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Glucose is a key regulator of VEGFR2/KDR in human epithelial ovarian carcinoma cells

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ABSTRACT

Epithelial ovarian cancer (EOC) is a serious gynecological cancer and there may be an increased risk of developing EOC in women with metabolic disruptions such as diabetes-related hyperglycemia, obesity or high glycemic load. Upregulation of vascular endothelial growth factor (VEGF) in ischemic conditions (e.g. hypoxia, hypoglycemia) induces tumor angiogenesis. We previously showed that EOC cells employ an autocrine VEGF/VEGFR2 signaling loop. Here we demonstrate the influence of glucose levels on VEGF and its receptors in the human EOC lines OVCAR-3 and CAOV-3. Glucose (but not pyruvate) deprivation induced significant increase in VEGF transcription and secretion, but a rapid reduction in VEGFR2 protein synthesis and glycosylation, combined with a reduction in co-receptor neuropilin-1 (NRP-1) protein levels. In contrast, mRNA for *KDR* and *NRP-1* was increased upon glucose depletion suggesting a mechanism of feed back upon protein reduction. The addition of the proteosome inhibitor epoxomycin restored VEGFR2 under glucose free conditions, suggesting degradation as the main mechanism of VEGFR2 reduction and transcriptional activation through the unfolded protein response (UPR) which was activated in glucose-starved cells through the upregulation of the Endoplasmic reticulum chaperon GRP-78. Our finding that glucose can regulate VEGF/VEGFR2 levels suggests that initiation and/or progression of ovarian surface epithelial cells towards a neoplastic phenotype might be modulated by dietary conditions, and that a patient's metabolic status may alter the effectiveness of the known anti-angiogenic therapies. This information provides opportunities to explore the biology of EOC progression and improve our understanding of the mechanistic insight of this interesting regulatory effect.

Introduction

Epithelial ovarian cancer (EOC) is one of the most serious gynecological cancers with significant worldwide mortality [1]. The angiogenic factor VEGF is highly expressed in ovarian carcinoma and associated with tumor angiogenesis and ascites formation [2,3]. Glucose starvation dramatically up-regulated VEGF mRNA in ID8 murine ovarian cancer cells, and mRNA expression and VEGF secretion increased in human lymphoma U-937 cells exposed to low glucose [4,5]. Upregulation of VEGF in ischemic conditions has important implications for tumor angiogenesis [4], and we have previously shown that hypoglycaemia can impact tumor progression by altering mismatch repair protein levels [6]. During ischemia VEGF and the glucose transporter GLUT-1 restore tissue homeostasis through angiogenesis and improved glucose utilization, respectively [7]. GLUT transporters are overexpressed in various tumors, including ovarian carcinomas, probably as a reflection of their glucose requirements [8]. Such studies focused on the impact of ischemia on VEGF (i.e. ligand) expression; to our knowledge, there are no previous reports on the relationship between glucose levels and VEGF/VEGFRs expression in ovarian cancer.

Activation of the unfolded protein response (UPR) is a common feature of tissues under nutrient stress (such as glucose deprivation), and evidence exists that the HSP/chaperone GRP-78 is involved in stress-induced cell survival in EOC cells [9]. Disruption of this mechanism in different types of tumors is an attractive approach for selective cancer killing [10]. Here we show a novel relationship between glucose exposure and expression at the transcriptional and translational levels of VEGF, VEGFR2, and NRP-1 in EOC cells. Our findings provide insight into the known correlation between diabetes, hyperglycaemia and ovarian cancer incidence [11], and may open new opportunities for investigating the synergistic effects of anti-angiogenic inhibitors along with the anti-hyperglycaemic drugs to target EOC and possibly other types of cancer.

Materials and methods

Cell culture. The human epithelial ovarian cancer cell lines OVCAR-3 and CAOV-3 were purchased from the American Type

Abbreviations: KDR, kinase insert domain receptor; HSP, heat-shock proteins.

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Culture Collection (Manassas, VA, USA) Cells were grown in complete DMEM (4500 mg glucose/L) (Sigma–Aldrich, Oakville, ON, Canada) supplemented with 10% heat-inactivated FBS, 50 μg/mL gentamicin and 1 mmol/L sodium pyruvate, or under hypoglycemic conditions in DMEM (0 mg glucose/L) at 37 °C in a humidified atmosphere containing 5% CO_2. Epoxomycin (Calbiochem, Gibbstown, NJ, USA) was used at a final concentration of 5 μM dissolved in DMSO.

