

REVIEW

## Tissue barriers and novel approaches to achieve hepatoselectivity of subcutaneously-injected insulin therapeutics

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### ABSTRACT

Current subcutaneously (s.c.)-injected insulin (INS) products result in a hyperinsulin exposure to peripheral tissues (skeletal muscle and adipose) while INS hardly accesses to liver after injection. This unphysiological distribution raises risks of hypoglycemia episode and causes weight gain after long term treatment. An ideal INS replacement therapy requires the distribution or action of exogenous INS to more closely mimic physiological INS in terms of its preferential hepatic action. However, there are 2 factors that limit the ability of s.c. injected INS to restore the liver: peripheral gradient in INS deficient diabetes patients: (1) the transport of INS in capillary endothelium and peripheral tissues from the injection site; and (2) peripheral INS receptor (IR) mediated INS degradation. In this review, the tissue barriers against efficient liver targeting of s.c. injected INS are discussed and current advances in developing hepatoselective insulin therapeutics are introduced.

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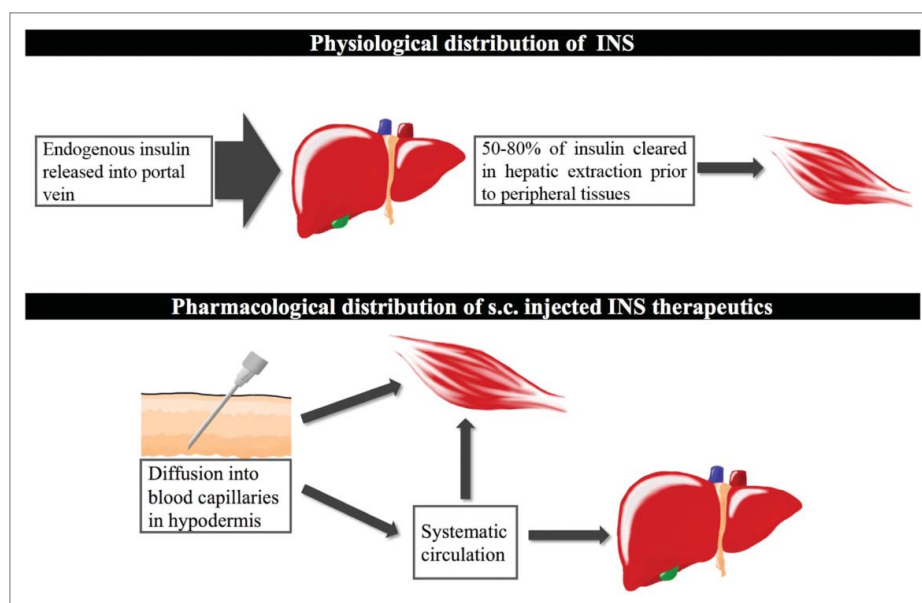
### KEYWORDS

diabetes; hepatic-directed vesicle INS; hepatoselectivity; IR-B selective INS analog; Peglispro; proinsulin-transferrin; subcutaneously-injected insulin; thyroxyl-INS analog

Liver is the primary action site of physiologically secreted insulin (INS). INS secretion bursts are synchronous with blood glucose (BG) fluctuations and the oscillations of intracellular calcium levels.<sup>1</sup> After exocytosis of secretory granules from pancreatic  $\beta$  cells, released INS is first delivered to the hepatic portal vein, where the concentration of INS fluctuates from 100 to 1000 pM, and then INS reaches the hepatic sinusoids. Hepatic sinusoids are unique capillary cells due to their open endothelial pores (50–300 nm diameter in humans) and discontinuous basal lamina lacking a diaphragm.<sup>2</sup> These sinusoids function as a loose molecular sieve allowing substance exchange between the hepatic artery through the perisinusoidal space to the hepatocytes. In the lumen of hepatic sinusoids, INS freely diffuses through the highly permeable endothelium and is exposed to insulin receptors (IR) on hepatocytes. A substantial amount of INS is degraded in the liver after IR mediated endocytosis,<sup>3</sup> with a hepatic clearance of 50~80%. The remaining INS is circulated systemically, where the range of INS is 10 to 30 pM at basal conditions,<sup>4</sup> and then reaches peripheral tissues including skeletal muscle and adipose tissue. Different from the microvessels aligning hepatocytes, peripheral blood

