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Insulin glargine affects the expression of *Igf-1r*, *Insr*, and *Igf-1* genes in colon and liver of diabetic rats

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ABSTRACT

Objective(s): The mitogenic effect of the analogous insulin glargine is currently under debate since several clinical studies have raised the possibility that insulin glargine treatment has a carcinogenic potential in different tissues. This study aimed to evaluate the *Igf-1r*, *Insr*, and *Igf-1* gene expression in colon and liver of streptozotocin-induced diabetic rats in response to insulin glargine, neutral protamine Hagedorn (NPH) insulin, and metformin treatments.

Materials and Methods: Male Wistar rats were induced during one week with streptozotocin to develop Type 2 Diabetes (T2D) and then randomly distributed into four groups. T2D rats included in the first group received insulin glargine, the second group received NPH insulin, the third group received metformin; finally, untreated T2D rats were included as the control group. All groups were treated for seven days; after the treatment, tissue samples of liver and colon were obtained. Quantitative PCR (qPCR) was performed to analyze the *Igf-1r*, Insr and *Igf-1* gene expression in each tissue sample.

Results: The liver tissue showed overexpression of the Insr and *Igf-1r* genes (P>0.001) in rats treated with insulin glargine in comparison with the control group. Similar results were observed for the Insr gene (P>0.011) in colonic tissue of rats treated with insulin glargine.

Conclusion: These observations demonstrate that insulin glargine promote an excess of insulin and IGF-1 receptors in STZ-induced diabetic rats, which could overstimulate the mitogenic signaling pathways.

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Introduction

Type 2 Diabetes (T2D) is considered a metabolic disorder of multifactorial etiology with relative or absolute deficiency of insulin secretion, involving several degrees of insulin resistance (1). Currently, some studies have reported an increased risk of cancer in T2D patients (2, 3). The reasons behind this association are subject to debate, and different factors could potentially be involved. Diabetes and cancer are indirectly associated trough common risk factors; however, causal relationships due to metabolic disturbance as hyperglycemia, hyperinsulinemia, or insulin resistance, and the different therapeutic schemes for diabetes are more directly involved. On the other hand, now it is known that insulin affects the cellular proliferation and differentiation (4), and through epidemiological analysis a consistent association of some insulin analogs with an increased risk of cancer has been demonstrated (5-14).

Although it has been described that AspB10 is the only insulin analog that promotes tumor growth (15), there exist reasonable doubts about the other types of insulin analogs. Insulin glargine is widely used as a long-acting analog and differs from the human insulin by the inclusion of asparagine by glycine at the position 21 of the A-chain and two arginine residues in the carboxy-terminal extension of the B-chain. Metabolic characteristics of glargine are equivalent to human insulin but display a slightly higher affinity to the insulin-like growth factor receptor 1 (IGF1R) (16, 17). The insulin glargine treatments increase the arrest to apoptosis in several cancer cell lines (18). This observation has allowed hypothesizing that these insulin analogs, with increased IGF1R affinity *in vitro,* promote augmented cell growth and reproduction (15). Both in animals and in humans, insulin glargine undertakes a fast and increased metabolism, generating the early formation of the critical metabolite M1, which has a mitogenic profile (*in vitro)* equivalent to human insulin (14, 16, 19). Such activity probably signifies the theoretical basis for the potential carcinogenicity of insulin glargine; however, the results obtained regarding the effect of insulin glargine on diverse types of cancer are inconsistent (20-26). Several studies report that neither glargine nor AspB10 manage to phosphorylate the IGF1R in different tissues from rats treated with high doses of these insulin analogs (14); however, results from another

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study showed that treatment with AspB10 resulted in higher phosphorylation levels and significantly longer phosphorylation duration of insulin receptor (IR) and protein kinase B (Akt) in several tissues, in comparison with human or glargine insulins, supporting the idea that AspB10 promotes tumorigenesis via persistent stimulation of the IR (27).

Insulin and IGF receptors are part of the superfamily of tyrosine kinase receptors, which shows an extensive structural homology. Both IGF-1Rs and insulin receptors (IRs) are dimeric molecules containing two ligand-binding extracellular alpha subunits and two transmembrane beta subunits in which the tyrosine kinase domain is included. When ligands bind to the receptor, transphosphorylation of kinase domains triggers the metabolic and mitogenic signaling pathways (14, 28, 29). Insulin-like growth factor-1 (IGF-1) is widely distributed in animal cells and exhibits multiple activities such as metabolism regulation, improvement of growth, and expansion of cells and tissues (30).

