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Potentiation of angiogenic switch in capillary endothelial cells by cAMP: A cross-talk between up-regulated LLO biosynthesis and the HSP-70 expression

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Abstract During tumor growth and invasion, the endothelial cells from a relatively quiescent endothelium start proliferating. The exact mechanism of switching to a new angiogenic phenotype is currently unknown. We have examined the role of intracellular cAMP in this process. When a non-transformed capillary endothelial cell line was treated with 2 mM 8Br-cAMP, cell proliferation was enhanced by ∼70%. Cellular morphology indicated enhanced mitosis after 32–40 h with almost one-half of the cell population in the S phase. Bcl-2 expression and caspase-3, -8, and -9 activity remained unaffected. A significant increase in the $Glc_3Man_9GlcNAc_2$-PP-Dol biosynthesis and turnover, Factor VIIIC N-glycosylation, and cell surface expression of *N*-glycans was observed in cells treated with 8Br-cAMP. Dol-P-Man synthase activity in the endoplasmic reticulum membranes also increased. A 1.4–1.6-fold increase in HSP-70 and HSP-90 expression was also observed in 8Br-cAMP treated cells. On the other hand, the expression of GRP-78/Bip was 2.3-fold higher compared to that of GRP-94 in control cells, but after 8Br-cAMP treatment for 32 h, it was reduced by 3-fold. GRP-78/Bip expression in untreated cells was 1.2–1.5-fold higher when compared with HSP-70 and HSP-90, whereas that of the GRP-94 was 1.5–1.8-fold lower. After 8Br-cAMP treatment, GRP-78/Bip expression was reduced 4.5–4.8-fold, but the GRP-94 was reduced by 1.5–1.6-fold only. Upon comparison, a 2.9-fold down-regulation of GRP-78/Bip was observed compared to GRP-94. We, therefore, conclude that a high level of $Glc_3Man_9GlcNAc_2$-PP-Dol, resulting from 8Br-cAMP stimulation up-regulated HSP-70 expression and down-regulated that of the GRP-78/Bip, maintained adequate protein folding, and reduced endoplasmic reticulum stress. As a result capillary endothelial cell proliferation was induced.

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Abbreviations

EMEM	minimal essential medium with Earle's salt
DMEM	Dulbeccos' minimal essential medium
NP-40	Nonidet P-40
SDS	sodium dodecyl sulfate
PBS	phosphate-buffer-saline
PAGE	polyacrylamide gel electrophoresis
HRP	horseradish peroxidase
Dol-P	dolicylmonophosphate
DMSO	dimethylsolfoxide
Con A	concanavalin A
WGA	wheat germ agglutinin
cAMP	adenosine 3′, 5′-cyclic monophosphate
PKA	cAMP-dependent protein kinase
ER	endoplasmic reticulum
LLO	$Glc_3Man_9GlcNAc_2$-PP-Dol
Dol-P-Man	dolichol-P-Mannose
Dol-P-Glc	dolichol-P-glucose
GPT	UDP-GlcNAc-dolichol-P GlcNAc-1-P transferase
DPMS	Dol-P-Man synthase
UPR	unfolded protein response

Introduction

Angiogenesis is the formation of new blood vessels from pre-existing vessels [1] and a 'key step' in tumor growth and invasion. The new vessels enhance the exchange of nutrients, gases, and waste products. In addition to this "perfusion effect", endothelial cells also release important "paracrine" growth factors for tumor cells [2–5]. The invasive chemotactic behavior of endothelial cells is also facilitated by their secretion of collagenases, stromelysin, urokinases, and plasminogen activator [6,7]. Thus, the additive impact of the "perfusion effect" and "paracrine" growth factors, plus the endothelial cell-derived chemotaxis facilitators all contribute to a phase of rapid tumor growth and signal a "switch" to a potentially lethal angiogenesis phenotype. The process of angiogenesis is characterized by vasodilation, increased vascular permeability, extracellular matrix degradation, endothelial cell proliferation-migration- differentiation, and periendothelial maturation. Examination of the molecular underpinnings of these events has revealed that angiogenesis is tightly regulated by an array of stimulators and inhibitors at multiple steps.

It is now clear that asparagine-linked (N-linked) glycans have essential roles as carriers of biological information. Information-carrying N-linked glycans on newly synthesized endoplasmic reticulum (ER) glycoproteins are continuously altered during folding and assembly to reflect the status of the glycoproteins and to promote interaction(s) with appropriate components of the quality control machinery [8]. $Glc_3Man_9GlcNAc_2$-PP-Dol (LLO) is transferred co-translationally by the multi-subunit enzyme oligosaccharyl transferase to sterically accessible asparaginyl residues in Asn-Xaa-Ser/Thr on nascent proteins in the ER lumen, but not necessarily to the completion of translation [9]. LLO synthesis occurs in a stepwise manner from dolichol-P (Dol-P), requiring four donor substrates in the order UDP-GlcNAc, GDP-mannose, dolichol-P-Mannose (Dol-P-Man), and dolichol-P-glucose (Dol-P-Glc). Dol-P-Man and Dol-P-Glc are formed by transfer of mannose or glucose from GDP-mannose or UDP-glucose, respectively, to dolichol-P by specific transferases. Elongation of $Man_5GlcNAc_2$-PP-Dol to $Man_9GlcNAc_2$-PP-Dol prior to acquisition of three glucose residues is completed by transferring mannose residues from Dol-P-Man [10,11].

LLO synthesis is primed by UDP-GlcNAc-dolichol-P GlcNAc-1-P transferase (GPT), which transfers GlcNAc-1-P from UDP-GlcNAc to dolichol-P to yield GlcNAc-PP-dolichol [9]. Several forms of regulation of GPT have been reported [12]. Considerable information is also available for the stimulation of GPT *in vitro* by Dol-P-Man [13,14]. Dol-P-Man synthase (DPMS) which catalyzes the transfer reaction Dol-P + GDP-mannose $\leftarrow Mn^{2+}\rightarrow$ Dol-P-Man + GDP has been found to be regulated by cAMP-dependent protein kinase (PKA) mediated phosphorylation signal [15–17].

Previous studies from our laboratory have shown that there is a clear link between the LLO biosynthesis of protein N-glycosylation and angiogenesis [18–21]. These studies have indicated treatment of capillary endothelial cells with a β-agonist, isoproterenol caused an up-regulation of mannosylphospho dolichol synthase activity [22], protein N-glycosylation [20] and, consequently, accelerated the lumen formation [23] due to an increased level of intracellular cAMP. Here, we provide evidence that treatment of capillary endothelial cells with 8Br-cAMP reduced the expression of ER chaperones, with no change in the cell cycle regulator Bcl-2 and apoptosis-inducing caspase expression. On the other hand, there was an up-regulation of the heat-shock proteins HSP-70 and HSP-90 expression, the DPMS activity, LLO biosynthesis and turnover, as well as the protein N-glycosylation. Together all these resulted in accelerated capillary endothelial cell proliferation.