capillaries are considered as continuous and tight (fenestration 6–12 nm).<sup>5</sup> The transendothelial movement of INS is non-receptor mediated and non-saturable. According to previous studies, the ratio of INS concentration in the plasma versus the interstitial fluid of skeletal muscles significantly decreased during INS infusion at higher pharmacological levels ( $1.37 \pm 0.25$ ) compared to lower physiological levels ( $1.98 \pm 0.21$ ).<sup>6</sup> The higher amount of infused INS delivered to skeletal muscles at the ultraphysiological level suggests the transport efficiency and capacity increased under higher plasma INS concentrations.

Current INS therapeutics administered via the s.c. route are unable to recapitulate physiological INS distribution (Fig. 1). The connective tissue septa comprise the first physical and catabolism barriers against INS absorption. INS, which is below the upper limit of capillary uptake (16 kDa), primarily accesses systemic circulation via either passive diffusion through small pores ( $< 30 \text{ \AA}$ ) or paracellular transport pathway in capillary endothelium.<sup>7</sup> The overall influence of "first-pass catabolism" on protein biologics disposition is not conclusive. However, studies have shown that the bioavailability (BA) of s.c. injected INS in humans was as high as 84%, indicating minimal



**Figure 1.** Comparison of physiological insulin distribution and exogenous insulin distribution. Under physiological conditions, insulin was secreted by pancreatic  $\beta$  cells and delivered to hepatic portal vein. 50-80% of insulin was cleared out in liver before its exposure upon peripheral tissues.<sup>3</sup> Exogenous insulin after s.c. injection appeared in blood capillaries through diffusion. It imposed hepatic hypoinsulinemia and peripheral hyperinsulinemia compared to the physiological hepatic/peripheral insulin gradient.

catabolic clearance.<sup>8</sup> Even so, BA may vary on a case-dependent manner for individual INS analogs. When evaluating the influence of the s.c. catabolic barrier for each INS analog, factors including injection site (abdominal vs appendicular side), retention time at injection site (fast-acting INS vs depot effect of long-acting INS), as well as amino acid (AA) sequence (native INS vs INS analogs with AA substitution) should be considered.<sup>9,10,11</sup> Once in systemic circulation, INS is almost equivocally distributed between liver and peripheral tissues. The plasma concentration of pharmacological INS is approximately 2.5-3.5 times of circulated INS in normal subjects.<sup>12</sup> This higher pharmacological insulin concentration predisposes peripheral tissues into hyperinsulinemia and leaves the liver under-insulinized. Liver hypoinsulinemia was reported in studies on rats, where it was shown that when arterial INS levels were clamped at twofold above basal levels during peripheral INS infusion, the portal vein INS concentration was still marked as "deficient," or below the basal level.<sup>13</sup> Peripheral IR mediated degradation and the short plasma  $T_{1/2}$  (5 min) of INS may presumably attribute to observations in those early studies, which showed that only 1% of exogenous INS was delivered to the liver.<sup>14</sup> Therefore, in order to obtain sufficient INS concentrations in the liver, peripheral hyperinsulinemia is

necessary. Moreover, s.c. INS administration shifts the main burden of INS metabolism from the liver to the kidney compared with endogenous INS. Subsequently, it also raises the hypoglycemia risk for renal impaired patients.<sup>3</sup> Further, the higher amount of INS exposed to the peripheral tissues is also responsible for many of the adverse metabolic side effects and increased risk of mitogenicity associated with exogenous INS therapy.<sup>15</sup>

