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17β-estradiol Changes the Human Cerebral Endothelial Cell Proteome Upon Exposure to Cyclic Stretch

(17β-estradiol Merubah Proteome Sel Endotelial Serebral Manusia yang Terdedah Kepada Regangan)

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ABSTRACT

Hypertension and estrogen deficiency in women have been identified as significant risk factors for cerebrovascular diseases. Hypertension causes excessive vascular stretch and contributes to the initiation of cellular injury in blood vessels while estrogen has been demonstrated to exert beneficial protective effects on the vascular system. Although the specific biological outcomes exerted by either excessive stretch or estrogen exposure are well established, the combined biochemical effects of both stimuli remain unclear. Therefore, this study was conducted for quantitative proteomics study on human cerebral microvascular endothelial cells (HCMECs) subjected to 20% "pathological" cyclic stretch for a period of 18 hour in the presence or absence of 17β-estradiol by isobaric Taqs for Relative and Absolute Quantification. The results showed that only some proteins responded to 17β-estradiol (e.g., thioredoxin reductase-1), stretch (e.g., 14-3-3 protein epsilon or acidic leucine-rich nuclear phosphoprotein 32 family member B) and interestingly, some proteins returned to control pre-treatment levels when exposed to both (e.g., d-dopachrome decarboxylase, thrombospondin-1). In addition, HCMECs that exposed only to estrogen had a very similar proteomic profile (i.e., up-regulation of structural, cellular adhesion and proliferation proteins) as to those exposed to estrogen with 20% stretching for 18 hour, suggesting that estrogen ablated the detrimental effects by the stretch alone. These findings sheds light on the molecular mechanisms by which the cerebrovascular protective actions of estrogen on HCMEC exposed to pathological levels of cyclic stretch which could provide a platform for future research in therapeutic approach.

Keywords: Cyclic stretch; estrogen; intracranial aneurysm; proteome expression; vascular endothelial cell

ABSTRAK

Tekanan darah tinggi dan kekurangan estrogen di kalangan wanita telah dikenal pasti sebagai faktor risiko penyakit serobrovaskular. Tekanan darah tinggi menyebabkan regangan salur darah berlebihan dan membawa kepada kecederaan sel salur darah. Estrogen telah menunjukkan kesan perlindungan pada sistem vaskular. Walaupun kesan spesifik biologi yang dihasilkan oleh regangan dan estrogen diketahui, kesan kombinasi kedua-dua stimuli ini masih tidak jelas. Justeru, kajian kuantitatif proteomik ini dilakukan pada sel endotelium serebral manusia pada 20% regangan patologi selama 18 jam dengan atau tanpa kehadiran 17β-estradiol. Sesetengah protein hanya bertindak balas kepada 17β-estradiol (thioredoxin reduktase-1); hanya kepada regangan (14-3-3 protein epsilon atau asid kaya-leusin- nuklear fosfoprotein 32 kumpulan B) dan protein yang kembali ke paras asal (d-dopakrom dekarboksilase, thrombospondin-1). Sel yang terdedah kepada 17β-estradiol mempunyai profil proteomik yang sama dengan pendedahan 17β-estradiol dan regangan patologi. Ini menunjukkan estrogen boleh menahan kesan yang dikaitkan dengan regangan patologi. Kajian ini memberikan gambaran mekanisme molecular perlindungan serebrovaskular terhadap estrogen pada sel yang terdedah kepada regangan patologi yang boleh digunakan untuk kajian masa depan dalam pendekatan teraputik.

Kata kunci: Regangan berulang; estrogen; aneurisma intrakranial; ekspresi proteomik; sel vaskular endotelial

INTRODUCTION

Intracranial aneurysm (IA) is the dilatation of a cerebral artery segment due to fragility in the arterial wall that usually arised at the bifurcation regions of Circle of Willis. It is often small in size and asymptomatic, however, it can be fatal when it ruptures which lead to bleeding, resulting in subarachnoid haemorrhage (SAH) (Keedy 2006). About 2-3% of the general population of the world developed IA (Juvela 2011; Rinkel et al. 1998). In addition, 5-15% of strokes are related to ruptured IA which are often classified as hemorrhagic stroke (Bederson et al. 2000). Several risk factors have been associated with IA such as hypertension, gender, family history, alcohol consumption and smoking (Cebral & Raschi 2000; Rinkel 2005).