Materials and methods

The capillary endothelial cells used in this study were from a clonal cell line isolated from bovine adrenal medulla and characterized in our laboratory. Minimal essential medium with Earle's salt (EMEM), glutamine, antibiotic mixture (penicillin-streptomycin-fungizone), phosphate-buffer-saline (PBS), pH 7.2 & pH 7.4, and trypsin-versine were obtained from BioSource, Camarillo, CA. Fetal bovine serum was a product of HyClone Laboratories, Logan, UT. Hydroxyurea, paraformaldehyde, dimethysulfoxide, nystatin, cholera toxin, 8Br-cAMP (sodium salt), prostaglandin E1, forskolin, and non-enzymatic cell dissociation solution were obtained from Sigma Aldrich, St. Louis, MO. Mouse monoclonal antibodies against Bcl-2, HSP-70, and GRP-94 were from EMD Biosciences, San Diego, CA. Kits for caspase-3, -8, and -9 enzyme assays were from R&D Systems, Minneapolis, MN. Rabbit polyclonal anti-human HSP-90, mouse monoclonal anti-human GRP-78/Bip, and horseradish peroxidase (HRP)-conjugated donkey anti-rat IgG were from Calbiochem, La Jolla, CA. Mouse monoclonal antibody to human Factor VIIIC was from Roche Molecular Biochemicals, Indianapolis, IN, and polyclonal sheep anti-human Factor VIIIC was from RDI, Flanders, NJ. FITC-concanavalin A (Con A) and Texas Red-wheat germ agglutinin (WGA) were obtained from EY Laboratories, San Mateo, CA. HRP-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG, and ECL chemiluminescence detection kit were from GE Healthcare, Piscataway, NJ. Cell culture supplies were from Sarstedt, Newton, NC. All electrophoresis reagents and biotinylated protein molecular weight markers were obtained from Bio-Rad Laboratories, Hercules, 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 EMEM containing 10% heat-inactivated fetal bovine serum (56°C for 20 min), glutamine (2 mM), penicillin (50 units/ml), streptomycin (50 μg/ml), fungizone (2.5 μg/ml), and nystatin (1,000 units/ml) at 37°C in a humidified incubator (5% CO_2-95% air) in tissue culture flasks or dishes without collagen underlay or other extracellular matrix components as previously described [24]. Cells were sub-cultured once a week upon reaching confluence.

To analyze cell cycle, cells were synchronized by incubating for 48 h in serum-free EMEM containing 2 mM hydroxyurea, glutamine (2 mM) and one-third concentration of standard antibiotic/antimycotic mix (low antibiotics), followed by incubation for additional 24 h in serum-free EMEM with low antibiotics [25]. Cells were routinely monitored by light microscopy, and the viability was determined by trypan blue (0.4%) exclusion.

Analysis of cell cycle by flow cytometry

The cell cycle was assessed by propidium iodide staining of fixed cell nuclei followed by analysis in a FacSort (Beckton Dickinson) flow cytometer according to the method of Krishna [26] as modified by Vindelov [27]. Briefly, cells were trypsinized and pelleted by centrifugation at 900 rpm for 5 min in a benchtop centrifuge (Sorvall T6000B). After washing twice with PBS, the cell pellet was resuspended in propidium iodide-buffer (PI-buffer; 10 mM Tris-HCl, pH 8.0 containing 10 mM NaCl, 0.7 μM PI, 700 units/liter RNase, and 0.1% NP-40), kept in ice for 30 min and then fixed with 2% paraformaldehyde. After FacSort calibration, 10,000 events were counted per sample. Acquisition and analysis of data were carried out by using Cell Quest flow cytometry software. Gates: M1 = G0/G1; M2 = S; M3 = G2 + M; M4 = programmed cell death.

Metabolic labeling of cells with D-[2-^{3}H]-mannose and quantification of [^{3}H] man-oligosaccharide-PP-dol:

Cells (5×10^5) were seeded in 60 mm petri dishes in 3 ml of EMEM containing 10% fetal bovine serum. At the end of the culture period, cells were washed twice with serum-free Dulbeccos' minimal essential medium (DMEM) (low glucose) and incubated at 37°C for 0 to 4 h with D-[2-^{3}H]mannose (50 μCi/ml) in the presence or absence of 2 mM 8Br-cAMP, or other cAMP-stimulating agents, or a β-agonist isoproterenol (10^{-9} M $-$ 10^{-3} M). At the end of the incubation, medium was separated, cells were washed three times with 0.5 ml of ice-cold PBS, pH 7.4, removed with a rubber policeman, and collected after centrifugation at 900 rpm for 5 m in a Sorvall T6000B centrifuge.

One-milliliter chloroform-methanol (2:1, v/v) was added into cell pellets and centrifuged for 5 min. The supernatant containing [^{3}H]Dol-P-Man was removed, and the pellets were extracted twice as above. The combined chloroform-methanol extracts were washed once with 0.2 volume of 0.9% NaCl and twice with 0.5 volume of chloroform-methanol-water (3:47:48, v/v/v) as previously described [21]. The lower organic phase containing Dol-P-Man was dried under nitrogen and quantified in a liquid scintillation spectrometer. The pellets were extracted three times with chloroform-methanol-water (10:10:3, v/v/v) after washing once with 0.9% NaCl and twice with water. The supernatants containing [^{3}H] Man-oligosaccharide-PP-Dol were pooled and quantified in a liquid scintillation spectrometer. The remaining pellet contained [^{3}H] Man-glycoprotein.

Analysis of cell surface glycans by flow cytometry

5×10^5 cells were seeded in 60 mm petri dishes and synchronized as above. At the end of the culture period (0, 12, 24, and 32 h), cells were removed by non-enzymatic cell dissociation solution, pooled, collected after a brief centrifugation (900 rpm for 5 min), and washed twice with ice-cold PBS, pH 7.4. 1×10^6 cells were incubated in 1 ml FITC-Con A (100 μg/ml), or with Texas Red-WGA (100 μg/ml) for 20 min at room temperature. Cells were then washed three times with PBS, pH 7.4 and resuspended in 300 μl of PBS, pH 7.4 for two-color flow cytometric analysis. Using FL-1 and FL-3 detectors the relative amounts of high mannose type glycans (FITC-Con A) and complex type glycans (Texas Red-WGA) were determined.