In this study, we evaluated the expression of the *Igf-1r, Insr,* and *Igf-1* genes in colon and liver of streptozotocin-induced diabetic rats in response to insulin glargine, NPH insulin, and metformin treatment.

Materials and Methods

Healthy Wistar rats were obtained from the Bioterium of University Center for Health Science at the University of Guadalajara, in Guadalajara, Mexico. The experimental animals were preserved under standard laboratory conditions (temperature 24 ± 2 ˚C; humidity 50 ± 5%, and 12-hr/12-hr light-dark cycles); they were fed *ad libitum* a standard rodent diet (Purina LabDiet® 5001). Male Wistar rats (200–250 g) were grouped and housed in separate stainless-steel cages under a controlled environment. Animal usage protocols and study procedures were strictly based on the Mexican Official Standard (NOM-062-ZOO-1999) for laboratory animal care and management. This study was approved by the Scientific and Ethics Committee 1305 (R-2008-1305-6) of the West Biomedical Research Center, Instituto Mexicano del Seguro Social (IMSS), Guadalajara, Mexico, and conducted respecting national and international ethical standards.

Experimental induction of T2D

Before diabetic induction, Wistar rats fasted for 8 hr. T2D induction method was based on a protocol previously described (31-33). Animals were injected intraperitoneally with a and single dose of streptozotocin (STZ, 65 mg/kg; Sigma, St. Louis, MO, USA) diluted in citrate buffer (10 mmol/l sodium citrate, pH 4.5) (34). Glucose levels were monitored after 48 hr of STZ induction to verify the development of T2D in rats. Only rats showing blood glucose values of ≥200 mg/dl were included (35, 36). 40% of STZ-induced T2D rats died during the first post-induction week. Surviving T2D rats were randomly distributed into four groups, with five rats in each one.

Experimental groups

Rats from first group (MET group) were treated with metformin (300 mg/kg, dissolved in saline at 0.9%) (37); the second group (GLAR group) with insulin glargine (2 IU), and the third group (NPH group) with neutral protamine Hagedorn (NPH) insulin (2 IU). Both glargine and NPH insulin doses were standardized separately, and the final doses were the half of those previously described by Stammberger *et al* (38). T2D rats received one dose per day during seven days of metformin (oral administration) or insulin NPH/glargine (subcutaneous administration). The control group consisted of streptozotocin-induced T2D rats and received only the usual saline solution, 0.90% w/v NaCl. Blood glucose was monitored from the first to the seventh day to safeguard glycemic decompensation in animals.

Glucose levels in diabetic and controls rats

In addition to daily glucose measurements, pre-treatment (as a reference value) and final (the day of the sacrifice) glucose determinations were achieved in all rats. For a more accurate estimation, blood samples were collected in dry centrifuge tubes from the retro-orbital venous plexus of anesthetized rats (ether), which were fasted overnight.

Serum was separated by centrifugation at 3000 rpm for 15 min at 4 ºC and immediately used for glucose level determination by the glucose oxidase-peroxidase enzymatic method (BioSystems, Spain) in a semi-quantitative spectrophotometer (BTS-330, BioSystems, Spain), following the manufacturer's instructions. Remaining serum was stored at -70 ºC. On the other hand, on the day of the sacrifice, tissue samples (liver and colon) were obtained for gene expression analysis.

RNA isolation, reverse transcription and gene expression

Total RNA was obtained from liver and colon tissues (nearly 30 mg) using the TOTALLY RNA™ Kit (Ambion® Applied Biosystems). Isolated RNA (5 µg) was converted into cDNA using *SuperScript™ III First-Strand Synthesis SuperMix for RT-PCR* Kit (Invitrogen™, Life Technologies); both procedures were performed according to the manufacturer´s instructions. *Igf-1r, Insr,* and *Igf-1* gene expression was realized by real-time PCR using Light Cycler® FastStart DNA Master Plus SYBR Green I Kit (Roche, Germany). Design of primers was done using Oligo 6 analyzer software. *Actb* was used as a housekeeping gene. Triplicate amplification reactions were performed in a 2.0 Light Cycler® (Roche, Germany), under the following cycling conditions: ***Actb* gene**: 95 ˚C for 10 min and 40 cycles of 95 ˚C for 10 sec, 60 ˚C for 10 sec, and 72 ˚C for 9 sec. Forward primer: 5´-TAC CAC TGG CAT TGT GAT GG-3´and reverse primer: 5´-AGG GCA ACA TAG CAC AGC TT-3´. ***Insr* gene**: 95 ˚C for 10 min and 40 cycles of 95 ˚C for 10 sec, 59 ˚C for 10 sec, and 72 ˚C for 11 sec. Forward primer 5`-TCT TCG AGA ACG GAT CGA GT-3`and reverse primer 5'-CAC AAA CTT CTT GGC GTT CA-3`. ***Igf-1* gene**: 95 ˚C for 10 min and 40 cycles of 95 ˚C for 10 sec, 59 ˚C for 10 sec, and 72 ˚C for 14 sec. Forward primer 5´-AAC CTG CAA AAC ATC GGA AC-3´and reverse primer 5´-GCA GCC AAA ATT CAG AGA GG-3´. ***Igf-1r* gene**: 95 ˚C for 10 min and 40 cycles of 95 ˚C for 10 sec, 60 ˚C for 10 sec, and 72 ˚C for 10 sec. Forward primer 5´-GAC AGT GAA TGA GGC TGC AA-3´and reverse primer 5´-CCA GCC ATC TGG ATC ATC TT-3´. As a negative control, sterile water rather than cDNA was used. The crossing

