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Insulin up-regulates a $Glc_3Man_9GlcNAc_2$-PP-Dol pool in capillary endothelial cells *not* essential for angiogenesis

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Endothelial cells line blood vessels, and their proliferation during neovascularization (*i.e.*, angiogenesis) is essential for a normal growth and development as well as for tumor progression and metastasis. Mechanistic details indicated that down-regulation of $Glc_3Man_9GlcNAc_2$-PP-Dol level reduced angiogenesis and induced apoptosis in capillary endothelial cells (Martínez JA, Torres-Negrón I, Amigó LA, Banerjee DK, *Cellular and Molec Biochem* 45, 137–152 (1999)). Unlike in any other insulin-responsive cells, insulin reduced capillary endothelial cell proliferation by increasing the cell doubling time. But, when analyzed, the rate of lipid-linked oligosaccharide-PP-Dol (LLO) synthesis as well as its turnover (*i.e.*, $t_{1/2}$) were increased in insulin treated cells. No major differences in their molecular size were observed. This corroborated with an enhanced glycosylation of Factor VIIIC, an N-linked glycoprotein (essential cofactor of the blood coagulation cascade) and a marker for the capillary endothelial cell. Increased LLO synthesis was independent of elevating either Dol-P level or Man-P-Dol synthase gene (dpm) transcription. Insulin however, enhanced 2-deoxy-glucose transport across the endothelial cell plasma membrane and caused increased secretion of Factor VIIIC, thus, supporting the existence of additional LLO pool(s), and arguing favorably that growth retardation of capillary endothelial cells by insulin turned a highly proliferative cell into a highly secretory cell.
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Keywords: **angiogenesis, capillary endothelial cells, insulin, lipid-linked oligosaccharide, glucose transport, N-linked glycoprotein, Man-P-Dol synthase, cell prolifeartion**

Abbreviations: **EMEM: minimal essential medium with Earle's salt; DMEM: Dulbecco's essential medium; PMSF: phenylmethylsulfonyl fluoride; NP-40: Nonidet P-40; TPCK: N-tosyl-l-phenylalanine cholоromethyl ketone; SDS: sodium dodecyl sulfate; HRP: horseradish peroxidase; FBS: fetal bovine serum; LLO: oligosaccharide-lipid; Dol-P: dolichylmonophosphate; DMSO: dimethylsulfoxide; SRM: standard reaction mixture; EDTA: ethylenediaminetetraacetic acid.**

Introduction

Glycosylation is a means of diversifying a protein without altering the DNA sequence, and it has the potential to both respond and reflect environmental changes. N-glycoproteins contain one or more glycan chains attached through N-glycosidic linkage to the asparagine residues present in the consensus sequence (or sequon) Asn-Xaa-Ser/Thr, where Xaa may be any amino acid with the possible exception of proline and cysteine [1]. The biological roles of these glycans span the spectrum from maintaining glycoprotein structure to being crucial for development, growth, function, or survival of an organism such as formation of new blood vessels, *i.e.*, neovascularization [2].

Neovascularization is formed by vasculogenesis or angiogenesis. The former is *de novo* vessel formation from mesoderm-derived endothelial cell precursors called angioblasts, and the angiogenesis is remodeling from the primary plexus in the embryo or from preexisting vasculature in the adult animal [3]. Remodeling and pruning processes are common to angiogenesis and vasculogenesis during embryonic development. However, the events that include both the growth of new vessels and regression of others are less understood [4]. In adult mammals, it is a tightly regulated and self-limited process [5,6] and takes place only during wound healing and in the female reproductive cycle. Nonetheless, it has a critical role in a pathological situation, such as diabetic retinopathy, arthritis, hemangioma,

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and psoriasis in addition to malignant tumor growth. Tumor cells require a vascular network to support growth by allowing "perfusion" of nutrients, oxygen, and waste products through a crowded cell population [7]. These cells also exploit the vessel network for cell intravasation and subsequent metastasis [8].

The cellular and molecular events leading to angiogenesis make up a complex multi-step process and occur in stages that orchestrate a network of cooperative interactions. These include (i) initiation, characterized by increased cell membrane permeability; (ii) progression, constituted by production of proteolytic enzymes that degrade the extracellular matrix and basement membrane of preexisting blood vessels and promote endothelial cell migration, and the entry of cells into either a proliferative or a "programmed cell death"; (iii) production of extracellular matrix allowing the reconstitution of the basement membrane; (iv) differentiation into new vessels; and (v) the stabilization and maturation of vessels by mediator molecules that recruit mesenchymal cells to vessel walls [5,6,9]. Several directly angiogenic and relatively specific growth factors have been isolated: vascular endothelial growth factor A (VEGF-A), VEGF-B, VEGF-C, and placental growth factor (PIGF) are among the best characterized. These glycoproteins display high amino acid similarity in the platelet-derived growth factor (PDGF) domain [5].

Glycosylation affects the structure and function of a protein in four major ways. The first two involve intramolecular interaction between sugar and protein, either directly or with a ternary complex. The others depend mainly on intermolecular interactions. First, oligosaccharides modify local structure and overall dynamics of the protein to which they are attached. Second, oligosaccharides may also modify the functional activity of a protein [10,11]. For example, processing of the glycan chains to a 'high-mannose' type or to a 'complex' type is essential for endothelial cell differentiation into capillary tubes [12–14]. The process is accelerated upon stimulation of endothelial cell proliferation and capillary lumen formation by cAMP, an intracellular signal enhancing glycan chain synthesis [15,16]. We hypothesize that availability of $Glc_3Man_9GlcNAc_2$-PP-Dol (LLO) is essential for survival of endothelial cells, and observed that down-regulation of (LLO) synthesis by blocking the GcNAc-1phosphate transfrease by a pyrimidine nucleoside, tunicamycin or tying up intracellular dolichylmonophosphate (Dol-P) by a lipopeptide, amphomycin reduced angiogenesis, and induced apoptosis in capillary endothelial cells [15,17].