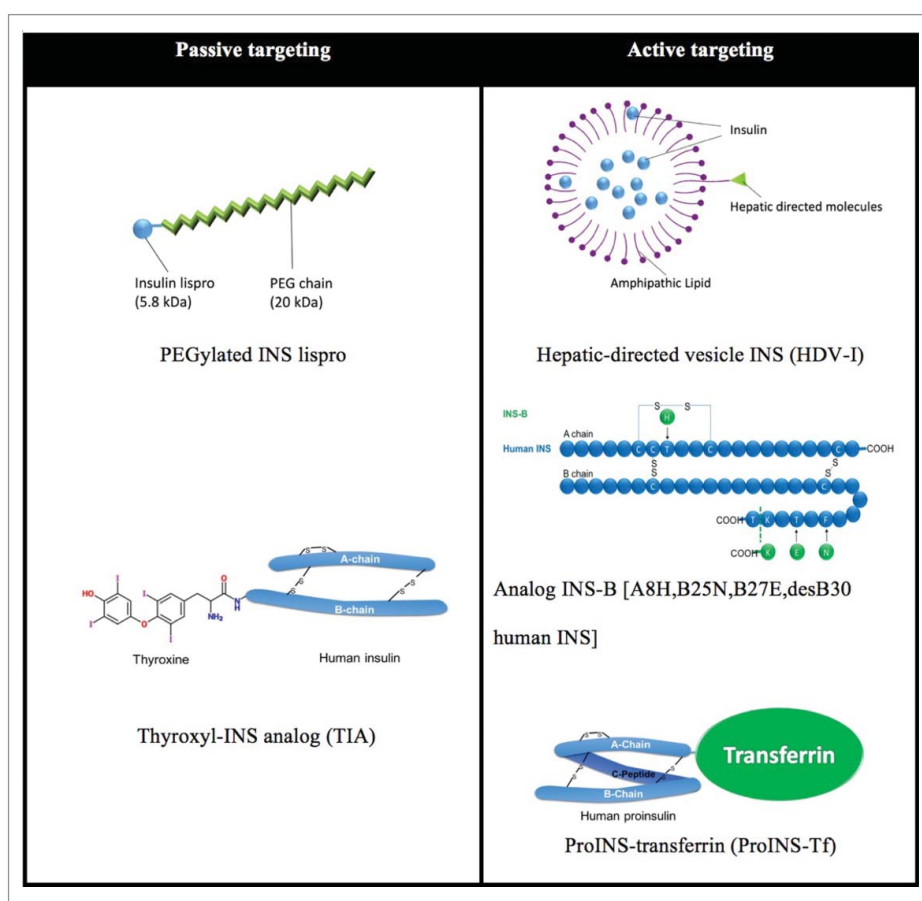
Direct delivery of INS to the hepatic portal circulation allows efficient liver-targeting. According to empirical calculations, continuous intraportal INS infusion (0.35–0.56 U/kg/day) together with peripheral INS supplementation (0.08–0.11 U/kg/day) can reproduce the physiological distribution of INS in type 1 diabetic patients.<sup>1</sup> However, such complicated delivery system is unfeasible and not suitable for self-administration by diabetic patients, where current administration via a s.c. bolus provides patient compliance and remains the main route for long-term INS treatment.<sup>16</sup> As mentioned, the physiological barriers of peripheral tissues and liver when using a remote administration route (s.c.) hamper the ability of exogenous INS to mimic portal vein INS secretion. This review summarized current INS analogs and formulation refinements aimed at achieving liver targeting of s.c. injected INS (Fig. 2). Some examples are emerging

novel concepts and some have been approved following clinical studies. By comparison and discussion of diverse targeting machineries, the aim of this review is to shed light on potential solutions to develop effective liver targeting INS therapy.