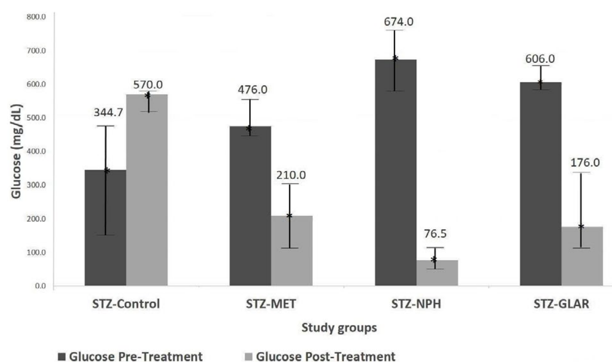

Figure 1. Pre and post-treatment determination of the serum glucose levels (mg/dl) in each study group. The "X" axis shows the study groups and the "Y" axis the glucose levels. To evaluate the glucose level differences in pre- and post-treatment, a Student t-test was used (P>0.05, 95%)

of threshold (Ct) values obtained for the target genes were normalized against housekeeping gene (*Actb*) Ct values. The relative quantification of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Melting curve analysis was performed to confirm the amplification of single amplicons for each gene analyzed (39).

Statistical analysis

A paired t-test was used to compare the pre- and post-treatment glucose levels. For the gene expression analysis, the mean is depicted in relative expression units (REU). Differences in the expression of each gene (*Insr, Igf-1r,* and *Igf-1*) were assessed between experimental groups by the Kruskal-Wallis test and *post hoc* adjustment Mann-Whitney U Test. PASW statistical software (ver. 18) was used for the data analysis (Chicago, IL, USA). The data were represented as mean ± SEM; P-value < 0.05 at (CI 95%) was taken as the level of significance.

Results

Pre and post-treatment glucose levels

Figure 1 shows the pre- and post-induction glucose levels as medians in all experimental groups. When comparing serum glucose levels between the pre- and post-treatment experimental groups (metformin, glargine/NPH insulins), in the GLAR group the reference value diminished 66% (day 0) (P<0.005), while in the NPH group it decreased 88% (P<0.001).

Gene expression analysis

All analyzed genes (*Igf-1, Igf-1r,* and *Insr*) showed a single melting peak under specific amplification conditions. The results are depicted as REU, which correspond to $2^{\wedge-(\Delta\Delta CT)}$ of each analyzed gene (Figure 2). In liver, the *Insr* gene was significantly overexpressed in the GLAR group (3.66 REU) (P<0.05) compared with the control group; whereas, in the NPH group, this gene showed significant subexpression (0.17 REU) (P<0.05). The *Igf-1* gene showed overexpression in the NPH group compared to the control group (P<0.05). In contrast, the *Igf-1* gene was under-expressed in the MET and GLAR groups (P<0.05). On the other hand, the *Igf-1r* gene was overexpressed in all groups of treated rats, but mainly