Quantitative monitoring of protein expression by western blotting

5×10^5 cells were seeded in 60 mm petri dishes and synchronized. At the end of each incubation period, cells were washed with PBS and boiled in lysis buffer (10 mM Tris-HCl, pH 7.4 containing 1% sodium dodecyl sulfate (SDS), and 1.0 mM sodium ortho-vanadate). Scraped cells were transferred to micro-centrifuge tubes, briefly sonicated, and boiled for 5 min. The samples were centrifuged, and total protein in each sample was quantified by Bio-Rad DC Protein Assay. An equal amount of protein from each sample was separated on 10% SDS (polyacrylamide gel electrophoresis)-PAGE at 180 volts for 45 min and trans-blotted onto nitrocellulose membrane for 1 h at 100 volts (transfer buffer: 25 mM Tris, 190 mM glycine, 20% methanol). After blocking for one hour (in 5% non-fat dry milk in 10 mM Tris-HCl, pH 7.5 containing 100 mM NaCl and 0.05% Tween 20) at room temperature,

the blots were treated (1 h each) with monospecific primary antibodies followed by HRP-conjugated secondary antibody at room temperature. The blots were developed according to the instructions provided with the ECL chemiluminescence kit and exposed to Hyperfilm™ until the desired intensity was achieved. The protein bands were scanned, and relative amounts were determined in a GS365 scanning densitometer (Bio-Rad).

Quantification of proteins by flow cytometry

Both direct and indirect labeling was performed depending on the availability of antibody to quantify protein on a single cell. After culturing, synchronization, and exposure to different experimental conditions, cells were harvested by trypsinization and washed twice with PBS, pH 7.4. Cells were fixed either by pre-cooled (−20°C) methanol (100%) or by 0.25% paraformaldehyde followed by 70% ice-cold methanol. Blocking was accomplished with 2% fetal bovine serum and 5% non-fat dry milk for 3 h at 4°C in rotating Eppendorf tubes. The washed cells were incubated with appropriately diluted conjugated primary antibody overnight, under similar conditions. The samples were washed three times in PBS, pH 7.4 prior to flow cytometry analysis. When conjugated secondary antibody was used, the incubation was for 2 h. At the end, cells were washed and pelleted three times in PBS, pH 7.4 before resuspending for flow cytometry.

Biosynthesis of factor VIIIC

Capillary endothelial cells cultured in complete EMEM were washed with methionine-free and serum-free medium, and labeled with [^{35}S]-methionine (40 μCi/ml) in a methionine-free and serum-free medium containing aprotinin (1 μg/ml) for 1 h at 37°C in the presence or absence of isoproterenol (10^{-7} M). At the end of the incubation period, medium was collected and the cells were washed in PBS, pH 7.4 and lysed on ice for 30 min in 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 until analyzed. Factor VIIIC in cell lysates and in the conditioned media were immunoprecipitated with anti-Factor VIIIC monoclonal antibody and analyzed by SDS-PAGE followed by autoradiography [28].

Determination of protein concentration, radioactivity, and statistical analysis

Total protein content in each sample was analyzed following Bradford's procedure [29] 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 using student's 't' test.

Quantification of mannosylphosphodolichol synthase activity

Microsomal membranes from control and 8Br-cAMP (2 mM) or isoproterenol treated (10^{-7} M) capillary endothelial cells were isolated as described before [15]. Enzymatic formation of Dol-P-Man 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 Dol-P-Man [30]. [^{14}C]Dol-P-Man was quantified by counting in a liquid scintillation spectrometer.

Results

Effect of 8Br-cAMP on capillary endothelial cell proliferation

Capillary endothelial cells were synchronized and incubated for 0 to 96 h at 37°C in a humidified CO_2 incubator (5% CO_2 plus 95% air) in EMEM containing 2% fetal bovine serum (heat-inactivated) in the presence or absence of 2 mM 8Br-cAMP. The cellular morphology appeared to be that the cells entered into mitosis after spending 32 to 40 h in 2 mM 8Br-cAMP (Fig. 1a). In fact, the cell population was increased by ∼70% after 48 h (Fig. 1b). This was also supported by the presence of a decreased cell number (53%) in G0/G1 phase with a concomitant increase in S phase (47%) after 32 h in EMEM containing 8Br-cAMP (Fig. 1c).

Bcl-2 expression and caspase activity in capillary endothelial cells-treated with 8Br-cAMP

Proteins belonging to the Bcl-2 family appear to regulate mitochondrial membrane permeability to ions and to cytochrome c [31]. Although these proteins themselves can form channels in membranes, the actual molecular mechanisms by which they regulate mitochondrial permeability and the solutes that are released are less clear. The Bcl-2 family is composed of a large group of anti-apoptosis members that, when over-expressed, prevent apoptosis, and a large number of pro-apoptosis members that, when over expressed, induce apoptosis. The balance between the anti-apoptotic and pro-apoptotic Bcl-2 family members may be crucial to determining if a cell undergoes proliferation or apoptosis. Thus, the

