Insulin glargine and insulin have identical effects on proliferation and phosphatidylinositol 3-kinase/AKT signalling in rat thyrocytes and human follicular thyroid cancer cells

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Abbreviations

FOXO Forkhead box O
FRTL-5 Fisher rat thyroid low serum 5% (cells)
PTEN Phosphatase and tensin homologue

To the Editor: The mitogenic potency of the analogous insulin glargine (A21Gly,B31Arg,B32Arg human insulin) is currently under heated debate [1], since several clinical studies have raised the possibility of insulin glargine treatment having a carcinogenic potential, especially in breast cancer [2]. Moreover, latest data from Shukla et al. [3] suggest that insulin glargine may indeed trigger cellular proliferation in MCF-7 breast cancer cells at lower dosages than normal insulin.

In the past years, the insulin/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signalling cascade has emerged as a central mediator of thyroid carcinogenesis [4]. Our group recently observed that PI3K/AKT mediated inactivation of the tumour suppressor forkhead box O (FOXO) 3 is a hallmark of follicular and papillary thyroid carcinogenesis [5]. Physiologically, FOXO3 accumulates in

the nucleus, where it controls the transcription of anti-proliferative and pro-apoptotic targets such as growth arrest and DNA-damage-inducible-protein 45 (GADD45), cyclin-dependent kinase inhibitor 1B (p27^{kip1}) and BCL2-like 11 (BIM). In response to PI3K/AKT activation, FOXO3 is phosphorylated and subsequently translocates to the cytoplasm, where it is proteosomally degraded [6]. Furthermore, loss of PTEN, a crucial inhibitor of PI3K activity, results in the post-translational inactivation of FOXO3 in murine thyroids and in tumour progression in a mouse model of thyroid follicular carcinogenesis [7].

Since few data on the mitogenic potency of insulin glargine exist for the thyroid, we wondered how insulin glargine influenced cellular proliferation in normal and malignant thyroid cells, and to what extent the activity of the tumour suppressor FOXO3 might be impaired by insulin glargine treatment, when compared with stimulation by normal insulin. We therefore investigated the proliferative impact of insulin glargine in epithelial rat thyrocytes (Fisher rat thyroid low serum 5% [cells]; FRTL-5), which are highly sensitive to insulin stimulation, which results in inhibition of FOXO3 and induction of cellular proliferation [5]. We also sought to uncover possible distinct influences of insulin glargine vs normal insulin on the proliferative properties of thyroid cancer cells with inherent overactivation of the insulin/PI3K/AKT signalling axis. To this aim, we studied the human follicular thyroid cancer cell line FTC-133, which exhibits low abundance of the PI3K inhibitor PTEN [8].

Incubating cells with different amounts of normal insulin or insulin glargine, we observed that stimulation with both types of insulin (range 1 ng to 10 µg) increased proliferation of FRTL-5 cells when compared with controls kept in insulin-free media (Fig. 1a). Treatment with insulin glargine did not significantly augment the number of cells in

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Fig. 1 Impact of normal insulin vs insulin glargine on (a) proliferation of thyroid epithelial cells (rat FRTL-5). Cell numbers were quantified by cell counting on days 2, 4 and 6 using a Neubauer counting chamber (Carl Roth, Karlsruhe, Germany) (plating 1×10^4 cells in 12-well plates). The insulin concentrations were adapted on their specific insulin molarity. Continuous line, normal insulin; dashed line, insulin glargine; black squares, control; upright triangles, 1 ng; inverted triangles, 10 ng; black diamonds, 100 ng; black circles, 1 μ g; white squares, 10 μ g. **b** Induction of pro-apoptotic, anti-proliferative FOXO3 target genes *Gadd45*, (c) *p27^{kip1}* and (d) *Bim*, fold changes compared with control cells (Ctr) kept in insulin-free medium. mRNA expression determined by real-time PCR as previously described [5]. Normal insulin, black bars; insulin glargine, grey bars. **e** Activation of PI3K/AKT signalling in rat FRTL-5 cells and human follicular thyroid cancer cells (data not shown) with endogenous loss of PTEN. PI3K/AKT activation was assessed by Western blot using specific antibodies against phosphorylated (p)AKT, pFOXO3A, FOXO3A and β -actin as previously described [5]. NI, normal insulin

comparison to samples stimulated with normal insulin (Fig. 1a). Notably, proliferation of FTC-133 cancer cells was not affected by normal insulin or by the insulin analogue, glargine (Fig. 1a), suggesting that loss of endogenous PTEN in these cells already results in maximal activation of PI3K/AKT even under insulin-free conditions.

To uncover the impact of insulin glargine vs normal insulin on the transcriptional activity of FOXO3, we next used RT-PCR to investigate the expression patterns of the anti-proliferative FOXO target genes *Gadd45* (also known as *Gadd45a*), *p27^{kip1}* (also known as *Cdkn1b*) and *Bim* (also known as *Bcl2l1*; after stimulation with long- and normal-acting insulins (all methods performed as previously described) [5]. In line with our previous findings, we observed that treatment of FRTL-5 cells with normal insulin lowered levels of the cell cycle arrest inducer *p27^{kip1}* and the pro-apoptotic FOXO-target *Bim* in a dosage-dependent manner (Fig. 1b–d). Insulin glargine did

not have a higher inhibitory potential on the transcriptional activity of FOXO3 in FRTL-5 cells as reflected by non-significant changes in FOXO target gene expression after treatment with insulin glargine compared with cells stimulated with normal insulin (Fig. 1b–d).

Similar to the lacking growth response, none of the insulins tested revealed significant alterations of mRNA expression of FOXO target genes in the FTC-133 cell line (data not shown). Finally, we wondered whether insulin glargine could activate the PI3K/AKT axis more potently than normal insulin. Therefore, the PI3K/AKT activation signature was studied after incubation with normal insulin and insulin glargine using Western blot analysis. FRTL-5 cells showed a dosage-dependent induction of AKT activation in response to insulin stimulation; however no significant difference could be observed between the two insulins studied (Fig. 1e). Being independent from exogenous insulin substitution, FTC-133 already featured high

levels of AKT activation in combination with high levels of phosphorylated FOXO3 in insulin-deficient conditions, which could not be further amplified by subsequent stimulation with insulin or insulin glargine.

In summary, insulin glargine has identical effects on cellular proliferation in benign and malignant thyrocytes to those of normal insulin. Additionally, insulin glargine does not differentially affect the transcriptional activity of the tumour-suppressor FOXO3, which physiologically allows detection even of subtle changes in the PI3K/AKT activation status because of FOXO3's pivotal function in mitogenic PI3K/AKT signalling. However, we urgently emphasise that the observed effects are most likely to be cell-specific and are not suitable to universally rule out a mitogenic potency of insulin glargine or other insulin formulations.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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