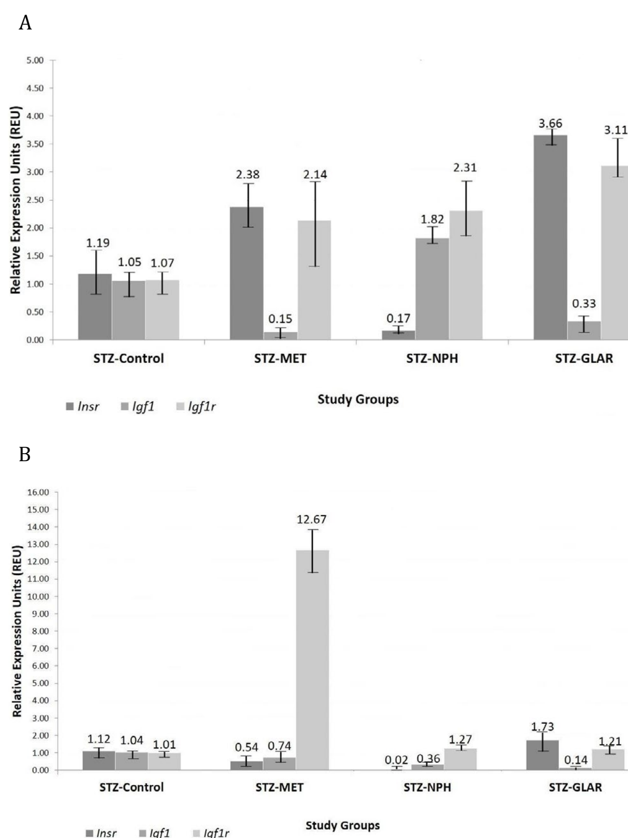

Figure 2. A) Relative expression of *Insr, Igf-1*, and *Igf-1r* genes in the hepatic tissue. B) Relative expression of *Insr, Igf-1,* and *Igf-1r* genes in the colon tissue. Bars show the mean and standard error of the mean values (SEM); *P<0.05

in the group treated with insulin glargine (3.11 REU) (P<0.05) (Figure 2A). In the colon, the *Insr* gene showed significant overexpression only in the GLAR group (1.73 REU). The *Igf-1* gene showed subexpression with no statistical significance in all treated groups (Figure 2B). Finally, the *Igf-1r* gene was overexpressed in the MET group (12.67 REU) (P<0.05) (Figure 2B).

Discussion

This report analyzes for the first time the effect of insulin glargine and other hypoglycemics agents on the expression of *Insr, Igf-1r,* and *Igf-1* in a model of STZ-induced diabetic rats. Insulin analogs are insulin molecules artificially modified by DNA recombinant technology; exhibiting some chemical variations compared with natural insulin, which result in an altered pharmacokinetic profile, as described for the insulin glargine (38). The NPH insulin is an intermediate-acting molecule characterized by a slow onset of action and by more prolonged activity regarding regular insulin (40). Metformin is also a hypoglycemic drug, widely used and with obvious benefits on the glucose metabolism and diabetes-related complications; the metformin treatment improves insulin sensitivity in sensitive tissues such as liver, fat, and muscles (41).

In humans, one of the conditions predisposing to T2D is obesity (2). In this study, the selection of an animal model that develops diabetes exclusively was crucial to eliminate or reduce the obesity effects, the inflammatory

factors and other possible confounding variables (42-43). All groups of STZ-induced rats developed hyperglycemia, which is a universal biochemical condition in T2D; likewise, all rats from each group (control, MET, NPH, and GLAR) kept the 200–250 g weight range during the study. Among the treated groups with hypoglycemic agents, the NPH group kept the lowest glycemic levels among groups treated with insulin glargine or metformin.

Gene expression analysis in this study (Figures 2A and 2B) revealed overexpression of the *Insr* and *Igf-1r* genes in hepatic and colonic tissues from rats treated with insulin glargine (GLAR group), although the *Igf-1r* overexpression in colonic tissue was also noteworthy in the group treated with metformin (MET group). Instead, the *Igf-1* gene was under-expressed in the GLAR and MET groups. A similar observation was reported in a previous study in diabetic rats (44); they proposed that the effect of diabetes on the *IGF-1* expression in liver initiated in early diabetic phases, results in down expression of *IGF-1*, and evolves with the severity and extent of diabetes.

It has been described that both IR and IGF-1R participate in an intricate biochemical network that controls diverse cell processes and is accountable for many of the actions in which insulin is involved, including their pleiotropic effects. This network occurs through phosphorylation and de-phosphorylation reactions of some substrates that take part in two principal signaling pathways: PI3K/AKT, which participate in the insulin metabolic actions, and Ras/MAPK/ERK, responsible of mitogenic actions of the hormone. Such delineation in two different ways for metabolic or mitogenic effects of insulin is highly complex because these two pathways are interconnected by minor pathways and involve hundreds of different isoforms (30).