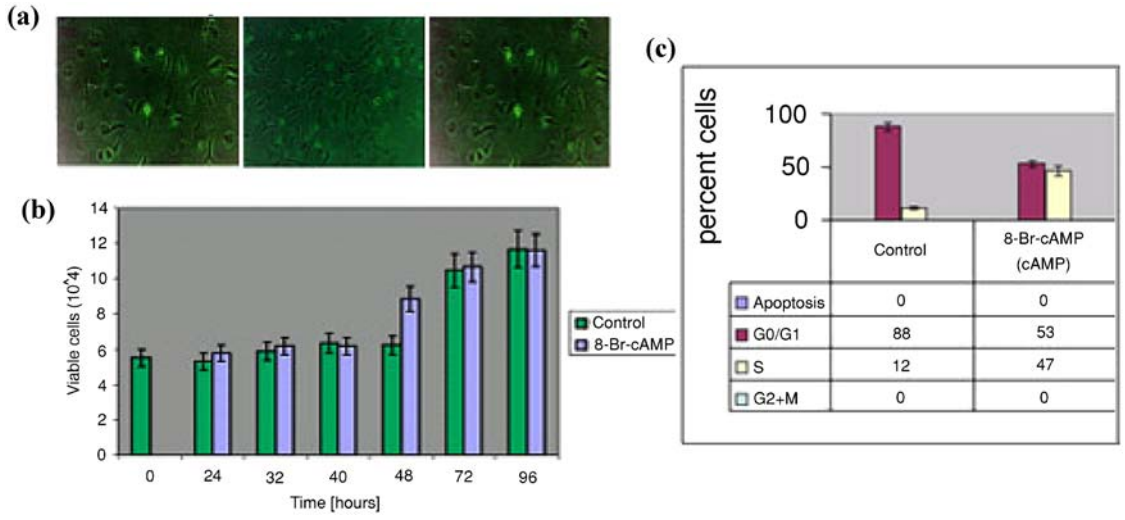

Fig. 1 (a) *Light microscopy of capillary endothelial cells before and after treating with 8Br-cAMP*. Cells were synchronized as mentioned in Materials and methods and cultured in EMEM with 2% serum with or without 8Br-cAMP (2 mM). At desired times (32 and 40 h) cells were examined under a Nikon AlphaShot inverted microscope and the photomicrographs were taken with a Nikon coolpix 990 camera. (b) *Effect of 8Br-cAMP on capillary endothelial cell proliferation*. Cells were synchronized as mentioned in Materials and methods and cultured in EMEM with 2% serum with or without 8Br-cAMP (2 mM) for 96 h. At desired times, cells were removed by trypsinization and counted in a hemocytometer. (c) *Cell cycle kinetics after exposure to 8Br-cAMP.* The synchronized cell population (85% to 90% in G0/G1 phase) was cultured in EMEM containing 2% serum with or without 8Br-cAMP (2 mM). After 32 h cells were harvested and processed for cell cycle analysis by flow cytometry as described in Materials and methods. Each sample represents the mean ± SEM from three experiments where 20,000 cells were analyzed

suppressor activity of the anti-apoptotic Bcl-2 family appears to be negated by the pro-apoptotic members. The expression of total Bcl-2 protein was then determined in capillary endothelial cells exposed to 8Br-cAMP by flow cytometry and Western blotting. Both methods gave almost identical results. Figure 2 explained that these capillary endothelial cells express a relatively high level of Bcl-2. 8Br-cAMP neither enhanced nor suppressed Bcl-2 expression throughout the time-course studied here (i.e., 24, 32, and 40 h), covering the entire G1 phase, the S-phase, and part of the G2 + M phase.

The caspase protease family plays a central role in the implementation of apoptosis in vertebrates [32–34]. Caspases

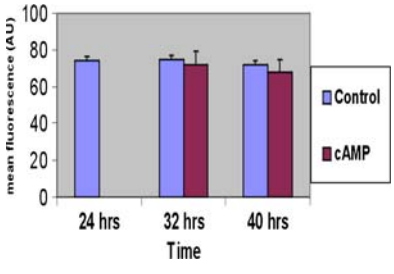

Fig. 2 *Bcl-2 expression in capillary endothelial cells before and after treating them with 8Br-cAMP.* Synchronized cells were incubated in EMEM containing 2% serum and with or without 8Br-cAMP (2 mM). After 24, 32, and 40 h, cells were harvested and processed for quantitative expression of Bcl-2 by flow cytometry as described in Materials and methods. The results are a graphical representation of the flow cytometry histograms. Each sample represents the mean ± SEM for $n = 6$, where 20,000 cells were analyzed in each case

are constitutively expressed in healthy cells, where they are synthesized as zymogens (procaspases). Caspases are activated upon cleavage of procaspases into their active mature fragments, in addition to N-terminal prodomain. The caspase family is broadly divided into two groups: initiator caspases (caspase-8, -9, and -12) and effector caspases (caspase-3, -6, and -7). Initiator caspases undergo auto processing for activation in response to apoptotic stimuli. Active initiator caspases in turn process precursors of the effector caspases responsible for dismantling cellular structures. To evaluate the status of caspases in 8Br-cAMP treated capillary endothelial cells, the enzymatic activity of caspase-3, -8, and -9 were determined in cell lysates. The results summarized in Fig. 3 indicated that the activity of all three caspases remained unaffected in 8Br-cAMP-treated capillary endothelial cells, explaining no induction of apoptosis.

Expression of ER and cytoplasmic chaperones in 8Br-cAMP-treated capillary endothelial cells

The glucose-regulated proteins 78 and 94 (i.e., GRP-78/Bip, GRP-94) are ER chaperones, and assist in the folding and trafficking of glycoproteins within the ER. During endoplasmic reticulum stress ("ER stress"), there is an increased expression of these chaperones, especially that of the GRP-78/Bip. A persistent high level of GRP-78/Bip indicates induction of the unfolded protein response (UPR) [35], and prolonged activation of the UPR results in apoptosis. Heat

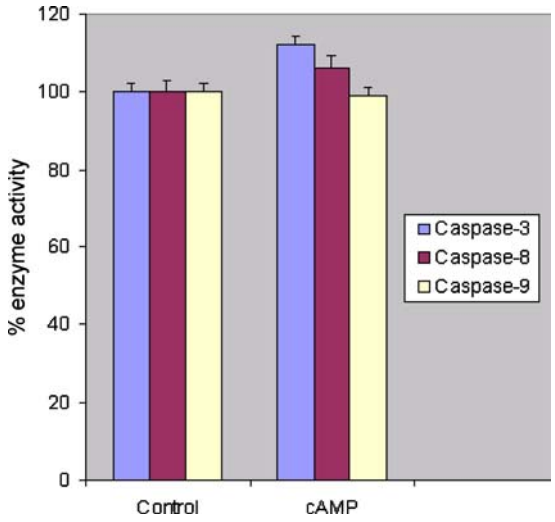

Fig. 3 *Effect of 8Br-cAMP on the activity of caspase-3, -8, and -9 in capillary endothelial cells.* Synchronized cells were exposed to 8Br-cAMP (2 mM) in EMEM containing 2% fetal bovine serum for 32 h. Cells were then harvested and approximately 2×10^6 cells were lyzed for measuring the enzyme activity of caspase-3, -8, and -9 spectrophotometrically as described in Materials and methods. Each sample represents the mean ± SEM for $n = 3$