A causal link between hypertension and aneurysms has been established, with previous studies showing it to be as high as 44% compared to normotensive individuals (24%) (Inci & Spetzler 2000). Interestingly, both hypertension and aneurysm share similar risk factors, that may indicate their close pathogenic relationship (Chen et al. 2015; Wei et al. 2015). Hypertension exerts excessive hemodynamic pressure on vascular walls. The increase in chronic pulsatile pressure could lead to overstretching of the vessels that

in turn may lead to cellular injury and eventually cause vascular weakness, endothelial dysfunction, scarring and an increased tendency for aneurysm formation in arteries (Anwar et al. 2012, Pascarella et al. 2008, Thubrikar & Robicsek 1995). One significant observation is that postmenopausal women have been reported to have high prevalence of IA and SAH compared to premenopausal women, and this has been shown to be due to low levels of estrogen (Longstreth et al. 1994). It has therefore been proposed that the risk of cerebrovascular disease rises as endogenous estrogen levels decline due to ageing and menopause in women.

To investigate proteins that are modulated, in cells of hypertension-induced stretch, an *in-vitro* approach has been developed where pathological cyclic stretch at 20% amplitude was applied (Anwar et al. 2012). A previous study has shown that pathological stretch can induce cell proliferation, apoptosis, production of reactive oxygen species (ROS) and inflammation which can contribute to cardiovascular diseases (Anwar et al. 2012). It is clear that the *in vitro* pathological cyclic stretch model is useful for understanding the range of pathophysiological biochemical and molecular changes occurring as a result of stretch in specific cell types allowing the study of factors such as estrogen exposure or drug exposure to be carried out.

Mechanical stretch on endothelial cells is associated with many negative effects and understanding how these negative effects can be mitigated using estrogen will be a therapeutic value (Anwar et al. 2012). This study aimed to investigate the concurrent effects of both stimuli (stretch and estrogen) on human cerebral microvascular endothelial cells (HCMEC). Although a number of stretch studies have been conducted on smooth muscle cells, this is the first study of its kind on cerebellar endothelial cells whose its biology is implicated in IA and SAH. We employed an approach using isobaric Taqs for Relative and Absolute Quantification (iTRAQ), 4-plex label quantitative proteomics approach on HCMECs that were subjected to physiological concentrations of 17β-estradiol at 20% cyclic stretch intensity separately and simultaneously for 18 hour to simulate chronic hypertension to allow a detailed view of the global changes occurring on these cells.

METHODS

CELL LINES

HCMECs (ABM, Richmond, Canada) at low passage were cultured on fibronectin-coated (20 µg/ml) silicone cubes in M199 media supplemented with 10% fetal bovine serum (FBS) at 37°C (5% CO_2). Fibronectin was selected as the coating material as the cells could attach well on the silicone cube. Upon reaching 70% confluence, the media was replaced with M199 phenol red free media supplemented with 10% charcoal-stripped FBS to prevent any inadvertent steroid hormone activity. Each experiment was conducted for the 4 groups at 37°C (5% CO_2); no stretch nor 17β-estradiol as a control (**C**); cells exposed to 1 nM 17β-estradiol (**E**), cells exposed to 20% stretch (**S**) and cells exposed to both stimuli (**E + S**). The media in each group was changed and the 17β-estradiol was added into the media for group (E) and (E + S) prior to the experiment.