### Current strategies in liver targeted insulin delivery

Current liver targeted INS analogs fall into either passive or active targeting mechanisms (Fig. 2). INS analogs, with increased size compared to INS (5.8 kDa), achieve hepatoselectivity by passive liver-targeting. These INS analogs exploit permeability differences between hepatic

sinusoids and peripheral blood capillaries. Large molecules, which have restricted transport across peripheral capillaries, can easily diffuse through the open-pored hepatic sinusoids. Moreover, molecules with a molecular size above the renal filtration threshold ( $> 50\text{--}60$  kDa) attain longer plasma  $T_{1/2}$  and protracted absorption as an s.c. depot. Thyroxyl insulin analogs<sup>17</sup> and insulin detemir<sup>18</sup> form larger complex via binding to plasma proteins: thyroxine-binding globulin (TBG; MW 54 kDa) and human serum albumin (HSA; 65 kDa), respectively. Another example is Peglispro (LY2605541, Eli Lilly and Company), which is composed of a 20 kDa PEG attached to lysine B28 on INS lispro. Each PEG monomer binds 3 molecules of water, resulting in a



**Figure 2.** Two targeting approaches are exploited in the development of liver targeted INS therapy. Passive targeting by size increment of INS molecule (5.8 kDa) takes advantage of the different endothelial vascular sieves in peripheral tissues vs. the liver. Large molecules, which minimally diffuse through peripheral capillary walls, are freely filtered through fenestrated hepatic sinusoids. PEGylated INS lispro (Peglispro, LY2605541) is covalently coupled with a 20 kDa polyethylene glycol (PEG) and attains a hydrodynamic radius as large as a 71–98 kDa globular protein. Thyroxyl INS analog (TIA) is semi-synthesized by covalently linking thyronine to the  $\epsilon$ -amino group of PheB1. In the active liver targeting area, hepatic-directed vesicle INS (HDV-I) encapsulates human INS in lipid vesicles ( $< 150$  nm in diameter) which have hepatocyte-targeting molecules (HTM) incorporated on the surface. HTMs include molecules targeting the asialoglycoprotein (galactose) receptor, hepatobiliary receptor or biotin receptor on hepatocytes. INS-B [A8H, B25N, B27E, desB30 human INS] is produced as an INS precursor in yeast with site directed mutagenesis, and the precursor is then enzymatically converted into 2-chain desB30 analogs.<sup>30</sup> ProINS-Tf is expressed in mammalian HEK293 cells by recombinant DNA techniques. MW increments of HDV-I and ProINS-Tf also presumably contribute to hepatoselectivity by passive targeting effect.

hydrodynamic radius as large as 71-98 kDa for Peglispro.<sup>19</sup> The preferential hepatic action of Peglispro has been substantiated by several studies by comparing the ratio of the peripheral glucose disposal (PGD) rate, Rd, to the hepatic glucose production (HGP) rate, Ra. In euglycemic clamp studies on conscious dogs, the change from baseline in glucose Rd-to-Ra ratio was 0.5-0.6 in the Peglispro infusion group compared with  $1.4 \pm 0.3$  in the INS group.<sup>20</sup> It is believed that the large hydrodynamic radius of Peglispro impedes its transport across peripheral blood capillaries to the interstitial fluid of skeletal muscles and adipose, which leaves more Peglispro available for filtration through hepatic sinusoids.<sup>19</sup> In addition, the reduced binding affinity of Peglispro to IR (less than 6% of INS lispro) possibly decreases peripheral IR-mediated clearance and contributes to a longer residence time in liver microcirculation.<sup>21</sup> However, hepatopreference of Peglispro was prominent only below a certain dose. When the amount of infused Peglispro increased, PGD also increased indicating decreased hepatoselectivity.<sup>20</sup>