Earlier we have observed that insulin imparted a negative growth response on proliferating capillary endothelial cells, and delayed the population doubling [18]. We, therefore, evaluated if insulin-mediated cell growth retardation followed a direct relationship with the LLO synthesis. The result was, insulin signaling up-regulated LLO biosynthesis in capillary endothelial cells. There was neither an activation of the Man-P-Dol synthase nor an increased expression of the synthase gene. Also, insulin did not up-regulate the intracellular Dol-P level. However, increased glucose transport in the presence of insulin made us to conclude that elevated levels of sugar-nucleotides are responsible for the enhanced glycan chain synthesis.

Materials and methods

The capillary endothelial cells used in this study were from a clonal cell line isolated from bovine adrenal medulla by differential plating. D-[2-^{3}H]-mannose (Sp. Act. 18.5 Ci/mmol), GDP-[U-^{14}C] mannose (Sp. Act. 307 mCi/mmol), [^{35}S]-methionine (Sp. Act. 1400Ci/mmol) and [^{14}C] methylated protein standards (10–50 Ci/mg protein) consisting of myosine (M_r 200,000 dalton), phosphorylase b (M_r 92,500 dalton), bovine serum albumin (M_r 69,000 dalton), ovalbumin (M_r 46,000), carbonic anhydrase (M_r 30,000), and lysozyme (M_r 14,300 dalton) were purchased from Amersham Pharmacia Biotech, Piscataway, NJ. *N*-tosyl-1-phenylalanine chloromethyl ketone (TPCK), leupeptine, aprotinin, phenylmethyl- sulfonyl fluoride (PMSF), soybean trypsin inhibitor, dolichyl monophosphate, insulin, bovine serum albumin (crystalline), HEPES, and blue dextran were obtained from Sigma Aldrich, St. Louis, MO. Nonidet P-40 was from Bethesda Research Laboratories, MD. Autofluor was a product of National Diagnostic, Manville, NJ. Minimal essential medium with Earle's salt (EMEM), glutamine, penicillin-streptomycin, and trypsin-versine were supplied by BioFluids Inc., Rockville, MD. Fetal bovine serum was a product of HyClone Laboratories, Logan, UT. Immobilon PVDF transfer membrane was a gift from Millipore Corporation, Bedford, MA. Mouse monoclonal antibody (IgG1k) to human factor VIIIC was obtained from Boehringer-Mannheim, Indianapolis, IN. Nitroblue tetrazolium chloride, 5-bromo-4-chloro-3-indoylphosphate-toluidine salt, sodium dodecyl sulfate, acrylamide, bis-acrylamide, TEMED, Tween-20, bromophenol blue, and 2-mercaptoethanol were purchased from Bio-Rad Laboratories, Hercules, CA. Horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin G was a product of Miles Laboratories, La Jolla, CA. Ready Protein, was from Beckman Instruments, Fullerton, CA. All other chemicals and solvents were of highest purity grade and obtained from commercial suppliers.

Culturing of capillary endothelial cells

The stock culture of capillary endothelial cells was maintained in minimal essential medium with Earle's salt (EMEM) supplemented with 10% fetal bovine serum (FBS, heat-inactivated), glutamine (2 mM), penicillin (50 units/ml) and streptomycin (50 μg/ml) at 37°C in a humidified incubator (5% CO_2–95% air) as described before [19]. Cells were sub-cultured once a week unless otherwise mentioned.

Metabolic labeling of cells with D-[2-^{3}H]-mannose and isolation of [^{3}H]Man-P-Dol, [^{3}H]Man-oligosaccharide -PP-Dol and [^{3}H]Man-glycoprotein fractions

Cells (1×10^6) were seeded in 60 mm petri dishes in 5 ml of EMEM containing 10% fetal bovine serum and cultured for 8 days. At the end of the culture period, cells were washed twice with DMEM (low glucose) containing no serum (serum-free) and incubated at 37°C for 0–4 h with D-[2-^{3}H]mannose (10 μCi/ml) in the presence or absence of insulin (1 μg/ml) in serum-free DMEM. At the end of the incubation, media were separated, cells were washed three times with 0.5 ml of ice-cold phosphate-buffered saline pH 7.4 (PBS), removed with a rubber policeman, and collected after centrifuging at 1,000 rpm for 5 min in a Sorvall T6000B centrifuge.

One milliliter chloroform-methanol (2:1, v/v) was added into cell pellets and after 5 min [^{3}H]Man-Dol-P was extracted following centrifugation at 2,000 rpm in a Sorvall T6000B centrifuge at room temperature. The resulting supernatant was collected and the pellets were extracted twice as above. The combined chloroform-methanol extracts were washed once with 0.2 volume of 0.9% saline and twice with 0.5 volume of chloroform-methanol-water (3:47:48, v/v/v) as previously described [20]. The lower organic phase containing mannosylphospho-dolichol (Man-P-Dol) was dried under nitrogen. The remaining pellets were washed once with 0.9% saline, and twice with water. The pellets were then extracted three times with chloroform-methanol-water (10:10:3, v/v/v). The supernatants containing [^{3}H-Man]-oligosaccharide-PP-Dol were pooled and dried under nitrogen. The remaining pellet contained [^{3}H]Man-glycoproteins.