Protein isolation and Western blotting. Cultured cells were lysed with 200 μl of whole cell protein lysis buffer (Cell Signaling, Boston MA, USA) with freshly added proteinase inhibitors, and lysates run on 7.5% polyacrylamide gels under reducing conditions. NRP-1 was immunoprecipitated using 50 μl Protein G dynabeads (Invitrogen, Burlington, ON, Canada) incubated with NRP-1 specific antibody (1:50; Santa Cruz Biotechnology, CA, USA) and 500 μg total protein of cell lysate. The eluted protein was run on a 7.5% polyacrylamide gel under reducing conditions. Proteins were transferred to PVDF membranes and blocked in 0.1% Tween 20, 5% skim milk protein in Tris Buffer Saline. Blots were probed with primary antibodies: VEGFR2, p21, p27 (1:1000; Cell Signaling), VEGFR1 (1:1000; Abcam, Cambridge, MA, USA), NRP-1 (1:1000; Santa Cruz Biotechnology), and GRP-78 (1:500; Santa Cruz Biotechnology), α-tubulin (1:200,000; Sigma–Aldrich), followed by secondary antibody POD-conjugated goat anti-rabbit (1:5000; Sigma–Aldrich) or goat anti-mouse (1:10,000; Sigma–Aldrich). Signal was developed using BM Chemiluminescence Western Blotting Substrate (POD) (Roche Applied Science, Madison, WI, USA) and exposed to ECL X-ray medical film (Konica Minolta, Mississauga, ON, Canada) to visualize bands; intensity of α-tubulin bands were used to determine equal protein loading.

VEGF-A ELISA. 5×10^4 OVCAR-3 or CAOV-3 cells were seeded in complete growth medium for 24 h then incubated in DMEM plus 1% FBS. Conditioned medium was collected at two different time points: 24 and 48 h. VEGF levels were determined using the Human VEGF ELISA kit (R&D Systems, Burlington ON Canada) according to manufacturer's protocol, and expressed as pg/ml.

Quantitative RT-PCR. Total RNA was isolated using Tri-pure reagent (Roche Applied Science) and reverse transcribed with SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR was performed with LightCycler FastStart DNA Master SYBR Green I kit, using the LightCycler 3 (Roche Applied Science). PCR conditions were as follows: 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, elongation at 72 °C for 10 s. After the last cycle, the melting curve was generated by beginning fluorescence acquisition at 72 °C and taking measurements every 0.1 °C until 95 °C was reached. Samples were run in triplicate and the experiment was repeated three times. The relative fold change $2-\Delta\Delta CT$ method was used to determine the quantitative gene expression compared with β-actin using the following primer sequences:

- NRP-1 [12]: HNRP-F: ′5-GCACGTGATTGTCATGTTCC-3′
- HNRP-R: ′5-GGTGGATGAATGTGATGACG-3′
- β-Actin: HBETA-ACTINF ′5-AAGATCAAGATCATTGCTCCTC-3′
- HBETA-ACTINR ′5-CAACTAAGTCATAGTCCGCC-3′
- VEGFR2: [12] HVEGFR2-F: ′5-ATCCCTGTGGATCTGAAACG-3′
- HVEGFR2-R: ′5-CCAAGAACTCCATGCCCTTA-3′
- VEGF: VEGFEX3F: ′5-ATCTTCAAGCCATCCTGTGTGC-3′
- VEGFEX8R: ′5-GCTCACCGCCTCGGCTTGT-3′

Statistical analysis. Statistically significant differences were determined by two-tailed unpaired Student's *t* test and one-way ANOVA, and were defined as $p < 0.05$. All experiments were repeated independently at least three times ($N = 3$); QRT-PCR and ELISA samples were analyzed in triplicate.