MECHANICAL STRETCH ON HCMEC

Cells grown on silicone cubes were subjected to cyclic mechanical stretch using the Shellpa Mechanical Cell Stretch System (Menicon Life Science, Japan). In detail, uniaxial strain was applied to the cells by a vacuum pump programmed at 60 cycle/min at a 20% stretch magnitude for 18 hour. The control cells were grown in the same condition and placed in the same incubator without being subjected to stretch.

iTRAQ FOUR PLEX LABELING

Isobaric Tag for Relative and Absolute Quantification (iTRAQ) kit (ABSciex, Framingham, USA) was used for protein labeling based on the manufacturer's instructions. The protein concentration was determined using BCA protein assay kit (Thermo Scientific, Rockford, USA). Briefly, 100 µg of protein from each condition was reduced with 50 mM methanethiosulfonate for 1 hour, followed by alkylation using 200 mM iodoacetamide for 30 minute. Proteins were then digested with trypsin at a ratio of 1:25 and incubated at 37°C overnight. The samples were dried in CentriVap Complete Vacuum concentrator (Labconco, Kansas, United States). Each iTRAQ tag was then added to the sample and incubated for 1 hour at room temperature. After labelling, 1 µl from each iTRAQ label was pooled in a new tube. The pooled sample was ZipTip-purified (Millipore) and spotted onto a matrix-assisted laser desorption/ionization (MALDI) plate with matrix (2,5-dihydroxy benzoic acid-DHB). The sample was first run under MALDI tandem time of flight (TOF/TOF) to ensure that iTRAQ reactions were successful. All samples were then pooled in new tubes and dried.

STRONG CATION EXCHANGE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Dried iTRAQ labeled samples were resuspended in loading buffer A (5 mM phosphate 25% acetonitrile, pH 2.7). After sample loading and washing the column (PolyLC polysulfoethyl A column 200 × 2.1 mm) with buffer A, buffer B (5 mM phosphate, 350 mM potassium chloride, 25% acetonitrile, pH 2.7) concentration was increased from 10% to 45% across a 70 minute gradient and then increased quickly to 100%. The buffer was held at 100% for 10 minute with a flow rate of 300 µl/minute. The eluent of strong cation exchange high performance liquid chromatography (SCX HPLC) was collected every 2 minute from the beginning of the gradient and at 4 minute intervals. Collected fractions were dried in vacuum centrifugation.

NANOLC ESI MS/MS DATA ACQUISITION

A total of 11 fractions were isolated from SCX HPLC. Each fraction was resuspended in 60 µL of loading/desalting solution (0.1% formic acid and 2% acetonitrile in water). The samples were analyzed by Triple TOF 5600 (ABSciex, Massachusetts, United States). Samples (40 µL) were injected into a peptide trap (Michrome Peptide Captrap) column for pre-concentration and desalting with 0.1% formic acid, 2% acetonitrile at 10 µL/min for 5 min. The peptide trap was then switched on line with an analytical column. Peptides were eluted from the column using a linear solvent gradient, from mobile phase A (0.1% formic acid): mobile phase B (90% acetonitrile/0.1% formic acid) (98:2) to mobile phase A: mobile phase B (65:40) at 550 nL/minute over 100 minute period. After peptide elution, the column was cleaned with 95% buffer B for 15 minute and then equilibrated with buffer A for 25 min before next sample injection.

The reverse phase nanoLC eluent was subjected to positive ion nanoflow electrospray ionization (ESI) analysis in an information-dependent acquisition mode (IDA). A TOF electrospray mass spectrometry (MS) survey scan was acquired (m/z 400-1500, 0.25 second), with the ten most intense multiply charged ions (counts > 150) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 200 ms in the mass range m/z 100-1500 with the total cycle time of 2.3 seconds.

DATA PROCESSING

The experimental nanoLC ESI MS/MS data were submitted to ProteinPilot V4.2b (AB Sciex) for data processing searching against the SwissProt 2014_04 Human database. The results were analysed using combined *p*-value analysis (Pascovici et al 2015). A protein is considered to be differentially expressed if the Stouffer *p*-value is less than 0.05 and all protein ratios in duplicate are in similar trend (both are up-regulated or down-regulated). To functionally annotate the proteins based on their biological processes, the up and down-regulated proteins were subjected to gene ontology annotation using PloGO (Pascovici et al 2012).

