (15 min at 1800 rpm at room temperature), a new washing was performed with 30 mL of PBS, and the pellet was stored at -20 ° C until further analysis. The expression of PTEN phosphatase was assessed in all patients enrolled using Western blot analysis, performed on a sodium dodecyl sulfate -polyacrylamide 10% gel using rabbit PTEN antibody as a primary antibody (Cell signaling, Mi, Italy; 1:1000) and mouse anti-rabbit unconjugated antibody (Mab) as secondary antibody (Millipore, Chermicon; 1:10000). All membranes were also probed with antibodies for β-actin as a loading control.

Statistical analyses

Continuous variables were expressed as mean ± standard deviation. Association between continuous variables was assessed by means of Spearman's correlation and related test. Categorical variables were described using proportions and were analyzed with the χ^2 test. For the analysis of time-to-event clinical outcomes we used Cox regression, logistic regression was used for binary outcomes, linear regression for Gaussian (possibly after transformation) continuous outcomes and Poisson regression for count outcomes. Specifically, we used Cox regression to evaluate the association between independent predictors (including PTEN) and (i) length of ICU stay, (ii) length of mechanical ventilation and (iii) mortality; logistic regression for indicators of specific complications; Poisson regression for complication counts; and linear regression for blood glucose levels and calories.

A P value < 0.05 was considered statistically significant. All statistical analyses were performed in R v. 3.0.2.

Results

Patients' demographic and clinical characteristics

A total of 21 patients were enrolled in the study. One male patient was excluded because of violation of the protocol (blood sample for PTEN analysis was not collected). At the end, 13 men and 7 women, with a mean age of 37.3 ± 12.7 years were studied. The characteristics of our sample are reported in Table 1.

The admission diagnosis was polytrauma in 14 patients, cranial trauma in 5 patients, and rachis trauma in 1 patient. Pre-existing comorbidities were not present in the entire cohort.

The mean PTEN value was 0.86 ± 0.62 and the median was 0.72.

Blood glucose levels, insulin dose and PTEN expression

The mean value of the daily blood glucose levels (measured 6 times per day) was calculated in each patient. At the end of the ICU stay, the mean glucose levels during the entire hospitalization were also calculated.

No correlation was found between the average plasma glucose levels during the entire ICU stay and PTEN values (r= -0.15, P= 0.55). Similarly, no correlation between glycemic variability (as standard deviation of the mean plasma glucose measurements) and PTEN expression was documented (r= -0.00, P= 0.99). Similarly, no correlation was found between the mean of plasma glucose levels, glycemic variability and PTEN expression according to the dose of insulin administered.

We additionally calculated the units of the insulin administered every day and no correlation was documented between these values and PTEN levels (r= -0.21, P= 0.37).

Artificial nutrition: association between calorie, amino acid administration, and PTEN expression

All patients received artificial nutrition. The average of the kcal/day administered in all patients, as well as the kcal/kg BW/day, and gN/day are shown in Table 1. One patient

received only enteral nutrition. Fifty-nine % of the mean calories daily administered were supplemented by parenteral nutrition because of intestinal intolerance.

We found a significant correlation between the average of the total kcal daily administered and PTEN expression (r= 0.56, P= 0.01) (Figure 1).

We next examined this relation based on PTEN median value (0.72) and we found that, in order to achieve the target glucose levels (110-180 mg/dL), patients with PTEN levels below the median were able to receive less kcal/day with respect to patients with PTEN levels above the median (1522.2 ± 135.2 vs 1683.2 ± 199.3) (P=0.048) (Figure 2, upper panel). In addition, when considering the kcal/kg BW/day, this association was more pronounced (19.99 ± 3.41 vs 23.39 ± 3.18) (P= 0.03) (Figure 2, lower panel). Finally, after adjusting for the mean of insulin (I.U.) daily administered, the association between the average of the total kcal daily administered and PTEN was confirmed (P= 0.02).