Results

Hypoglycaemia down regulates glycosylation and protein levels of VEGFR2

Upregulation of vascular endothelial growth factor (VEGF) in ischemic conditions (e.g. hypoxia, hypoglycemia) is known to induce angiogenesis in solid tumors [4]. We previously showed that human EOC cells employ an autocrine VEGF/VEGFR2 signaling loop [13], so here we explored the impact of glucose concentration on VEGFR expression in these cells. We observed a loss of VEGFR2 and its co-receptor NRP-1 after 48 h of glucose deprivation; addition of pyruvate in the absence or presence of glucose did not alter VEGFR2 expression patterns confirming that this is a glucose-specific effect (Fig. 1A). There were no detectable changes in VEGFR1 levels (Fig. 1A). Glycosylation studies have shown that the two expressed bands of VEGFR2 represent two forms of the protein, the 230 kDa band is the glycosylated form and the 200 kDa is non-glycosylated [14,15]. In this report we noticed that VEGFR2 glycosylation and loss was highly sensitive to glucose concentration (Fig. 1B). When cells were cultured with 0.5–1 mM of glucose, only the un-glycosylated (200 kDa) VEGFR2 molecule was detected; upon increasing glucose to 1.5 mM both this and the mature form of the protein (230 kDa) were expressed (Fig. 1B). However, the levels of VEGFR2 protein did not change significantly under normal physiological (5 mM) or superphysiological (15–25 mM) concentrations of glucose (Fig. 1B). Paradoxically, we observed upregulation of both *VEGFR2* and *NRP-1* mRNA quantified by QRT-PCR upon glucose withdrawal in a dose dependent fashion (Fig. 1C and D).

The effect of glucose withdrawal on loss of VEGFR2 protein and accumulation of mRNA was inversely related and rapid in both OVCAR-3 and CAOV-3 cells

After identifying this novel ability of glucose to regulate the expression of VEGFR2 in both EOC lines, we were interested in evaluating the time course and sensitivity of these responses. Both CAOV-3 and OVCAR-3 rapidly reduced their levels of VEGFR2 upon glucose withdrawal (Fig. 2). Through out this study and from our previous work with the different EOC lines [13] we detected higher expression of VEGFR2 in OVCAR-3 compared to CAOV-3 when both were grown in super physiological concentrations of glucose (25 mM). The changes in VEGFR2 levels were faster in CAOV-3 cells compared to OVCAR-3. After 1 h of glucose withdrawal CAOV-3 barely expressed detectable VEGFR2, while there was no change in VEGFR2 produced in OVCAR-3 at the same time points (data not shown) and it took 4 h for VEGFR2 protein levels in OVCAR-3 cells to significantly reduce (Fig. 2A). Similar to what was seen previously with different concentrations of glucose, *KDR* expression changes upon glucose withdrawal were inversely related to protein levels. Gene transcription in OVCAR-3 cells increased after 4 h of hypoglycaemia, and mRNA continued to accumulate with increasing time up to the 48 h of our experiment, while in CAOV-3 cells the increase in *KDR* mRNA accumulation began after 1 h of glucose deprivation (Fig. 2B).

Induction of VEGF protein and mRNA under hypoglycaemia in EOC

In contrast to the behavior of the receptors, VEGF expression at both the protein and mRNA levels was increased in hypoglycaemic conditions. ELISA assay specific to human VEGF showed increased VEGF protein secretion to the conditioned media by both CAOV-3 and OVCAR-3 (Fig. 3). There were, however, differences between the two cell lines, in that CAOV-3 cells responded faster to low glucose by secreting significantly higher