RESULTS AND DISCUSSION

A total of 2,772 proteins were identified in all samples with a protein false discovery rate (FDR) of < 1%. Out of this, 137 proteins were identified as being significantly and differentially expressed ($p < 0.05$) when cutoff ratios were arbitrarily applied between < 0.82 and > 1.2 with respect to controls listed in Table 1. Gene ontology (GO) analysis using PloGO was conducted on all proteins that changed significantly ($P \leq 0.05$) in E, S and E + S groups to gain a clearer understanding of protein function and possible implications. Exposure to 17β-estradiol (i.e., the E group) of HCMEC up-regulated proteins involved in cell processes such as cell proliferation, cell adhesion, and structural molecule activity (Table 2). However, the S group demonstrated different protein profiles as several proteins were not significantly changed in comparison to E group. These included proteins that were involved in cell proliferation [catenin β-1 (CTNNB1) and thioredoxin reductase 1 (TXNRD1)], cell adhesion [alpha-actinin-1 (ACTN1), integrin alpha-3 (ITGA3), alpha-parvin (PARVA)] and structural molecule activity [actin-related protein 2 (ACTR2)]. The proteins then were analyzed based on its trends at each group conditions (Figure 1).

TABLE 1. List of significantly different proteins ($p < 0.05$) against normal with a cutoff ratio > 1.2 and < 0.82 against the control. The proteins were grouped based on trends observed in the experiment; trend I: protein upregulated in E and E + S; trend II: proteins downregulated in E and E + S; trend III: proteins upregulated in S and E + S; trend IV: proteins downregulated in S and E + S; trend V: protein downregulated in E and upregulated in S, trend VI: proteins upregulated in E and downregulated in S; trend VII: proteins downregulated in E, upregulated in S and downregulated in E + S; and other proteins that were not included the trend. The red colour indicates the up-regulated proteins, the green colour indicates down-regulated protein and other indicates proteins that did not change.

Protein	Gene	Estrogen only (E)	Stretch only (S)	Stretch and estrogen (E + S)
Trend I				
ATP synthase subunit alpha	ATP5A1	1.23	0.98	1.23
Catenin beta-1	CTNNB1	1.24	1.06	1.24
Importin-5	IPO5	1.24	0.98	1.20
Dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit 2	RPN2	1.26	1.08	1.24
Probable ATP-dependent RNA helicase	DDX5	1.29	1.12	1.38
Actin-related protein 2	ACTR2	1.29	0.93	1.24
Switch-associated protein 70	SWAP70	1.36	1.10	1.39
Alpha-actinin-1	ACTN1	1.36	1.16	1.33
Isochorismatase domain-containing protein 1	ISOC1	1.45	1.19	1.25
Keratin, type I cytoskeletal 10	KRT10	1.46	0.83	1.45