Preparation of dolichol-linked oligosaccharides for chromatographic analysis

[^{3}H-Man]-oligosaccharide-PP-Dol was treated with 0.1 N hydrochloric acid in 80% tetrahydrofuran at 50°C for 30 min to release oligosaccharide units [20]. Analysis of [^{3}H-Man]-oligosaccharide was carried out by gel filtration on a Bio-Gel P-4 (200–400 mesh) column (0.9 × 53 cm) using 50 mM ammonium formate, pH 8.0 containing 0.02% sodium azide (w/v).

Biosynthesis of factor VIIIC

The capillary endothelial cells cultured for 8 days in complete EMEM were washed with methionine-free and serum-free medium, labeled with [^{35}S]-methionine (40 μCi/ml) in methionine-free and serum-free medium containing aprotinin (1 μg/ml) at 37°C for 2 h in the presence and absence of insulin (1 μg/ml). At the end of the incubation period, media were removed and the cells washed in PBS were lysed on ice for 30 min with 1 ml of cell lysis buffer (20 mM Tris-HCl, pH 8.0 containing 0.15 mm NaCl, 1% NP-40, 1 μg/ml aprotinin). The cell lysates were centrifuged at 100,000 × g for 40 min at 4°C (using a Beckman 50 Ti rotor), and the supernatants were kept frozen till analyzed. Factor VIIIC in cell lysates and in the conditioned media was immunoprecipitated with anti-Factor VIIIC monoclonal antibody and analyzed by SDS-PAGE followed by autoradiography [21].

2-Deoxy-D-[2,6-^{3}H]-glucose uptake in capillary endothelial cells

The capillary endothelial cells were cultured for 8 days in 24 well clusters (16 mm well diameter) with an initial seeding density of 5×10^4 cells per well in 1.5 ml complete EMEM. At the end of the culture period, cells were washed twice with 1 ml of standard reaction mixture (SRM; 118 mM NaCl/1.2 mM $MgSO_4$/6.7 mM KCl/2.2 mM $CaCl_2$/10 mM mannitol/25 mM HEPES-NaOH, pH 7.4) and incubated at 37°C for 5–120 min in 0.5 ml of SRM containing 0.1–5.0 mM of 2-deoxyglucose plus 2-deoxy-D-[2,6-^{3}H]-glucose (1 μCi/ml) in the presence and absence of insulin (1 μg/ml). At the end of the incubation, cells were washed 3 × 1 ml with PBS and counted for radioactivity.

Determination of protein concentration, radioactivity, and statistical analysis

Total protein content in each sample was analyzed following Bradford's procedure [22] using bovine serum albumin as a standard. To measure radioactivity, samples were mixed with 5 ml of liquid scintillation fluid "Ready protein" and analyzed in a Beckman LS-3801 liquid scintillation spectrometer. The statistical analysis was carried out by the student 't' test.

Activity assay for mannosylphospho dolichol (Man-P-Dol) synthase

Microsomal membranes from control and insulin treated (1 μg/ml for 1 h at 37°C) capillary endothelial cells were isolated as described before [23]. Enzymatic formation of Man-P-Dol was assayed by incubating microsomal membranes in 5 mM Tris-HCl, pH 7.0 containing 12.5 mM sucrose, 5 μm EDTA, 5 mM $MnCl_2$, 4 mM 5'AMP, 1% dimethyl sulfoxide, Dol-P (0–50 μg), and 2.5 μM GDP-[U-^{14}C]mannose (Sp. Act. 318 cpm/pmol) in 100 μl for 5 min at 37°C, unless otherwise mentioned. Each assay was initiated with GDP-[U-^{14}C]mannose and stopped at the desired time to extract Man-P-Dol [24].

Results

Effect of insulin on [^{3}H-Man]-oligosaccharide-PP-Dol synthesis and turnover in capillary endothelial cells

$Glc_3Man_9GlcNAc_2$-PP-Dol is an obligatory requirement for asparagine-linked (N-linked) protein glycosylation. When the capillary endothelial cells were incubated *in vitro* with ^{3}H-mannose in the presence of insulin, a time-dependent increase in ^{3}H-mannose incorporation was observed in Man-P-Dol, oligosaccharide-PP-Dol, and in cellular glycoprotein fractions

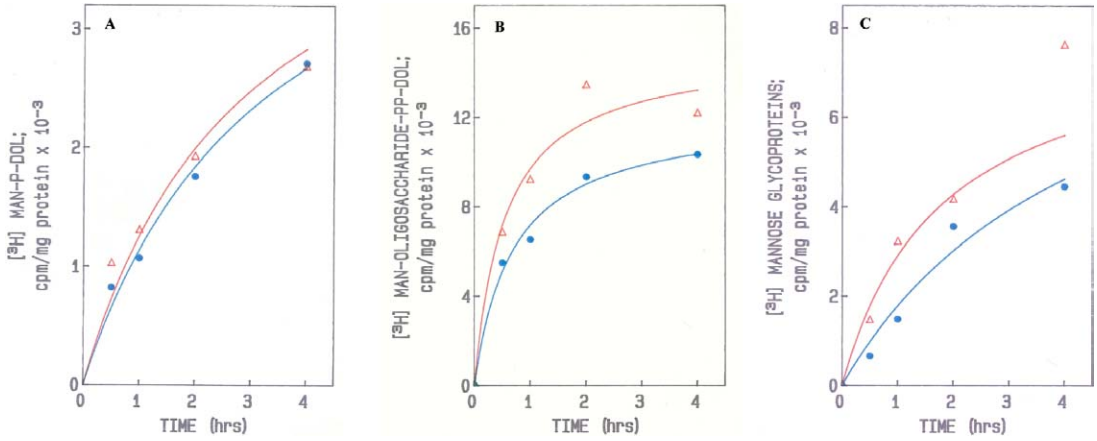

Figure 1. Effect of insulin on the incorporation of D-[2-^{3}H]-mannose in Man-P-Dol, oligosaccahride-PP-Dol and glycoproteins. Capillary endothelial cells were seeded at a density of 1 $\times$ 10^6 cells/60 mm dish and cultured for 8 days. The cells were labeled with D-[2-^{3}H]-mannose (50 μCi/ml) in a serum-free low glucose media in the absence (●) and presence (△) of insulin (1 μg/ml), and radio-labeled Man-P-Dol, oligosaccharide-PP-Dol and glycoproteins were isolated as described under Materials and Methods. The results are expressed as cpm/mg protein.