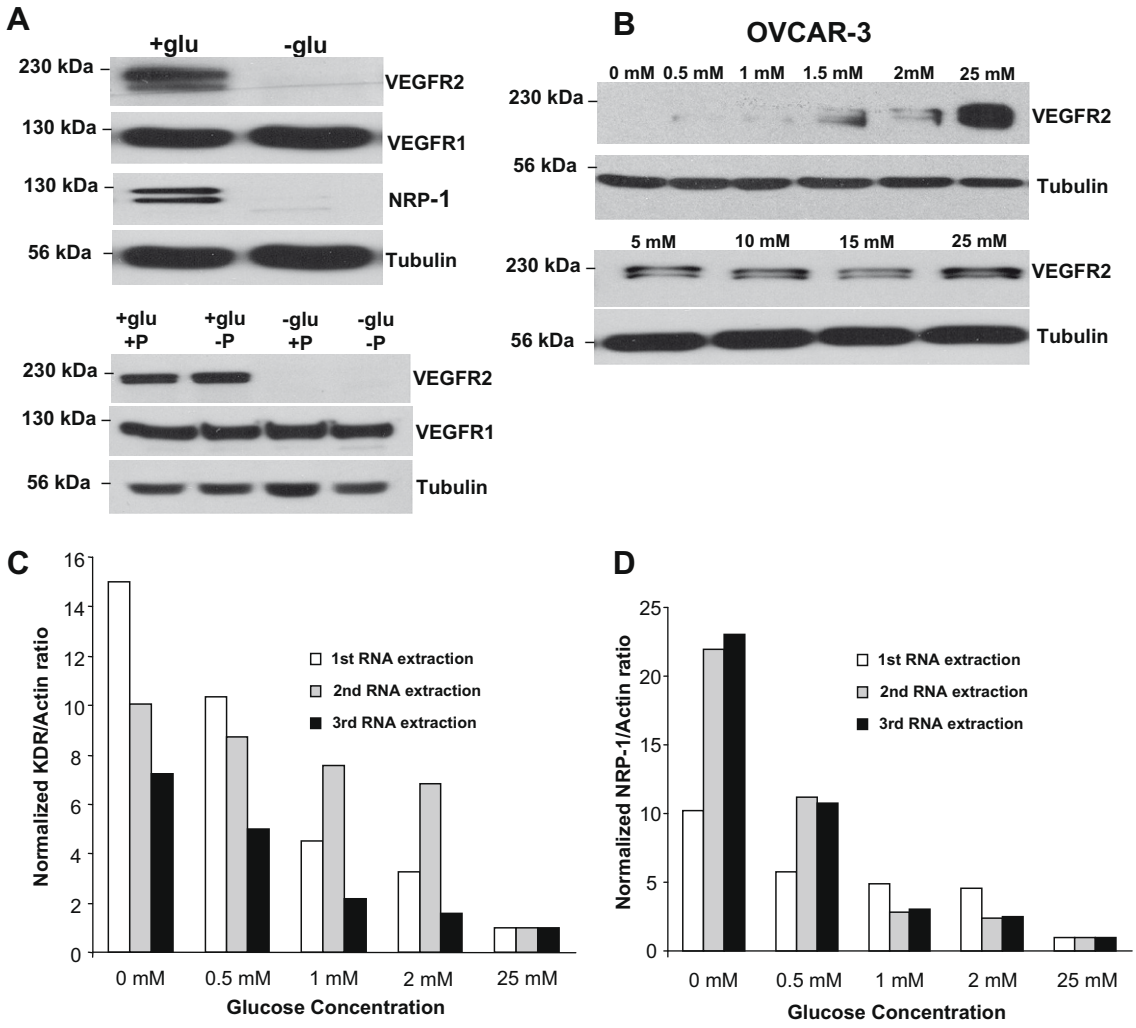

Fig. 1. Effect of glucose deprivation on VEGF receptors in EOC cells. (A) Western blots showing absence of VEGFR2 and NRP-1 but no change of VEGFR1 protein in OVCAR-3 cells after 48 h in glucose free environment. Pyruvate levels did not affect VEGFR2 levels in OVCAR-3 cells under glucose starvation. (B) Expression of VEGFR2 protein is glucose concentration dependant. (C,D) QRT-PCR of KDR and NRP-1 gene expression in three independent RNA isolations. (C) Exposure to different concentrations of glucose for 12 h leads to increased KDR mRNA levels inversely proportional to glucose concentration, with highest message levels at 0 mM and lowest at 25 mM of glucose. (D) NRP-1 mRNA accumulation at 12 h showed the same transcriptional pattern as KDR, with mRNA levels inversely related to glucose concentration.

amounts of VEGF after 24 and 48 h of glucose deprivation, while for OVCAR-3 cells significant increases in VEGF secretion were only detectable after 48 h of hypoglycaemia (Fig. 3A and B). Quantification of *VEGF* mRNA in OVCAR-3 cells demonstrated similar sensitivity to glucose concentration, where dose related alterations in *VEGF* mRNA occurred, reaching maximum production under glucose free conditions (Fig. 3C). The accumulation of *VEGF* mRNA was also increased by increased exposure to low glucose conditions, reaching a maximum at 48 h of hypoglycaemia (Fig. 3D).