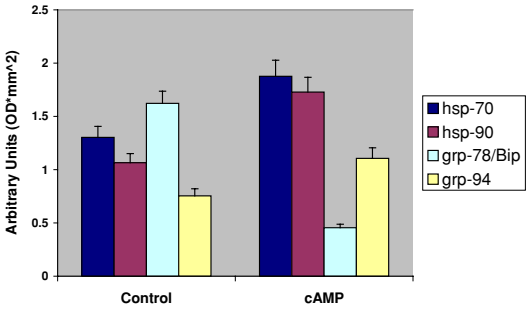

Fig. 4 *Expression Modulation of stress-related proteins by 8Br-cAMP.* Synchronized cells were exposed to 8Br-cAMP (2 mM) in EMEM containing 2% serum for 32 h. Cells were harvested, lyzed, and total protein in each lysate was quantified. The proteins were separated on 10% SDS-PAGE and transblotted onto nitrocellulose membranes. The blots were treated with monospecific antibodies against HSP-70, HSP-90, HSP-78/Bip, and HSP-94. The blots were developed with HRP-conjugated secondary antibody and detected by ECL chemoluminiscence kit. **Top panel:** A graphical representation of quantitative expression of stress-related proteins. Each sample represents the mean ± SEM for $n = 3$. **Bottom panel:** A representative immunoblot of stress-related proteins in capillary endothelial cells. C = control

shock proteins 70 and 90 (i.e., HSP-70 and HSP-90), on the other hand, are cytoplasmic chaperones and expressed during "cytoplasmic stress", *e.g.*, at elevated temperatures, oxidative stress, amino acid deprivation, etc. HSP-70 binds to regions of unfolded polypeptides that are rich in hydrophobic residues, preventing inappropriate aggregation. HSP-70 also blocks the folding of certain proteins which must remain unfolded until they have been translocated across membranes [36]. HSP-90 is mainly involved in the folding of proteins that are involved in signal transduction [37]. To understand the role of each of these two classes of chaperones in angiogenesis, we studied their expression in cells treated with 8Br-cAMP. The results in Fig. 4 indicated that the expression of HSP-70 and HSP-90 remained almost identical in untreated controls but their expression were enhanced by 1.4 to 1.6-fold in the presence of 8Br-cAMP. The expression of GRP-78/Bip was 2.3-fold higher compared to that of the GRP-94 in control cells. But, in the presence of 8Br-cAMP there was a reversal of this observation. The expression of GRP-78/Bip was nearly 3-fold lower compared to GRP-94 in 8Br-cAMP treated cells. Furthermore, the expression of GRP-78/Bip in untreated controls was 1.2 to 1.5-fold higher compared to HSP-70 and HSP-90, but that of the GRP-94 was 1.5 to 1.8-fold lower. When a similar comparison was made in cells treated with 8Br-cAMP there was a 4.5 to 4.8-fold reduction in GRP-78/Bip expression, but the GRP-94 expression was reduced by 1.5 to 1.6-fold only. Making a similar comparison for GRP-78/Bip and GRP-94 expression, GRP-78/Bip expression was down regulated 2.9-fold compared to GRP-94.

Dol-P-Man synthase activity in 8Br-cAMP-treated capillary endothelial cells

Earlier studies have suggested that Dol-P-Man synthase in capillary endothelial cells is activated by cAMP-dependent protein phosphorylation signal [22]. The present study indicated that 8Br-cAMP accelerated the capillary endothelial cell proliferation and reduced the ER stress, convincing us to analyze the DPMS activity in cells treated with 8Br-cAMP. In this experiment cells were collected from the untreated control as well as those treated with 2 mM 8Br-cAMP for 2, 24, 32, and 48 h, and the DPMS activity was measured *in vitro* in isolated microsomes. The DPMS activity was 30% and 20% higher in cells treated with 2 mM 8Br-cAMP for 24 and 32 h, respectively, over the untreated control (Fig. 5). There could be several explanations for the reduction of DPMS activity between 32 h and 48 h in 8Br-cAMP treated cells: (i) the cells are in the G2 + M phase; (ii) the level of intracellular cAMP concentration was depleted due to degradation by a phosphodiesterase; and/or (iii) a serine/threonine phosphatase dephosphorylated the DPMS.

Expression of surface glycans and factor VIIIC in capillary endothelial cells treated with 8Br-cAMP

It has been suggested that the relative distribution of cell surface glycans (i.e., the ratio of high-mannose-type to complex-type) can be as significant as the total amount of cell surface glycans expressed in terms of functional phenotypic modulation of some cell types. The ratio of high-mannose to

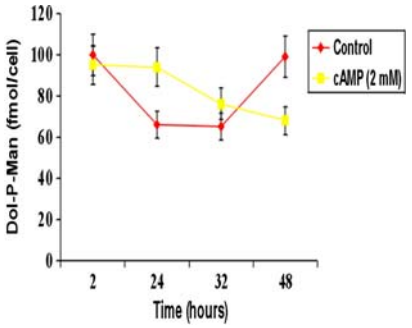

Fig. 5 *Dol-P-Man synthase activity in capillary endothelial cells treated with 8Br-cAMP.* Synchronized cells were incubated in EMEM containing 2% serum and with or without 8Br-cAMP (2 mM). Cells were harvested at 2, 24, 32, and 48 h and the DPMS activity as assayed *in vitro* using the microsomal fraction as the enzyme source. Each sample represents the mean ± SEM for $n = 3$. ●--●, Control; ◆--◆, 8Br-cAMP

complex-type glycans was then compared between the control and 8Br-cAMP treated capillary endothelial cells. The results in Table 1 indicated that during the first 12 h there was no change in the ratio; but as the cells continued to be exposed for a longer period of time, *e.g.*, 32 h, the fluorescence for Con A was reduced by 13% in control cells as compared to a 15% increase in cells treated with 8Br-cAMP for the same period of time. On the other hand, the fluorescence signal for WGA was increased 1% in control cells and 2% in cells treated with 8Br-cAMP, respectively. Thus, there was an overall reduction in Con A to WGA ratio in capillary endothelial cells treated with 8Br-cAMP (Table 1).

Factor VIIIC is a 270 kDa N-linked glycoprotein constitutively expressed in these capillary endothelial cells [28]; it maintains a temporal relationship with the cell proliferation [25]. When examined by flow cytometry, a 10% increase in the expression of Factor VIIIC was observed in capillary endothelial cells treated with 8Br-cAMP for 32 h (Fig. 6a). This increase only represented the intracellular amount of Factor VIIIC and was consistent with the expression of a constitutively secreted protein where a balance between synthesis and secretion maintains a constant intracellular pool. To corroborate these observations, we studied *de novo* biosynthesis of Factor VIIIC after activating the cell surface β-adrenoreceptor with isoproterenol (1×10^{-7} M). In these capillary endothelial cells the β-adrenoreceptors are coupled to adenylate cyclase and enhance intracellular cAMP when stimulated [20]. The results indicated that [^{35}S]-methionine incorporation in Factor VIIIC was substantially enhanced in isoproterenol-treated capillary endothelial cells compared to the untreated control (Fig. 6b). In addition, much more Factor VIIIC was found in the conditioned media than in the cell lysate. It has been mentioned above that there was an increase in Con A positive cells in 8Br-cAMP-treated cultures. Therefore, to analyze further, cells were doubly labeled with [^{35}S]-methionine and [2-^{3}H] mannose for 2 h at 37°C in a methionine-free, serum-free, low-glucose DMEM. Factor VIIIC was immuno-precipitated from equal amounts of cellular and media proteins, separated on 10% SDS-PAGE, and detected by autoradiography. The radioactivity of both radioisotopes in the excised Factor VIIIC bands was counted. The results (Table 2) indicated a nearly two-fold increase in the ratio of [^{3}H] mannose to [^{35}S]methionone in 8Br-cAMP-treated cells, thus confirming that 8Br-cAMP enhanced protein N-glycosylation in these cells.