(Figure 1). The incorporation of ^{3}H-mannose into each fraction was higher in the presence of insulin as compared to the untreated controls. The incorporation of ^{3}H-mannose into Man-P-Dol and oligosaccharide-PP-Dol was linear for about 30–60 min in both control and agonist-treated cells. The radioactive incorporation into glycoproteins, however, was linear for 60–120 min in untreated controls, but it was reduced to about 30–60 min in insulin treated cells.

To confirm the increased rate of protein glycosylation in the presence of insulin, the turnover of oligosaccharide-PP-Dol was examined. The results (Figure 2) indicated that the turnover rate of oligosaccharide-PP-Dol was increased in the presence of insulin. The $t_{1/2}$ for [^{3}H-Man]-oligosaccharide-PP-Dol was reduced to 5 min in the presence of insulin as compared to 20 min for untreated controls. These results strongly supported that insulin enhanced oligosaccharide-PP-Dol biosynthesis and its turnover in capillary endothelial cells.

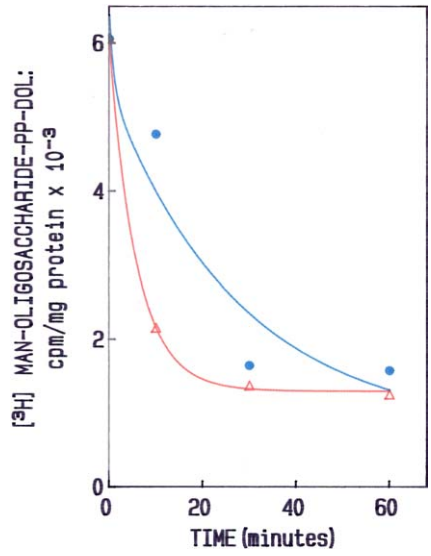

Figure 2. Effect of insulin on the turnover of [^{3}H]Man-oligosaccharide-PP-Dol. Cells were pulse-labeled for one hour with D-[2-^{3}H]-mannsoe (50 μCi/ml), washed, and resuspended in a medium containing 20 mM unlabeled mannose. The cells were then chased for 0–60 min in the absence (●) and presence (△) of insulin (1 μg/ml). Extraction of oligosaccharide-PP-Dol and its measurement were done as described under Materials and Methods. The results are expressed as cpm/mg protein.

Effect of exogenous dolichol monophosphate on the incorporation of D-[2-^{3}H]-mannose into oligosaccharide-PP-Dol

Increased [^{3}H-Man]-oligosaccharide-PP-Dol synthesis in the presence of insulin can be due to either (i) an increased level of endogenous Dol-P, or (ii) an increased GDP-mannose pool, or (iii) increased glycosyltransferase activity or its gene expression. To test these, oligosaccharide-PP-Dol synthesis was first studied as a function of exogenous Dol-P. The results in Figure 3A demonstrated that there was a stimulation of [^{3}H-Man]-oligosaccharide-PP-Dol biosynthesis in varying degrees when 10, 20, or 50 μg Dol-P was added in the incubation medium. To examine, if insulin mediated its effect by enhancing the endogenous Dol-P level, the cells were incubated together with insulin and Dol-P (20 μg). Separate incubations with Dol-P or insulin served as controls. The results in Figure 3B explained that Dol-P and insulin alone stimulated the oligosaccharide-PP-Dol biosynthesis between 60% and 127%, but with the two together the increase was 187%. The effect was additive and supported that insulin-mediated increased oligosaccharide-PP-Dol biosynthesis cannot be due to an increase in endogenous Dol-P level.