VEGFR2 degradation in EOC occurs via 26S proteasome activity

The rapid down-regulation in VEGFR2 protein suggests protein degradation upon glucose withdrawal. We therefore treated OVCAR-3 and CAOV-3 cells with a 26S proteasome inhibitor (epoxomycin) for 12 h, and observed that only the lower (200 kDa, un-glycosylated) form of VEGFR2 was detectable in these glucose-starved cells when epoxomycin was present (Fig. 4A). To ensure that proteasome activity was fully inhibited we confirmed the induction of cell cycle proteins p21 and p27, known to accumulate upon proteasomal inhibition (Fig. 4A). Endoplasmic reticulum (ER) stress can be induced by nutrient (e.g. glucose) depletion and may trigger the so-called unfolded protein response (UPR) through the expression of the ER chaperon GRP-78. We determined that GRP-78 levels are low in unstressed OVCAR-3 cells, increased in the absence of glucose, and moderately expressed in epoxomycin treated glucose-starved cells (Fig. 4B).

Discussion

VEGFR2 and its co-receptor NRP-1 are the main angiogenic receptors in the VEGF pathway and expressed by many types of cancer including EOC cell lines [13,16,17]. Previous reports demonstrated the biochemical link between these two receptors, specifically that protein modification of one leads to alteration of the other [17]. Due to the essential role this pathway plays in protecting EOC cells from anoikis [13], and our previous findings on ischemic contribution to tumor progression [6], we determined the impact of hypoglycaemia on the regulation of this pathway. We observed that hypoglycaemia reduces levels of VEGFR2 and its co-receptor NRP-1 with no change in VEGFR1; this result is consistent with previous findings that levels of VEGFR1 were unaffected by transient knockdown of VEGFR2 [18].