Clinical outcomes and complications

The mean length of ICU stay was 19.6 ± 9.7 days and all patients were mechanically ventilated for 15.2 ± 7.2 days. Twelve patients developed infectious complications and 2 patients developed acute renal failure (Table 1). We observed an ICU mortality rate of 30% (n=6 patients). No association between PTEN expression and length of stay, days of mechanical ventilation, numbers and type of complications, including mortality, was documented. However, we found a significant association between glycemic variability and mortality (HR= 1.10, P= 0.007), independently from PTEN values.

Discussion

In this study, involving non-diabetic adults admitted in the ICU for trauma, we did not find significant relationships between plasma glucose levels and PTEN levels, nor between

glycemic variability and PTEN levels. Similarly, no association was documented between insulin units administered and PTEN. However, we found that patients with lower expression of PTEN were able to receive, by artificial nutrition, reduced amount of calories per day in order to achieve blood glucose control [2, 17, 18]. The reduced amount of calories received by patients with lower PTEN levels was also confirmed when adjusted for the insulin dose administered.

The phosphatase PTEN antagonizes the phosphatidylinositol 3-kinase (PI3K) pathway, has a role in cell-cycle and metabolic pathways, and it has been indicated as a major player in insulin signaling and its sensibility and apoptosis [19]. Recent data showed that higher PTEN expression may represent a potential therapeutic strategy able to control cancer development and enhance calorie expenditure to reduce adipose tissue accumulation [20]. In particular, *Garcia-Cao et al* described that PTEN elevation regulates metabolic switch by controlling PI3K -dependent and -independent pathways and negatively impacting on glutaminolysis and Warburg effect [20]. In an another experimental condition, PTEN loss during pancreas development led to increased islet number and size, as well as total beta-cell mass, indicating that a potential modulation of PTEN-controlled signaling pathway might represent a therapeutic option for beta-cell protection and regeneration treatment [21]. In this light, PTEN was hypothesized as a potential molecule involved in the pathophysiology of insulin resistance and type 2 diabetes in humans. However, variability in PTEN expression was not found to be a cause of type 2 diabetes in a Caucasian population [22]. More recently, *Grinder-Hansen et al.* [13] documented that PTEN variation was related with insulin resistance and with the risk of developing type 2 diabetes. However, the association with decreased insulin sensitivity was not justified by reduced insulin signaling in muscle.

On the other hand, a different mechanism may be at least in part present in non-diabetic patients developing insulin-resistance in ICU after severe injury. Activating mutations may

exist in humans, and taking into account that PTEN is an essential component of the insulin signaling cascade, it could be possible that tight glucose control and calorie target may be achieved and may improve the outcome(s) only when pursued in those critically ill patients whose insulin signaling is constitutively activated. In addition, the controversial data available on tight glucose control on clinical outcomes have been described in study participants with likely different genetic background [3,15,23-25]. Factors that might contribute to these results may be determined also by different insulin treatment protocols, glucose goals and the use of artificial nutrition. In our cohort, patients with lower PTEN expression were able to receive less calorie supplementation in order to achieve the desired blood glucose concentration and, in particular, to reduce the development of hyperglycemia. This aspect is also clinically relevant considering that the reduction of calorie supplementation may determine or worsen alterations of nutritional status.

Moreover, a strong association has been documented between increased glycemic variability as well as hypoglycemia and poor outcomes in the ICU patients [26-29]. More importantly, there is evidence on the relevance of closely and effectively controlling blood glucose concentration during ICU stay to reduce its variability [30]. In this view, glucose and calorie infusion led to higher insulin requirements to avoid hyperglycemia, with increased risk for glycemic variability [30]. Based on these evidences, international guidelines indicate to reduce blood glucose variability in patients on parenteral nutrition, with a target blood glucose of 90-150 mg/dl [31].

In our results, we reported a significant association between higher glycemic variability and mortality, although our sample size was limited. However, we did not find a relation between PTEN expression and glycemic variability.