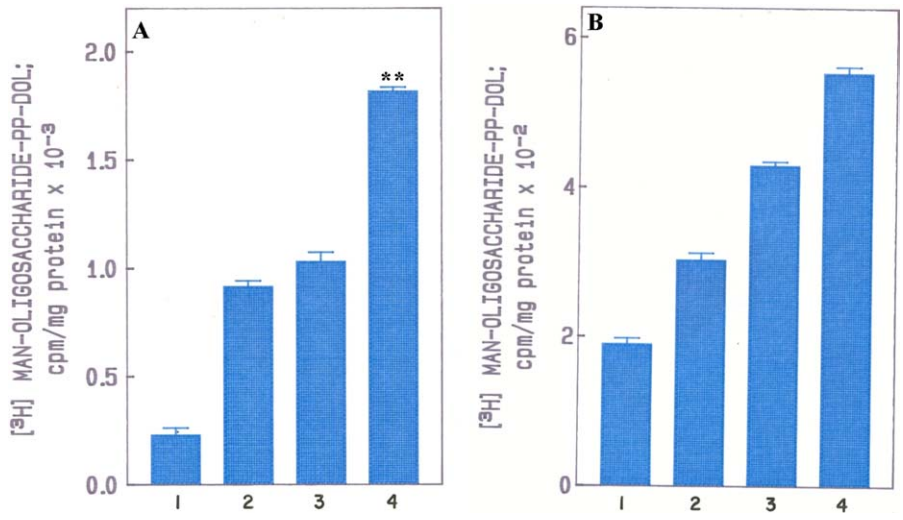

Figure 3. Effect of exogenous Dol-P on the incorporation of D-[2-^{3}H]-mannose into oligosaccharide-PP-Dol in the presence of insulin. The capillary endothelial cells were seeded in 60 mm dish at a density of 1 $\times$ 10^6 cells per dish and cultured for 8 days. The cells were washed and labeled with D-[2-^{3}H]-mannose (10 μCi/ml) at 37°C for one hour in a serum-free low glucose medium in the absence and presence of insulin (1 μg/ml). The extraction and quantification of oligosaccharide-PP-Dol were carried out as described in Materials and Methods. (A): 1 = Control; 2 = Dol-P (10 μg); 3 = Dol-P (20 μg); 4 = Dol-P (50 μg). (B): 1 = Control; 2 = Dol-P (20 μg); 3 = insulin (1 μg/ml); 4 = Dol-P (20 μg) + insulin (1 μg/ml). Values represent mean $\pm$ SEM. ($**$) Very significant difference ($P < 0.002$) v/s control.

Sizing of [^{3}H-Man]-oligosaccharide species

$Glc_3Man_9GlcNAc_2$-PP-Dol is assembled by a sequential addition of sugar residues from specific sugar-nucleotides, or from respective lipid-intermediates. If insulin accelerates the assembly process, then it is more logical to assume that the major species will be the tetradecasaccharide. The sizes of dolichol-linked oligosaccharides synthesized in the presence of insulin were therefore examined and compared with those synthesized in its absence. To achieve this goal, the oligosaccharides were released from their dolichol backbone and subjected to gel permeation chromatography on a Bio-Gel P-4 column. The elution profiles of glycan chains from control and insulin treated cells are shown in Figure 4. The sizing analysis confirmed the presence of both full-length and shorter glycan chains in both preparations. Although, insulin-treated cells contained a quantitatively higher amount of oligosaccharide, there was no apparent size difference between the control and the experimental groups.

Biosynthesis and N-glycosylation of factor VIIIC in insulin treated cells

Factor VIIIC in capillary endothelial cells is expressed as a M_r 270 kDa N-linked glycoprotein where the heavy chain M_r 215 kDa and the light chain M_r 46 kDa are joined together by a disulfide bridge [21]. It is located in the perinuclear region of the cell and maintains a close relationship with the cell proliferation [17,25]. During apoptotic induction in capillary endothelial cells by tunicamycin, the level of $Glc_3Man_9GlcNAc_2$-PP-Dol was not only reduced, but there was also an accumulation of unglycosylated factor VIIIC [17]. To evaluate if increased oligosaccharide-PP-Dol synthesis in insulin-treated cells correlates with increased factor VIIIC biosynthesis and N-glycosylation, cells were labeled with ^{35}S-methionine (40 μCi/ml) for two hours in the presence or absence of 1 μg/ml of insulin. The newly synthesized factor VIIIC in cell lysates and in the conditioned media was then analyzed by immunoprecipitation followed by SDS-PAGE and autoradiography. The results in Figure 5 explained that factor VIIIC synthesis was enhanced in insulin-treated cells. A large fraction of factor VIIIC was secreted in the culture media from insulin-treated cells as compared to the untreated controls. To address if up-regulation of the glycosylation machinery was responsible for the increased factor VIIIC biosynthesis, cells were doubly labeled with ^{35}S-methionine (20 μCi/ml) and ^{3}H-mannose (10 μCi/ml) for two hours, and factor VIIIC was immunoprecipitaed and separated on SDS-PAGE. The bands corresponding to factor VIIIC were excised from the gel, and the radioactivity was counted in a liquid scintillation spectrometer to calculate the ^{3}H-mannose to ^{35}S-methionine ratio in factor VIII species. The results in Table 1 indicated that there was an 82% increase in the ^{3}H-mannose to ^{35}S-methionine ratio in the cellular pool of factor VIIIC in the presence of insulin. No changes, however, were detected in the secreted factor VIIIC under the experimental condition.