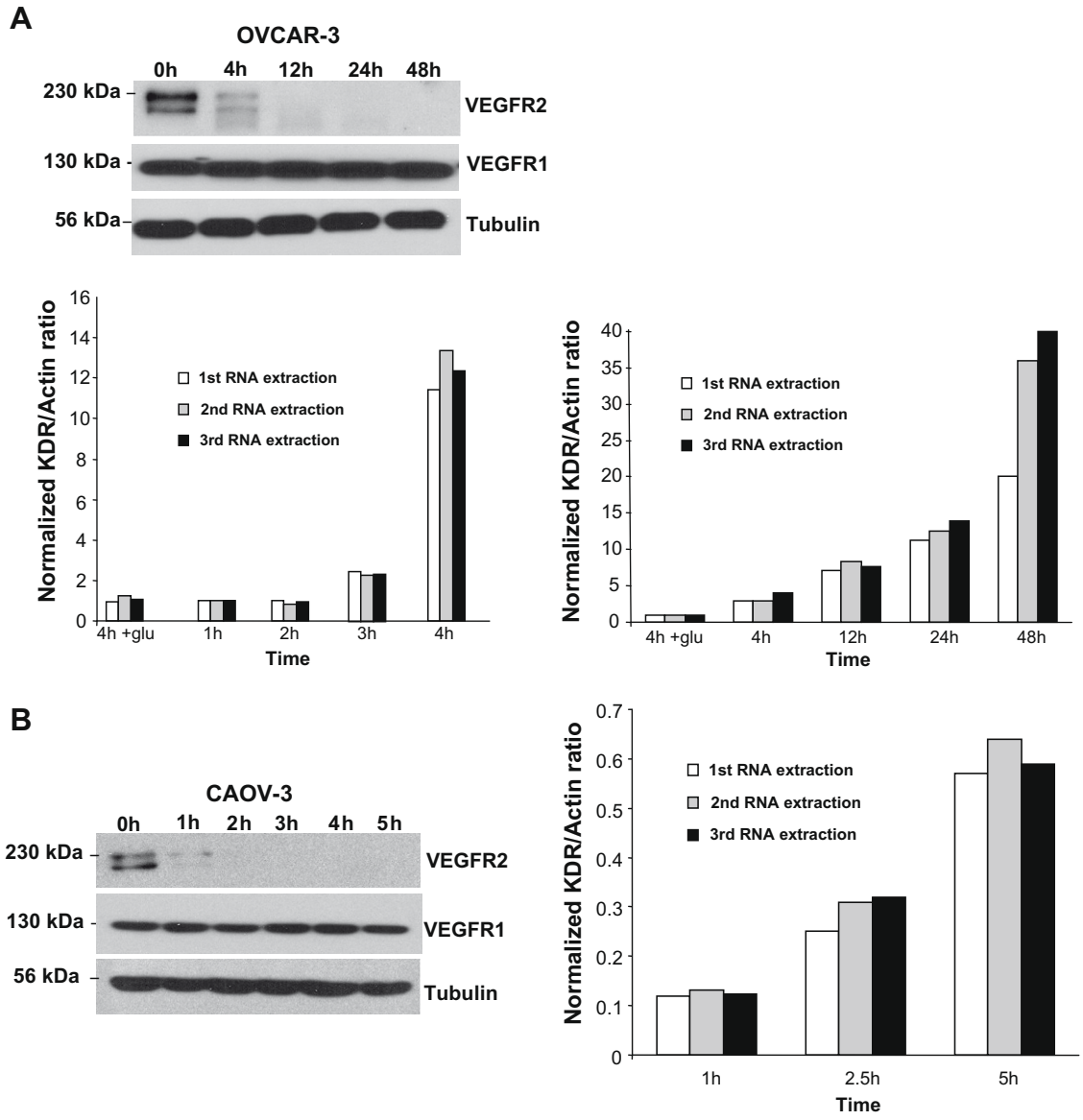

Fig. 2. VEGFR2 protein degradation and mRNA accumulation is rapid in EOC exposed to hypoglycaemia. (A) Western blot showing VEGFR2 protein degradation begins in OVCAR-3 cells 4 h after switching from complete DMEM (25 mM glucose) to glucose free DMEM. VEGFR2 mRNA accumulation increases significantly after 4 h (left) and continues to increase with increasing time in hypoglycaemia (right), as shown by Q-RT-PCR. (B) VEGFR2 protein degrades rapidly in CAOV-3 cells, with barely detectable levels after 1 h of glucose deprivation while KDR mRNA production significantly increases after 1 h and continues to increase with increasing the time of hypoglycaemia, as shown by QRT-PCR.

Another consequence of hypoglycaemia we saw was the reduction in glycosylation of VEGFR2, which is highly sensitive to glucose concentration. The extracellular domain of VEGFR2 contains 18 potential N-linked glycosylation sites and VEGFR2 is expressed in two different sizes depending on the level of glycosylation [14]. OVCAR-3 and CAOV-3 cells expressed both 230 and 200 kDa versions of VEGFR2, the higher molecular weight representing the fully glycosylated form. In the absence of glucose both forms were lost and their re-expression was achieved in a glucose dose-dependant fashion. VEGFR2 protein loss and message increase occurred sooner in CAOV-3 than in OVCAR-3, suggesting differences in sensitivity to glucose deprivation possibly reflective of the lower VEGFR2 protein levels in CAOV-3 cells under hyperglycaemic conditions.

In contrast to its receptors, VEGF-A at both the transcriptional and translational levels was increased in a time and dose-dependant fashion under glucose deprivation in OVCAR-3 and CAOV-3 cells, consistent with previous studies that showed higher VEGF mRNA and protein expression under hypoglycaemia in human U-937 lymphoma cells and murine ID8 cells [4,5]. Induction of VEGF gene expression by hypoglycaemia in rat C6 glioma cells is due to transcriptional activation and mRNA stabilization [7]. The high levels of secreted VEGF protein seen here confirm hypoglycaemia acts as an important trigger for angiogenic factor expression in human EOC cells. Elevated serum VEGF develops early in diabetic patients, is modulated by glycemic control, and contributes to diabetic microvascular complications [19]. Our results support the possibility that hyperglycemia participates in EOC initiation and/or progression via the VEGF/VEGFR2 autocrine signalling loop we previously reported in these cells [13].