Upregulation of lipid-linked oligosaccharide in capillary endothelial cells treated with 8Br-cAMP

Dol-P-Man is a "key" intermediate in the elongation of $Man_5GlcNAc_2$-PP-Dol to $Man_9GlcNAc_2$-PP-Dol [10,11] prior to the acquisition of three glucose residues and completing the glycan chain to LLO [9]. Increased DPMS activity as well as enhanced Factor VIIIC glycosylation in cells treated with 2 mM 8Br-cAMP suggested an increased LLO synthesis and turnover in these cells. We have, therefore, labeled cells with [2-^{3}H] mannose for 0–4 h in the presence or absence of a β-agonist, isoproterenol (1×10^{-7} M), which generates cAMP intracellularly), and analyzed the level of LLO along with the Dol-P-Man and glycoproteins. Results (Fig. 7) indicated the rates, as well as the extent of ^{3}H-mannose in-

Table 1 Effect of 8Br-cAMP on cell surface N-glycans. The synchronized cultures of capillary endothelial cells were exposed to 2 mM 8Br-cAMP for 12 to 32 h at 37°C. Cells were removed by non-enzymatic cell dissociation solution and collected after a brief centrifugation. After washing twice with ice-cold PBS, pH 7.4, 1×10^{6} cells were incubated with FITC-Con A (100 μg/ml), or with Texas-Red WGA (9100 μg/ml) for 20 min at room temperature. After washing three times with PBS, pH 7.4 cells were resuspended in 300 μl of PBS, pH 7.4 and subjected to two-color flow cytometry as mentioned in the Materials and Methods

Experimental condition	Con-A/WGA	Total fluorescence change		Change in Con-A/WGA
		Con-A	WGA	
Control (12 h)	1.020	Unchanged		None
8Br-cAMP (2 mM) (12 h)	1.030	Unchanged		None
Control (32 h)	0.876	13% ↓	1% ↑	16% ↓
8Br-cAMP (2 mM) (32 h)	0.832	15% ↑	2% ↑	15% ↓

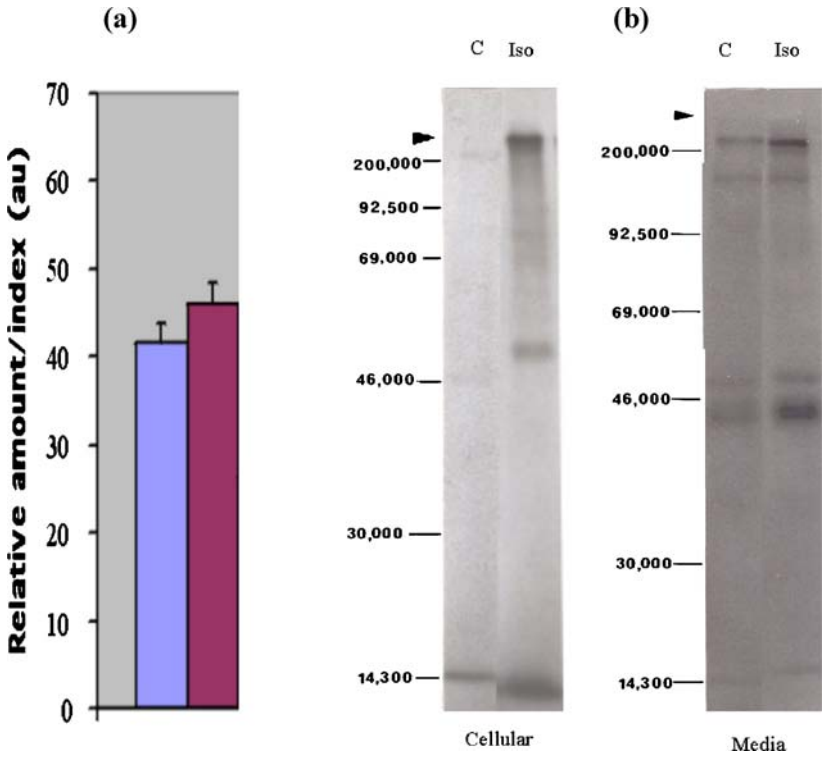

Fig. 6 *Factor VIIIC expression in capillary endothelial cells before and after treatment with 8Br-cAMP.* (a) Flow cytometric analysis: Synchronized cultures of capillary endothelial cells were exposed to 8Br-cAMP (2 mM) in EMEM containing 2% serum for 32 h. At the end of the incubation, cells were harvested and processed for two-color flow cytometric analysis for Factor VIIIC expression as mentioned in Materials and methods. Each sample represents the mean ± SEM for $n = 3$ where 20,000 cells were counted in each experiment. (b) Autoradiographic determination of Factor VIIIC: Cells were labeled with [^{35}S]-methionine (40 μCi/ml) for 1 h at 37°C in a methionine-free/serum-free DMEM with or without isoproterenol (1×10^{-7} M). At the end of the incubation, cells were separated from the media and lyzed. Equal amount of proteins from the cell lysate and the conditioned media were immunoprecipitated with a mouse monoclonal antibody to human Factor VIIIC. The washed immunoprecipitates were separated on 10% SDS-PAGE, dried, and exposed to X-ray films

corporation into Dol-P-Man, LLO and glycoproteins, were higher in isoproterenol-treated cells compared to their untreated controls. To analyze if increased LLO synthesis was associated with its turnover, the cells were labeled with [2-^{3}H]-mannose for one h in serum-free low-glucose DMEM and chased with 20 mM unlabeled mannose for 0 to 60 min in the presence or absence of isoproterenol (1×10^{-7} M). Results indicated that cells turned over LLO in 5–7 min in the presence of isoproterenol, whereas the untreated cells needed approximately 12 min (Fig. 8). To verify that the high level of LLO biosynthesis by isoproterenol was in fact due to intracellular cAMP, the cells were labeled as above but in the presence of other stimulators for cAMP such as forskolin (1 μM), cholera toxin (100 ng/ml), and prostaglandin E1 (10 μM). The results in Fig. 9 supported, beyond any doubt, a significant increase of ^{3}H-mannosylated oligosaccharide-PP-Dol in capillary endothelial cells treated with cAMP-related stimuli.