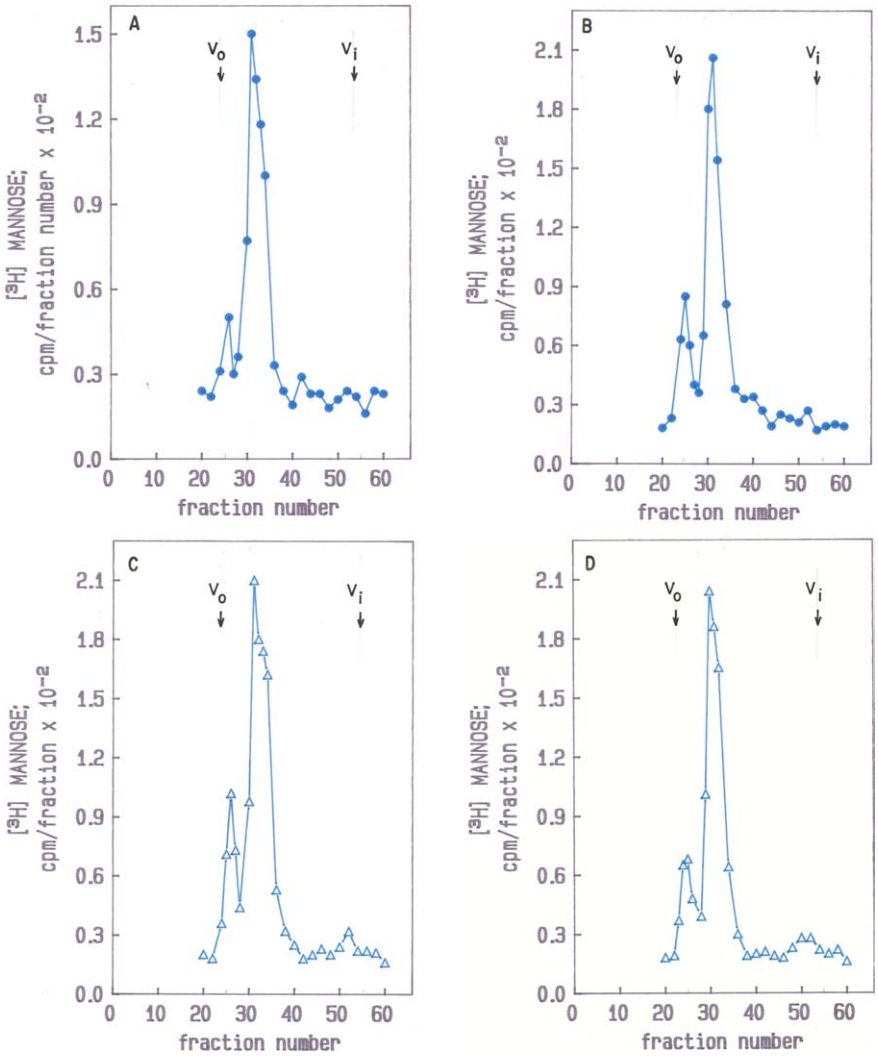

Figure 4. Bio-Gel P-4 column profile of mild-acid releasable mannosylated oligosaccharide from their pyrophosphoryl dolichol derivatives from insulin-treated cells. After culturing for 8 days, cells were incubated in a serum-free low-glucose medium containing D-[2-^{3}H]-mannose (10 μCi/ml) in the absence (●) and presence (△) of insulin (1 μg/ml). The cells were pelleted by centrifugation (500 × *g* for 5 min at 4°C) and immediately resuspended in chloroform-methanol (2:1, v/v) and processed as described in Materials and Methods. The oligosaccharide lipid obtained after extraction with chloroform-methanol-water (10:10:3, v/v/v) was hydrolyzed, and the released oligosaccharide was analyzed by gel filtration on a Bio-gel P-4 column (0.9 × 52 cm). The column was equilibrated and washed with 50 mM ammonium formate buffer, pH 8.0 and 0.5 ml fractions were collected. (A and C) = Cells were labeled for 30 min and (B and D) = Cells were labeled for 60 min. ●---●, Control; △---△ insulin (1 μg/ml).

Mannosylphosphodolichol synthase activity in microsomal membranes from insulin-treated cells

Mannosylphosphodolichol synthase (*i.e.*, Man-P-Dol synthase) is a 'key' enzyme of the dolichol pathway of protein N-glycosylation [20,26]. Therefore, Man-P-Dol synthase activity and/or its gene expression were evaluated to address whether it contributed toward enhanced [^{3}H-Man]-oligosaccharide-PP-Dol synthesis in insulin-treated cells. After exposing the capillary endothelial cells to insulin (1 μg/ml) for one hour either alone or in the presence of actinomycin D (10^{-7} M), Man-P-Dol synthase activity was measured in microsomal membranes. The results (Table 2) indicated that the Man-P-Dol synthase activity was 58% higher in insulin treated cells. Addition of actinomycin D alone increased the synthase activity by ~23%. But, when actinomycin D was present along with insulin, the synthase activity was enhanced by 2-fold. These results supported that insulin treatment does not enhance the synthase activity by gene expression.

Effect of insulin on 2-deoxyglucose uptake in capillary endothelial cells

The above results clearly demonstrate that insulin stimulated increased oligosaccharide-PP-Dol biosynthesis caused neither by enhancing the endogenous Dol-P level nor by enhancing

Table 1. Effect of insulin on the ratio of [^{3}H]-mannose to [^{35}S]-methionine incorporation into Factor VIIIC[a]

	Cellular		*Media*		
Sample	*Mean ± SEM*	*% Increase over control*	*Mean ± SEM*	*% Increase over control*	*Total Cellular + Media*
Control	1.00 ± 0.14	–	1.00 ± 0.27	–	–
Insulin (1 μg/ml)	1.82 ± 0.15	82	1.00 ± 0.13	0	82

[a]Capillary endothelial cells (1×10^6 cells/60 mm dish) were cultured for 8 days in a regular EMEM. At the end, the cells were washed and incubated in the presence and absence of insulin (1 μg/ml) in 1 ml of low glucose, methionine-free medium containing aprotinine (1 μg/ml) together with [^{35}S]-methionine (20 μCi/ml; Sp. Act. 1400 Ci/mmol) and D-[2-^{3}H]-mannose (10 μCi/ml; Sp. Act. 18.5 Ci/mmol) at 37°C for 2 h in a CO_2 incubator. After separating the culture medium, the cells were lysed in the lysis buffer as described in the Materials and Methods. Factor VIIIC was immunoprecipitated from media and cell lysates, and subjected to SDS-PAGE followed by autoradiography. The radioactivities in factor VIIIC bands were quantified by counting in a liquid scintillation spectrometer, and the ratio of [^{3}H] to [^{35}S] was determined. The results are mean ± SEM from three experiments.