The down-regulation in VEGFR2 protein under hypoglycaemia suggests protein degradation. EOC cell lines (including OVCAR-3) express abnormally high levels of 26S proteasome in vivo and in vitro [20]. The presence of only the un-glycosylated band of

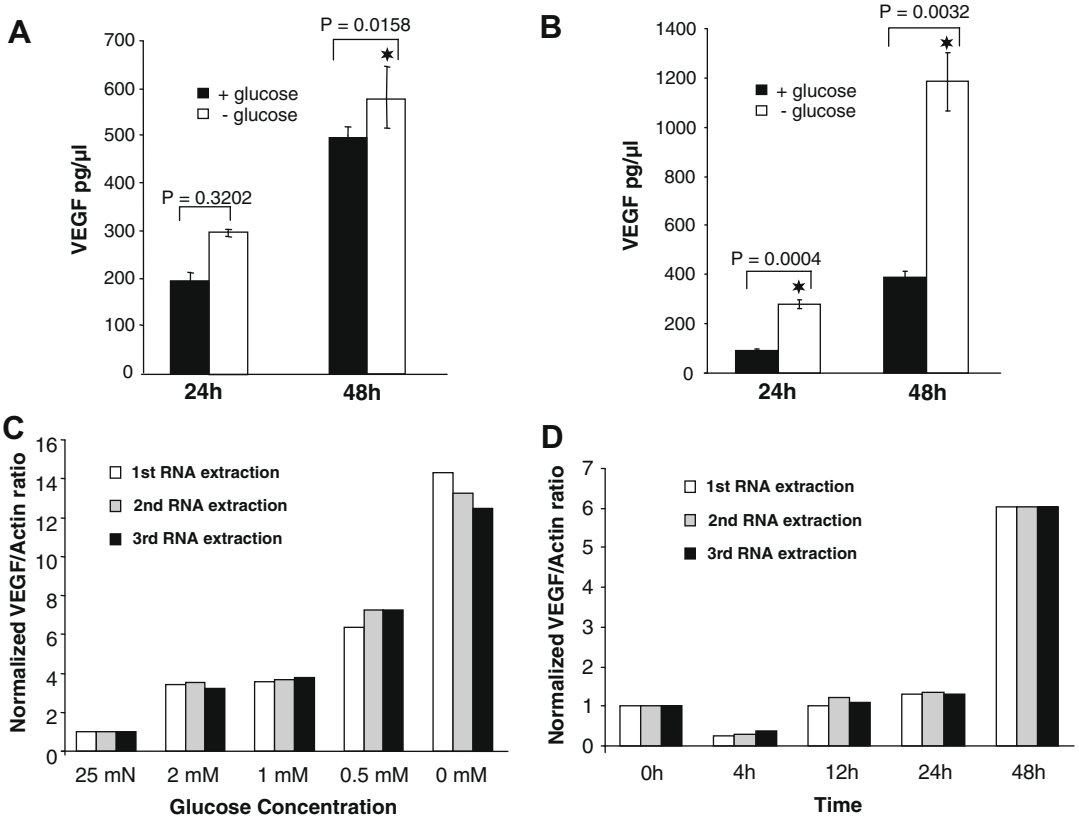

Fig. 3. Increase in VEGF protein secretion and mRNA accumulation in hypoglycaemic cells. (A) Quantification of VEGF in OVCAR-3 conditioned medium by ELISA shows a significant increase in production after 48 h of hypoglycaemia ($N = 3$). (B) In contrast, CAOV-3 cells secret significantly increased amounts of VEGF after 24 h of hypoglycaemia, and substantially more after 48 h, compared to control ($N = 3$). (C) QRT-PCR for VEGF mRNA shows an inverse relationship between glucose concentration and VEGF mRNA in OVCAR-3 cells. (D) QRT-PCR shows VEGF mRNA accumulation in OVCAR-3 cells increases with increased time of hypoglycaemia.