Table 2 Effect of isoproterenol on the ratio of [2-^{3}H]-mannose to [^{35}S]-methionine in Factor VIIIC. The capillary endothelial cells were double-labeled with [2-^{3}H]-mannose (50 μCi/ml) and [^{35}S]-methionine (40 μCi/ml) for 1 h at 37°C in the presence or absence of isoproterenol (1×10^{-7} M). At the end of the incubation, cells were separated from the conditioned media, and lysed as mentioned in Materials and Methods. Equal amount of cellular and media protein were immunoprecipitated with an anti-Factor VIIIC monoclonal antibody (human), separated on SDS-PAGE, and detected by autoradiography. The bands corresponding to the heavy and light chains were excised and counted in a liquid scintillation spectrometer

	Cellular		Media		
Experimental condition	Mean ± SEM	% Increase over control	Mean ± SEM	% Increase over control	Total = cellular + media
Control	1.0 ± 0.14		1.00 ± 0.27	–	–
Isoproterenol (1×10^{-7} M)	1.73 ± 0.08	73	1.45 ± 0.08	45	118

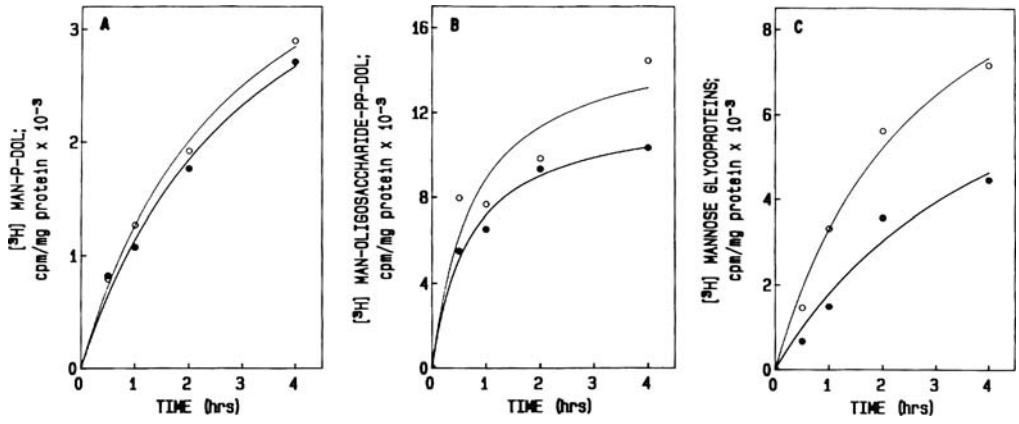

Fig. 7 *Effect of isoproterenol on the incorporation of D-[2-^{3}H]-mannose in Dol-P-Man, LLO, and glycoproteins.* The capillary endothelial cells were labeled with D-[2-^{3}H]-mannose (50 μCi/ml) for 1 h at 37°C in serum-free low-glucose DMEM with (○) or without (●) isoproterenol (1×10^{-7} M). Dol-P-Man, LLO, and the glycoproteines were separated as mentioned in the Materials and methods, and quantified by counting in a liquid scintillation spectrometer. The results are expressed as cpm/mg protein, and from a representative experiment done in triplicate

Discussion

Tumor growth is angiogenesis dependent. In malignant breast tissue the endothelial cells of blood capillaries proliferate 45 times faster than that of the surrounding benign breast [6]. Furthermore, in invasive breast carcinoma patients with metastasis, the mean microvessel count is 101 ± 49.3 per 200x, whereas those without metastasis is 45 ± 21.1 per 200 x field. The adult endothelium is essentially quiescent, but in response to physiological or pathological stimuli, such as during tumor growth, the endothelial cell can alter to a proliferating and organized population of cells. It is therefore extremely important to understand the signal/signals responsible for switching the endothelial cells to their new angiogenic phenotypes. This will help the development of novel anti-angiogenic therapeutics to treat solid tumor growth, *e.g.*, breast cancer.

In this study, we have used intracellular cAMP enhancing agent(s) to understand the role of cAMP in the angiogenic switch. The phase contrast microscopy of capillary endothelial cells exposed to 2 mM 8Br-cAMP for 32 to 40 h exhibited proliferating cell morphology, *e.g.*, the presence of enlarged and flattened cells. Analysis of cell cycle kinetics by flow cytometry supported enhanced cellular proliferation

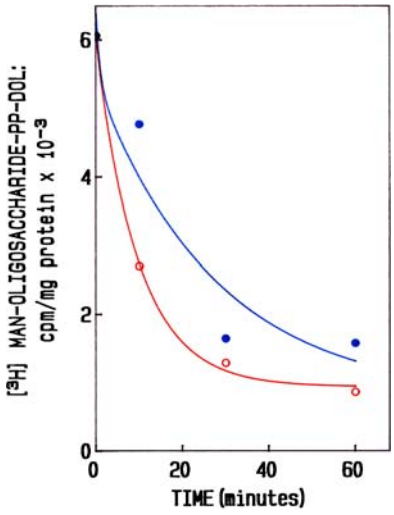

Fig. 8 *Effect of isoproterenol on the turnover of LLO.* Cells were pulse-labeled for 1 h at 37°C with D-[2-^{3}H]-mannose (50 μCi/ml) in serum-free low glucose DMEM, washed, and resuspended in serum-free low glucose DMEM containing 20 mM unlabeled mannose in the absence (○) or presence (●) of isoproterenol (1×10^{-7} M) for 0 to 60 min. LLO was extracted as mentioned in the Materials and methods and quantified in a liquid scintillation spectrometer. The results are cpm/mg protein