Continued

TABLE 1. *Continue*

Protein	Gene	Estrogen only (E)	Stretch only (S)	Stretch and estrogen (E + S)
Glucose-6-phosphate isomerase	GPI	1.47	0.91	1.23
Phospholipase D3	PLD3	1.48	0.89	1.46
Integrin alpha-3	ITGA3	1.50	1.18	1.43
Thioredoxin reductase 1, cytoplasmic	TXNRD1	1.55	1.18	1.44
Transketolase	TKT	1.59	1.16	1.33
Transgelin	TAGLN	1.74	1.17	1.23
	Trend II			
Dynamin-2	DNM2	0.60	0.86	0.70
Cyclin-dependent kinase 1	CDK1	0.71	1.15	0.80
Heterogeneous nuclear ribonucleoprotein A3	HNRNPA3	0.73	0.84	0.72
Vigilin	HDLBP	0.75	0.83	0.76
Alpha-parvin	PARVA	0.76	0.91	0.72
Transforming acidic coiled-coil-containing protein 1	TACC1	0.79	1.01	0.79
Rho GTPase-activating protein 17	ARHGAP17	0.79	1.09	0.71
Cytoplasmic dynein 1 intermediate chain 2	DYNC1I2	0.80	0.87	0.83
Heterogeneous nuclear ribonucleoprotein M	HNRNPM	0.81	0.91	0.78
	Trend III			
Transcription initiation factor IIB	GTF2B	0.99	1.72	1.27
Protein bicaudal D homolog 2	BICD2	1.04	1.36	1.45
Transmembrane 9 superfamily member 4	TM9SF4	1.06	1.48	1.40
Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	PDHA1	1.06	1.37	1.36
Retinal dehydrogenase 2	ALDH1A2	1.11	1.42	1.35
Nuclear pore complex protein Nup93	NUP93	1.14	1.32	1.28
Serine/arginine repetitive matrix protein 2	SRRM2	1.16	1.31	1.20
	Trend IV			
Signal transducer and activator of transcription 1-alpha/beta	STAT1	0.84	0.75	0.68
Serine hydroxymethyltransferase, mitochondrial	SHMT2	0.85	0.55	0.69
X-ray repair cross-complementing protein 6	XRCC6	0.86	0.73	0.80
Cytoplasmic dynein 1 heavy chain 1	DYNC1H1	0.87	0.79	0.81
14-3-3 protein epsilon	YWHAE	0.87	0.79	0.75
Acidic leucine-rich nuclear phosphoprotein 32 family member B	ANP32B	0.93	0.59	0.66
Citrate synthase, mitochondrial	CS	0.95	0.78	0.77
GTP-binding nuclear protein Ran	RAN	1.11	0.70	0.67
	Trend V			
D-dopachrome decarboxylase	DDT	0.66	1.93	1.06
	Trend VI			
Thrombospondin-1	THBS1	1.30	0.75	0.95
ATP synthase subunit beta, mitochondrial	ATP5B	1.37	0.68	0.97
	Trend VII			
Protein FAM49B	FAM49B	0.73	1.22	0.78