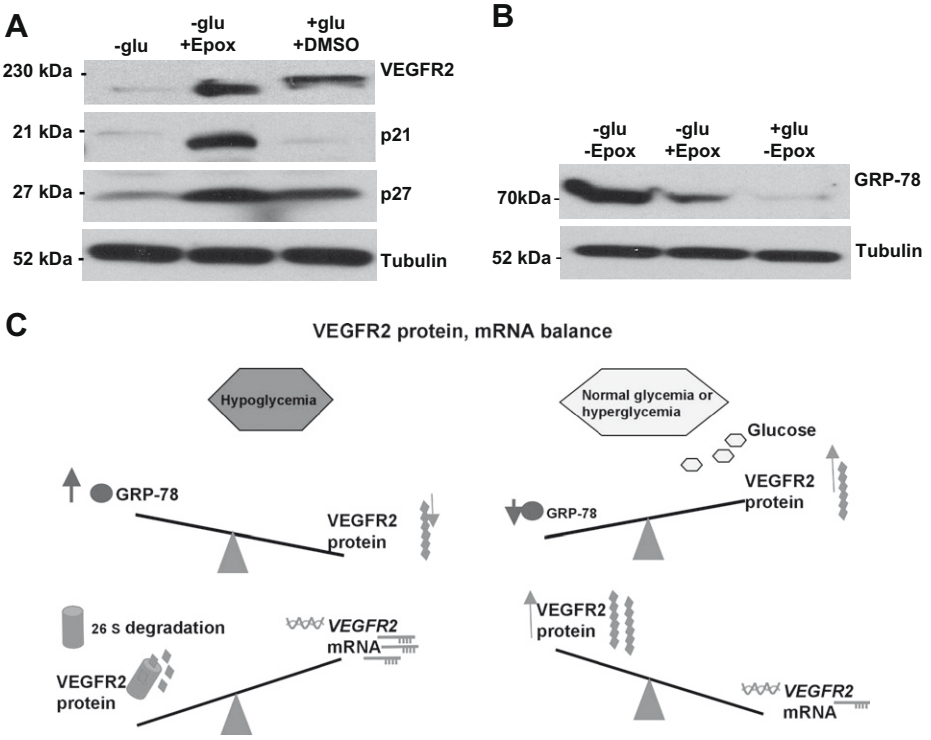

Fig. 4. Proteasomal degradation of VEGFR2 protein under hypoglycaemia. (A) Western blot of OVCAR-3 cell lysates showing the re-expression of the un-glycosylated VEGFR2 upon the addition of 5 mM epoxomycin under hypoglycemic conditions for 12 h. Cells growing in normal physiologic concentration of glucose (5 mM) in the presence of DMSO expressed full length VEGFR2. Cell cycle proteins p21 and p27 accumulated in hypoglycemic epoxomycin treated cells. (B) GRP-78 protein was highly up-regulated in OVCAR-3 cells in the absence of glucose, with lower expression upon proteasome inhibition and very low expression in normal glycemic conditions. (C) Schematic summary of our observations on removal of glucose from the growth media and its effects on VEGFR2 protein and mRNA levels expressed by EOC.

VEGFR2 in glucose-starved epoxomycin treated cells implies that VEGFR2 protein glycosylation requires adequate cytosolic glucose levels. Endoplasmic reticulum (ER) stress can be induced by nutrient (e.g. glucose) depletion and may trigger the so-called unfolded protein response (UPR). Key features of the UPR are the transcription program activator, which allows the cell to survive under stress conditions, and the regulation of the molecular chaperones such as GRP-78 involved in protein folding in the ER [10,21]. Our findings suggest that glucose stress, which occurs in ischemic conditions within solid tumors, can modulate the VEGF/VEGFR2 autocrine pathway in these cells [13].

Conclusion

Our results are summarized in Fig. 4C. Taken together, they suggest a new avenue for exploring how diabetes-related hyperglycemia may contribute to EOC initiation and/or progression. This represents a paradigm shift in how we view the role of angiogenic factors such as VEGF in progression of EOC, and possibly in other types of cancer. Our finding that glucose can regulate VEGF/VEGFR2 levels suggests that initiation and/or progression of ovarian surface epithelial cells towards a neoplastic phenotype might be modulated by dietary conditions, and that a patient's metabolic status may alter the effectiveness of the known anti-angiogenic therapies. This information provides opportunities for exploring the mechanisms underlying this interesting regulatory effect, leading to enhanced understanding of the biology of EOC progression.

Acknowledgments

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