Experimental studies have shown that overexpression of *Igf-1r* induces tumor growth and metastasis (45). IGF-1R is a receptor tyrosine kinase which triggers the phosphatidylinositol-3 kinase (PI3k)/AKT signaling pathway (46) achieving many essential roles in several physiological processes including cell growth, proliferation, and survival (47). Within the IGF signaling pathway, the insulin receptor (IR) and the IR-A and IR-B isoforms ratio also exert cancer-promoting functions through total insulin levels (48-50). The IGF axis organizes an interactive network of the peptide-ligands IGF1 and IGF2 and insulin, and the receptors IGF1R, IGF2R, and IR as IGF binding proteins (IGFBPs) (50, 51). IGF1R and IR show a significant structural similarity, which allows the construction of IGF1R/INSR hybrid receptors, which are active in both IGFs and insulin ligands (50, 52-55).

Several studies have tried to elucidate the possible mitogenic effect of insulin glargine in diabetic patients, experimental diabetes, and cancer models. Kurtzhals *et al*. (56) found that glargine possesses 6–8 times higher affinity for IGF-1R and that this mitogenic potential correlates with results found in the liver tissue (1.92 REU). Likewise, in colorectal cell lines (HCT-116), prostate cancer (PC-3), and breast adenocarcinoma (MCF-7), insulin analogs (glargine and detemir) had similar effects on IGF1 as those reported for cell proliferation and resistance to apoptosis (14). Besides, it has been demonstrated that glargine induces phosphorylation of IR, IGF1R, ERK, and AKT in cultured cancer cells, suggesting the possibility of stimulation of the MAPK and PI3K-AKT pathways (18).

The overexpression of *Insr* and *Igf-1r* genes in liver and colon of rats treated with insulin glargine observed in this study probably related with the increased affinity of glargine toward these receptors and with the subsequent increase of both metabolic and mitogenic pathways in hepatocytes and colonocytes. On the other hand, it is known that different isoforms of IR (A and B) are differentially expressed in diverse tissues; thus, IR-A is highly expressed in the kidney and the brain, while IR-B is expressed in the human liver (57). This alternative splicing is hormonally regulated, but it can be altered during development and under some pathological conditions such as T2D and cancer (57). Therefore, the *Insr* gene overexpression in the liver tissue, observed in this study, perhaps is related predominantly with the IR-B isoform.

Although it is proposed that insulin glargine has a potential mitogenic effect due to higher affinity for IGF-1R and IR and its consequent activation of metabolic and mitogenic signaling pathways, the molecular mechanism is still unidentified. Based on our results, which show an evident overexpression of both IGF1R and IR receptors, mainly in hepatic tissues of rats treated with glargine, it is reasonable to suppose that glargine, in addition to inducing metabolic and mitogenic signaling, might induce by a different and unknown pathway, the transcription of the *Igf-1r* and *Insr* genes, giving rise consequently to an increased number of receptors in the cytoplasmic membrane that stimulates these signaling pathways, inducing a vicious circle that finally begins a neoplastic process.

We also observed a dominant *Igf-1r* overexpression in the colonic tissue in the group treated with metformin (MET group). Although it has been reported that metformin inhibits cell proliferation by activating AMPK, which counteracts the PI3K/AKT and MAPK pathways downstream of the insulin-IGF1 receptors (58), and moreover, that metformin downregulates the insulin/IGF-I signaling pathway (59-61), researchers (62) observed overexpression of the *IGF1R* gene (4.7-fold change, $P < 0.001$), but no overproduction of IGF1R in human endometrial stromal cells in response to treatment with metformin and insulin. In that sense, it is expected that the increased expression of the *IGF1R* gene, observed in the MET-group rats in our study, could not be translated in receptors; however, this assumption should be tested.

Leibiger *et al*. (63) described that the overexpression of A-type isoform of the insulin receptors led to a noticeable activation of the insulin promoter in response to either glucose or insulin stimulus. Such an observation allowed suggesting that insulin activates the transcription of its gene by signaling through the A-type insulin receptor. In the present study, rats from the different groups, including the control group, were STZ-induced T2D animals that showed variable degrees of hyperglycemia; however, the IR and IGF-1R however, the IR and IGF1R expression are significantly increased in the GLAR group respect the control group. Such a difference probably means that the glycemic level is

not a major stimulus to reach the overexpression of these receptors, at least in comparison with the insulin glargine.

Conclusion

Although the molecular mechanisms causing neoplastic transformation in the presence of IR and IGF1R overexpression are not fully understood, our observations showed that insulin glargine promotes an excess of insulin and IGF-1 receptors in a model of diabetic STZ-induced rats, which could play a central role over stimulating the mitogenic signaling pathways.

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Conflicts of Interest

The authors declare no conflicts of interest

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