The role of PTEN on modulating insulin sensitivity in humans is complex, and controversial data exist with respect to experimental models. In particular, *Pal et al.* showed that PTEN

haploinsufficiency is a genetic cause of profound constitutive insulin sensitization [32]. However, these results appear divergent in the same cohort of patients studied, considering that PTEN insufficiency was also associated with an obese phenotype [32]. These data additionally highlight concerns that therapeutic approaches aimed at enhancing PTEN levels will determine lower insulin sensitivity possibly increasing the risk for developing type 2 diabetes [33].

We acknowledge the limitations of our study. In particular, the reduced sample size of the patients enrolled and the lack of association between glycemic variability and PTEN levels, independently from the calorie administration. Moreover, we did not investigate specifically PTEN function at molecular levels and its modifications overtime but only PTEN levels at single-point measurement. Additionally, we did not measure patients' resting energy expenditure by indirect calorimetry which is the most accurate tool to assess energy needs.

Conclusions

Based on our results, the identification of different PTEN expression in larger cohorts of patients, rather than intensive calorie and glucose control per se, might be a possible novel key factor involved in the control and management of calorie supplementation in critically ill patients.

Abbreviations: ICU, intensive care unit; PI3K, phosphatidylinositol-3 kinase; PTEN, phosphatase and tensin homologue; BMI, body mass index; APACHE, acute physiology and chronic health evaluation; ESPEN, European Society for Clinical Nutrition and Metabolism; BW, body weight; PBS, phosphate buffered saline; Mab, mouse anti-body.

Ethics approval and consent to participate

All procedures were in accordance with the ethical standards of the Helsinki Declaration issued in 1975, and later amendments. We have obtained the approval of the local Ethics Committee (Azienda Policlinico Umberto I, Sapienza University of Rome, Italy) and obtained the written informed consent.

Competing interests

None

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Authors' Contributions

A.M. designed research, conducted research, analyzed data, wrote the paper;

F.A. conducted research and collected data;

P.M. provided essential reagents necessary for research;

D.D. collected data;

A.F. performed statistical analysis;

M.I.A. collected data and analyzed data;

A.L. designed research, reviewed the paper, and had primary responsibility for final content.

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Table 1 Patients' characteristics and outcomes

All patients *N= 20*	Mean ± SD*
Sex (male:female)	13:7
Age, years	37.3 ± 12.7
Body weight, kg	75.9 ± 16
BMI, weight (kg)/height^2 (m)	24.4 ± 3.9
Blood glucose level (baseline), mg/dl	160 ± 47.8
Insulin dose, units/day	16.8 (11.1 to 34.9)*
Nonprotein calories administered, Kcal/day	1602.7 ± 185.18
Nonprotein calories administered, Kcal/kg BW/day	21.69 ± 3.65
Gram of nitrogen per day, g/day	11.24 ± 1.74
Days in ICU	19.6 ± 9.7
Days of mechanical ventilation	15.2 ± 7.2
APACHE score	12.2 ± 3.6

*Median (interquartile range) is shown for non-normally distributed variable (insulin daily administered). Abbreviations include: BMI, body mass index; BW, body weight; ICU, intensive care unit; APACHE, acute physiology and chronic health evaluation.

Figure Legends

Figure 1 Upper panel: linear regression between kilocalories per day supplemented by artificial nutrition and PTEN. The average of the total kcal daily administered significantly correlated with PTEN (expressed as percentage of control) (r= 0.56, P= 0.01). Lower panel: representative western blots quantifying the band densities of four PTEN blots, normalized against β-ACTIN, calculated as percentage of control. Abbreviations: Kcal/day, kilocalories per day; PTEN, phosphatase and tensin homologue.

Figure 2 Left panel: total kcal/day administered according to PTEN median values. Total kcal/day were significantly higher in patients with PTEN levels above the median with respect to patients with PTEN levels below the median (P= 0.048). Right panel: total kcal/Body Weight (BW)/day. Kcal/BW/day were significantly higher in patients with PTEN levels above the median with respect to patients with PTEN levels below the median (P= 0.03). Data are expressed as means ± SD. Abbreviations include: Kcal/day, kilocalories per day; PTEN, phosphatase and tensin homologue; BW, body weight.