In active liver-targeting, one approach is to preferentially target IR isoform B (IR-B), which is the dominant isoform expressed on human liver compared to the other isoform of IR, IR-A. The INS analog INS-B [A8H, B25N, B27E, desB30 human insulin]<sup>22</sup> displays a 2 to 4-fold higher binding affinity toward IR-B than IR-A. In *ex vivo* rodent studies, INS-B exhibited higher potency in hepatic glycogen accumulation (75% potency of human INS) than glycogen synthesis in skeletal muscles (45% potency of human INS). The hepatoselectivity of INS-B largely depends on tissue-specific expression of IR isoforms. However, IR-B is also prominently expressed on skeletal muscles in humans.<sup>23</sup> This discrepancy obligates further investigation of INS-B's liver-targeting in alternate animal models which are more human-relevant. Another example in actively targeting INS to the liver is hepatic-directed vesicle INS (HDV-I). HDV-I entraps INS inside vesicles, along with hepatocyte-targeting molecules (HTM) attached to the amphipathic lipid layers. After absorption and vesicle release, free INS transits to systemic circulation and triggers peripheral IR activation. Meanwhile, lipid layer-associated INS is driven to hepatocytes by virtue of the HTM/hepatic receptor mediation. INS is then dissociated from the lipid complex over time and exerts INS action in the liver.<sup>24,25</sup> HTM is composed of molecules targeted either the

asialoglycoprotein (galactose) receptor, hepatobiliary receptor or biotin receptor on hepatocytes. The amount of INS delivered to liver has been enhanced in the HDV-I formulation. Still, the vast majority of HDV-I was the free form encapsulated inside of vesicles (99% free INS).<sup>25</sup> In addition, vesicles with increased diameter over 90 nm exhibit increased uptake by nonparenchymal cells which decreases the specificity to hepatocytes.<sup>26</sup> The final example of active liver targeting is Proinsulin-transferrin (ProINS-Tf) fusion protein, which consists of the inactive human INS precursor, ProINS, and Tf. In cultured adipocytes and after short-term incubation in hepatocytes, ProINS-Tf itself displayed weak INS potency, similar to ProINS. However, following prolonged incubation with hepatocytes, ProINS-Tf was converted to immuno-reactive INS-transferrin (irINS-Tf). The resultant irINS-Tf acquired enhanced INS potency and was recognized by human INS specific radioimmunoassay.<sup>27</sup> When evaluated in a type 1 diabetes mouse model (streptozotocin-treated mice), ProINS-Tf exerted a delayed but prolonged BG lowering effect *in vivo*.<sup>28</sup> The delayed effect was attributed to the requirement of conversion from ProINS-Tf to irINS-Tf to achieve bioactivity, while the prolonged effect was attributed to Tf, a stable protein established for its ability to prolong the  $T_{1/2}$  of fused proteins.<sup>29</sup> Compared with native INS, ProINS-Tf led to a higher ratio of hepatic/peripheral IR activation and enhanced hepatic glycogen accumulation after an *s.c.* bolus injection. Uniquely, ProINS-Tf is the first described liver targeted INS prodrug with low stimulation of PGD.<sup>27</sup> In contrast, the aforementioned INS analogs and formulation refinements still bring circulated active INS to peripheral tissues. Consequently, the efficiency of their hepatopreference may be attenuated during dosage escalation (smaller Ra to Rd ratio), which is a potential drawback. However, compared with most INS analogs listed in this review, the anti-diabetic effect of ProINS-Tf has only been tested on rodent models so far. Therefore, its hepatic action on human and liver toxicity needs further exploration.

## Conclusion

INS therapy with improved glycemic control without compromising safety issues remains a critical challenge for chronic treatment in diabetes. Currently applied s.

c. INS analogs or formulations work mostly to achieve an anti-hyperglycemic effect via promoting PGD, and reverse the physiological portal: peripheral INS gradient. Liver targeted INS mimicking endogenous INS distribution may be able to restore normal glucose control. The use of liver targeted INS also reduces the required peripheral INS dose, thereby reducing the risk of subsequent hypoglycemia. Liver-targeted INS analogs may serve as the next generation of INS therapy in diabetes treatment with improved safety.

## Abbreviations

Rd peripheral glucose disposal rate  
Ra hepatic glucose production rate  
PGD peripheral glucose disposal

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