Table 2. Effect of insulin on Man-P-Dol synthase activity in capillary endothelial cells[a]

Treatment	*Man-P-Dol synthase activity (pmol/mg protein/5 min)*
Control	8.4 (7.5; 9.3)
Insulin (1 μg/ml)	13.3 (18.1; 9.06)
Actinomycin D (2×10^{-3} M)	9.7 (11.6; 7.9)
Insulin (1 μg/ml) + Actinomycin D (2×10^{-3} M)	17.1 (12.4; 22.4)

[a]Microsomal membranes from control and insulin treated (1 μg/ml) capillary endothelial cells were incubated in 5 mM Tris-HCl, pH 7.0 containing 12.5 mM sucrose, 5 μm EDTA, 5 mM $MnCl_2$, 4 mM 5'-AMP and 2.5 μM GDP-[U-^{14}C]mannose (Sp. Act. 412 cpm/pmol) in 100 μl for 5 min at 37°C, and Man-P-Dol was extracted as described in Materials and Methods. The results are an average from two measurements. The numbers in the parentheses are the two numbers).

the Man-P-Dol synthase activity. Therefore, the attention was turned toward addressing the size of the sugar nucleotide pool. Since sugar nucleotide synthesis requires the availability of intracellular sugar molecules, we, therefore, studied the entry of 2-deoxy-D-[2,6-^{3}H]-glucose into the cell in the presence and absence of insulin. As shown in Figure 6, the transport of 2-deoxy-D-[2,6-^{3}H]-glucose across the endothelial cell plasma membrane was a time-dependent process. It also explained that the transport was linear approximately for 15 min and then reached a steady state. Addition of insulin (1 μg/ml) increased the rate of 2-deoxyglucose transport into the capillary endothelial cells.

Discussion

Insulin, a growth factor, has been found to down-regulate the proliferation of capillary endothelial cells [18]. To delineate this reciprocal relationship, we have studied the modulation of $Glc_3Man_9GlcNAc_2$-PP-Dol assembly in the presence of insulin. The results suggested that insulin signaling of capillary endothelial cell growth inhibition was not mediated by down-regulation of LLO biosynthesis. In fact, LLO biosynthesis was up-regulated in insulin treated capillary endothelial cells.

The roles of glycoprotein glycans span from protein structure to cell function. The glycan chain assembly, therefore, must respond to environmental as well as humoral factors. For example, activation of β-adrenoreceptor enhanced capillary endothelial cell proliferation and capillary lumen formation by activating the protein N-glycosylation pathway mediated by intracellular cAMP [15,16]. A recent report from our laboratory also supported that down-regulation of $Glc_3Man_9GlcNAc_2$-PP-Dol synthesis in capillary endothelial cells by inhibiting GcNAc-1phosphate transferase with a pyrimidine nucleoside, tunicamycin, or tying up intracellular dolichylmonophosphate (Dol-P) with a lipopeptide, amphomycin, reduced angiogenesis by arresting cells in G1 phase and induced apoptosis. The cells used a signaling mechanism of *Unfolded Protein Response* [27]. In addition, fatal disorders of glycoprotein biosynthesis such as congenital disorders of glycosylation (CDG) are a result of defects in the synthesis of N-linked glycans [28–30]. This defect may result in the absence of entire N-linked glycan chains from many proteins or from under-glycosylation due to synthesis of a truncated oligosaccharide-lipd (LLO) precursor because of a shortage of Man-P-Dol [15,20,27,31–34].

The capillary endothelial cells line blood vessels. Therefore, angiogenesis is an essential physiological event during growth and development, as well as during wound healing. Failing to induce capillary endothelial cell proliferation by insulin [18], irrespective of the presence of 210,000 high affinity receptors per cell [35], raised a question about the modulation of the dolichol cycle of the protein N-glycosylation by insulin. The results presented here clearly demonstrate the presence of a reciprocal relationship between the inhibition of capillary endothelial cell proliferation and the $Glc_3Man_9GlcNAc_2$-PP-Dol biosynthesis in cells treated with insulin. The exact molecular mechanism of the growth inhibitory action of insulin in capillary endothelial cells is currently not understood, but it is believed that increased cell-doubling time is the main contributing