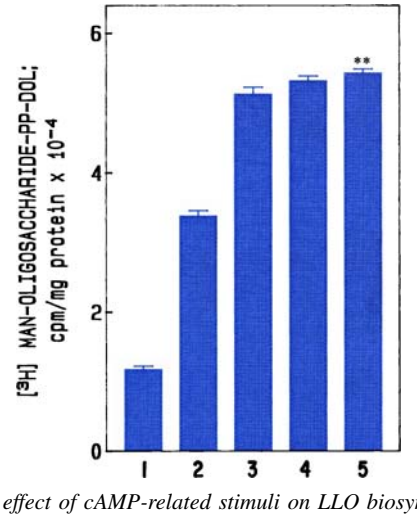

Fig. 9 *The effect of cAMP-related stimuli on LLO biosynthesis.* The cells were incubated in a serum-free low glucose DMEM containing D-[2-^{3}H]-mannose (50 μCi/ml) for 1 h at 37°C in the presence or absence of 8Br-cAMP (2 mM), forskolin (1 μM), cholera toxin (100 ng/ml), and PEG1 (10 μM). LLO was extracted as mentioned in the Materials and methods, and quantified in a liquid scintillation spectrometer. 1 = Control; 2 = 8Br-cAMP; 3 = Forskolin; 4 = Cholera toxin; 5 = Prostaglandin E1. The results are mean ± SEM. ** = $P < 0.002$

by 8Br-cAMP and indicated the shortening of G1 phase. Normally, the G1 phase is 36 h long when cultured in 2% serum-containing EMEM [25]. Upon stimulation with 8Br-cAMP ~47% cells entered into S phase indicating cells have not only progressed through the cell cycle, but their G1 phase was similar to that of culture condition with high serum, *i.e.*, 10% [25].

The primary action of cAMP in eukaryotic cells is to activate PKA [38]. The active kinase, then free to phosphorylate, substrates on serine/threonine residues, which are present in the consensus sequence Arg-Arg-Xa-Ser/Thr or Lys-Arg-Xaa-Ser/Thr. Consequently, several regulatory mechanisms are in place to ensure that cAMP levels and kinase activity are highly controlled [39]. PKA exists in two isoforms: type I and type II. Type I PKA holoenzyme (consisting either RIα or RIβ) is predominantly cytoplasmic, whereas >75% of the type II PKA holoenzyme is targeted to certain intracellular sites through association of the RII substrates (RIIα or RIIβ) [40]. Functions and importance of the PKA have been studied extensively [41–46]. We have also observed that by enhancing intracellular cAMP, capillary endothelial cell proliferation was also enhanced [19,20,23].

The present study revealed that treatment of capillary endothelial cells with 8Br-cAMP enhanced the LLO biosynthesis and protein N-glycosylation due to activation of the DPMS. DPMS from these endothelial cells has now been cloned in our laboratory, and the DNA sequence indicated the presence of a conserved site to be phosphorylated by PKA (Banerjee, DK and Baksi, K; unpublished results). In addition, we have recently shown that removal of the PKA site (*i.e.*, serine-141) from the recombinant *S. cerevisiae* DPMS by PCR site-directed mutagenesis resulted in half-maximal activation of the recombinant DPMS, when phosphorylated by PKA *in vitro* [17]. In an analogous study, we have also observed low expression of LLO as well as down-regulation of DPMS activity in PKA-deficient CHO cells [16]. Furthermore, PKA type I is required for normal maintenance of LLO and DPMS activity in cAMP-responsive cells (Banerjee, DK; unpublished results).

Maintaining higher DPMS activity as well as higher N-glycosylation profile in cells treated with 8Br-cAMP suggests that the ER function in these cells is intact. Absence of the "ER stress" is evidenced by the low expression of GRP78/Bip. Also, no change in the expression of Bcl-2 as well as the activity of caspase-3, -8, and -9 supported the absence of apoptosis. As a result, all glycoproteins (secretory or structural) entering into the ER lumen are fully N-glycosylated, thus, preventing the dimerization/oligomerization of non-glycosylated proteins and induction of *UPR*-mediated apoptosis.

Up-regulated expression of HSP-70 and HSP-90 is considered to be a major contributing factor for capillary endothelial cell proliferation during 8Br-cAMP treatment. HSP-70 chaperone, with its co-chaperone, comprise a set of abundant cellular machines to assist a large variety of protein-folding processes in almost all cellular compartments. Historically, they were identified by induction under conditions of stress (*e.g.*, heat shock), during which they are now known to provide an essential action of preventing aggregation and assisting refolding of misfolded proteins. But they also play an essential role under normal conditions, including (i) assisting folding some newly translated proteins; (ii) guiding translocating proteins across organelle membranes through action at both the *cis* and *trans* sides; (iii) disassembling oligomeric protein structures; (iv) facilitating proteolytic degradation of unstable proteins; and in selected cases, (v) controlling the biological activity of folded regulatory proteins including transcription factors [36,47,48]. All of these activities rely on the ATP-regulated association of HSP-70 with short hydrophobic segments in substrate polypeptides [49, 50], which prevent further folding or aggregation by shielding these segments.

In HSP-70-assisted folding reactions, substrates undergo repeated cycles of binding/release [51,52], frequently at a stoichiometry of a single HSP-70 monomer per substrate molecule. HSP-70 binding does not appear to induce global conformational changes in the substrate but, rather, appears to act locally. Substrates released from the chaperone undergo kinetic partitioning between folding to native state, aggregation, rebinding to HSP-70, and binding to other chaperones or proteases as part of a multi-directional folding network. HSP-70 family of proteins share common domains: a highly conserved NH_2-terminal, an ATPase domain of 44 kDa, and a COOH-terminal region of 25 kDa, divided into a conserved substrate binding domain of 15 kDa and a less-conserved immediate COOH-terminal domain of 10 kDa. Thus, HSP-70 is able to interact with a wide spectrum of non-native proteins and help to target some proteins to mitochondria and other organelles [53–55]. The molecular ratchet model proposes that HSP-70 binds to segments of the polypeptide as these emerge on the *trans* side of the membrane by random oscillation within the transport channel [56,57]. On the other hand, translocation motor model proposes that the translocating polypeptide binds to HSP-70, that this binding stimulates ATP hydrolysis by HSP-70, and that ATP hydrolysis causes a conformational change in HSP-70, which actively pulls a segment of the bound chain across the membrane. By generating an inward force, HSP-70 can thus act as an unfoldase for a protein domain that has not yet crossed the membrane. Thus, a high level of HSP-70 plays a significant role in maintaining the mitochondrial integrity and preventing the release of cytochrome c and activation of caspase-cascade pathway for apoptotic induction. Therefore, no changes in the status of all three caspases (i.e., caspase-3, -8, and -9) in 8Br-cAMP treated cell fully justify the up-regulated expression of HSP-70. No change in the Bcl-2 expression under the experimental condition is intriguing. One possibility may lie to the fact that

total Bcl-2 expression was followed instead of its phosphorylated form. The overall conclusion is that LLO helps protect cells from undergoing unfolded protein response-mediated apoptosis through increased expression of HSP-70 and that cAMP plays a critical role in this process. It is expected that our on-going studies will provide further information in the near future.

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