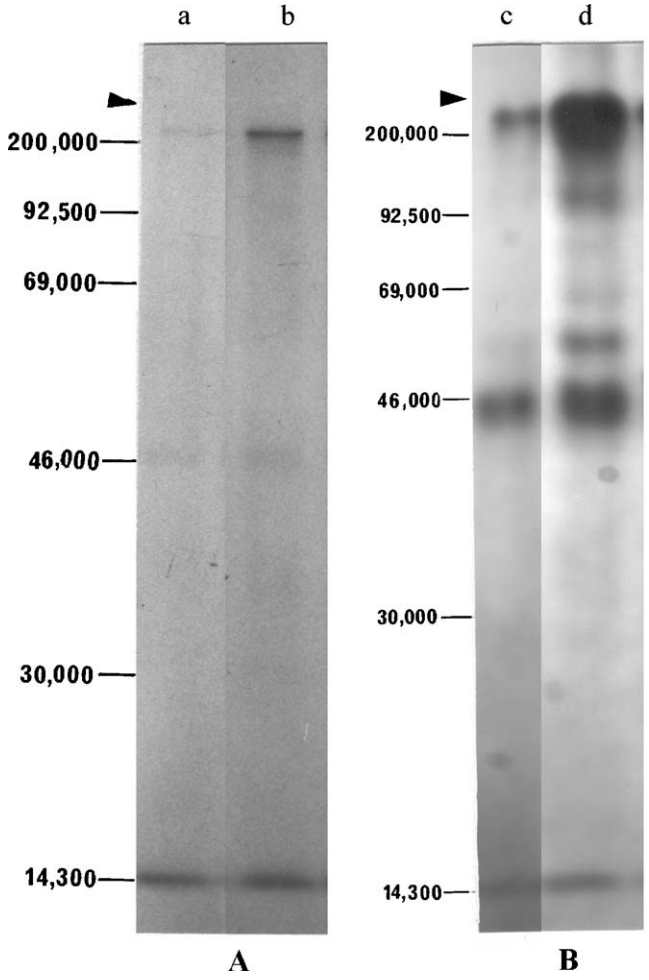

Figure 5. Effect of insulin on cellular and secretory factor VIIIC. The cells were seeded at a density of 1×10^6 cells per 60 mm dish in EMEM containing 10% serum and cultured for 8 days. At the end of the culture period cells were washed and labeled with [^{35}S]-methionine (40 μCi/ml) in the absence and presence of insulin (1 μg/ml) in 1 ml methionine-free and serum-free medium at 37°C for 2 h. The medium and cells were separated. After washing with PBS, pH 7.4 cells were lysed by incubating in 2 ml of lysis buffer (20-mM Tris-HCl, pH 8.0 containing 0.15 M NaCl and 1% NP-40) for 30 min on ice. The cell lysates were centrifuged at 100,000 $\times$ *g* using a Beckman 50Ti rotor for 40 min at 4°C. The supernatants were collected and saved at −20°C until used. To immunoprecipitate factor VIIIC, aliquots of radiolabeled media and cell lysates were incubated with anti-Factor VIIIC mouse monoclonal antibody (1:20 dilution) for 4 h at 4°C in the presence of protease inhibitors (PMSF (1 mM)/leupeptine (1 μM)/TPCK (200 μM)/aprotenin (1 μg/ml)/trypsin inhibitor (1 μg/ml) in a total volume of 200 μl. At the end of the incubation, 50 μl of 10% Protein A-Sepharose Cl-4B was added and the samples were re-incubated overnight at 4°C. The immunoprecipitates were washed three times with NET buffer (50 mM Tris-HCl, pH 7.4/150 mM NaCl/5 mM EDTA/0.02% NaN_3/2% BSA), three times with a washing buffer (100 mM Tris-HCl, pH 7.4/1% beta-mercaptoethanol/50 mM LiCl) and once with PBS, pH 7.4. The samples were then prepared for SDS-PAGE and autoradiography. A = Cellular; B = Media.

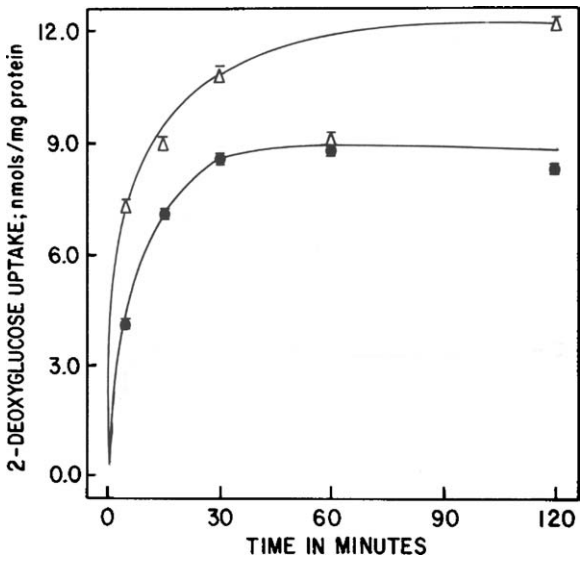

Figure 6. Insulin stimulated 2-Deoxy-D-[2,6-^{3}H]-glucose transport in capillary endothelial cells. 5×10^4 cells were seeded in 24 well clusters in EMEM containing 10% fetal calf serum (heat inactivated). After 8 days the cells were washed with SRM and incubated with 1 μCi/ml of 2-Deoxy-D-[2,6-^{3}H]-glucose in the absence (●) and the presence (△) of insulin (1 μg/ml) for 0–120 min. The cell-associated radioactivity was measured in a liquid scintillation spectrometer as described in Materials and Methods. Each point represents the mean ± SEM.

factor. Furthermore, the morphological analysis did not show any sign of apoptosis. Increased $Glc_3Man_9GlcNAc_2$-PP-Dol biosynthesis as well as glycosylation of factor VIIIC glycoprotein without enhancing the intracellular Dol-P level or Man-P-Dol synthase gene expression at an insulin-concentration inhibitory to cell growth is certainly intriguing. Increased factor VIIIC glycosylation may be due to an availability of more glycosylation sites in the factor VIIIC molecule in the presence of insulin, or due to an increased protein synthesis since it has been enhanced now that LLO synthesis is linked to cellular proliferation and its reduction is anti-angiogenic due to an induction of apoptosis [17]. A 58% increase in Dol-P-Man synthase activity in insulin-treated cells is believed to be related to an enhanced GDP-mannose pool. Since, a number of sugar molecules (*e.g.*, mannose) share the glucose transporter in capillary endothelial cells (unpublished observation). The evidence presented here supports the hypothesis that increased glucose transport is a possible driving force for enhanced LLO biosynthesis and factor VIIIC N-glycosylation. It has also been observed that more factor VIIIC is secreted in the culture media when insulin is present. Thirteen glucose transporters have been described in the literature [36,37] but only GLUT-4 (present in muscle cells and adipose tissue cells) is activated by insulin [38,39]. The presence of an insulin-sensitive glucose transporter in capillary endothelial cells can, therefore, be added to the growing list of glucose transporters. This perhaps explains why insulin exhibited no increase in the LLO level in other model systems when tried

in the past. Most likely, these models (*i.e.*, the cell types) did not have an insulin-linked glucose transporter. Insulin treatment indeed converted these capillary endothelial cells into a pseudophenotype, where a highly proliferative cell transformed into a highly secretory cell type. The secretory activity needs glucose because, when the cells were doubly labeled with [^{35}S]-methionine and [^{3}H]-mannose in a methionine and glucose-free medium to monitor the glycosylation status of factor VIIIC, factor VIIIC accumulated inside the cell as explained in Figure 5. The physiological significance of this newly identified $Man_9GlcNAc_2$-PP-Dol pool is currently being studied.

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