Continued

TABLE 1. *Continue*

Protein	Gene	Estrogen only (E)	Stretch only (S)	Stretch and estrogen (E + S)
Other Proteins				
Interferon-induced GTP-binding protein Mx1	MX1	0.20	0.43	0.28
Septin-11	SEPT11	0.22	0.17	0.20
Septin-9	SEPT9	0.33	0.52	0.39
Septin-2	SEPT2	0.47	0.56	0.52
Septin-7	SEPT7	0.47	0.64	0.53
Retinal dehydrogenase 1	ALDH1A1	0.50	0.51	0.51
Unconventional myosin-Ic	MYO1C	0.57	0.59	0.65
Lamina-associated polypeptide 2, isoform alpha	TMPO	0.59	0.80	0.67
Prelamin-A/C	LMNA	0.59	0.57	0.53
DNA-dependent protein kinase catalytic subunit	PRKDC	0.62	0.65	0.61
Caveolin-1	CAV1	0.64	0.66	0.62
Elongation factor 1-gamma	EEF1G	0.65	0.67	0.64
Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	ACADVL	0.71	0.75	0.71
Mesencephalic astrocyte-derived neurotrophic factor	MANF	0.74	0.77	0.75
Heterogeneous nuclear ribonucleoprotein A1	HNRNPA1	0.75	0.77	0.69
Protein FAM98B	FAM98B	0.77	0.65	0.79
Protein-glutamine gamma-glutamyltransferase 2	TGM2	0.81	0.67	0.66
Acyl-coenzyme A thioesterase 9, mitochondrial	ACOT9	0.83	1.03	1.04
Trifunctional enzyme subunit alpha, mitochondrial	HADHA	0.85	0.93	0.92
Spectrin alpha chain, non-erythrocytic 1	SPTAN1	0.88	0.94	0.88
Staphylococcal nuclease domain-containing protein 1	SND1	0.88	1.13	0.99
Translational activator GCN1	GCN1L1	0.90	1.06	0.86
26S proteasome non-ATPase regulatory subunit 1	PSMD1	0.90	0.99	0.96
Cytoplasmic aconitate hydratase	ACO1	0.94	0.98	0.99
Ubiquitin-like modifier-activating enzyme 1	UBA1	0.95	0.94	1.02
Filamin-A	FLNA	1.01	0.88	0.89
Rho GTPase-activating protein 18	ARHGAP18	1.02	0.99	1.05
DNA replication licensing factor	MCM5	1.03	0.92	1.05
U5 small nuclear ribonucleoprotein 200 kDa helicase	SNRNP200	1.06	1.06	1.01
Neuroblast differentiation-associated protein	AHNAK	1.07	1.10	0.91
COP9 signalosome complex subunit 4	COPS4	1.14	0.91	1.08
Endoglin	ENG	1.19	0.89	0.95
Band 4.1-like protein 3	EPB41L3	1.20	0.97	1.19
Palmdelphin	PALMD	1.20	1.62	1.35
Protein-lysine 6-oxidase	LOX	1.22	1.70	1.51
Calpain-2 catalytic subunit	CAPN2	1.27	1.27	1.52
Extended synaptotagmin-1	ESYT1	1.27	1.23	1.23
LIM domain only protein 7	LMO7	1.29	1.60	1.35
Membrane-associated progesterone receptor component 2	PGRMC2	1.35	1.21	1.74
Aldehyde dehydrogenase family 1 member A3	ALDH1A3	1.35	1.59	1.41
Serine palmitoyltransferase 2	SPTLC2	1.36	1.36	1.55
Cell surface glycoprotein MUC18	MCAM	1.37	1.33	1.40
Guanine nucleotide-binding protein G(s) subunit alpha isoforms XLas	GNAS	1.38	1.23	1.38
Cadherin-2	CDH2	1.38	1.25	1.59
UDP-N-acetylhexosamine pyrophosphorylase	UAP1	1.39	1.34	1.39
Glutathione S-transferase Mu 3	GSTM3	1.41	1.21	1.60

Continued

TABLE 1. *Continue*

Protein	Gene	Estrogen only (E)	Stretch only (S)	Stretch and estrogen (E + S)
E3 ubiquitin-protein ligase	HECTD1	1.41	1.55	1.58
Acylamino-acid-releasing enzyme	APEH	1.42	1.25	1.42
BTB/POZ domain-containing protein	KCTD12	1.43	1.37	1.39
Fibronectin	FN1	1.43	2.11	1.43
Prostaglandin reductase 1	PTGR1	1.43	1.59	1.53
4F2 cell-surface antigen heavy chain	SLC3A2	1.47	1.44	1.57
Heat shock 70 kDa protein 1A/1B	HSPA1A	1.59	1.45	1.66
Ferritin light chain	FTL	1.59	1.68	1.70
Lysosome-associated membrane glycoprotein 1	LAMP1	1.82	1.27	1.67
Annexin A2	ANXA2	1.86	1.85	1.49
Annexin A6	ANXA6	2.11	1.54	1.74
Heme oxygenase 1	HMOX1	2.63	2.27	2.50
Apolipoprotein E	APOE	6.72	1.66	3.78
Peptidyl-prolyl cis-trans isomerase	FKBP3	0.63	0.76	0.86
Serpin B9	SERPINB9	0.79	0.80	0.85
Structural maintenance of chromosomes protein 4	SMC4	0.72	0.85	1.05
AP-2 complex subunit mu	AP2M1	0.76	1.01	1.02
Phenylalanine--tRNA ligase alpha subunit	FARSA	0.76	0.96	0.93
Structural maintenance of chromosomes protein 2	SMC2	0.78	0.97	0.90
Phenylalanine--tRNA ligase beta subunit	FARSB	0.83	1.00	0.89
PRKC apoptosis WT1 regulator protein	PAWR	0.83	1.08	0.89
Protein DJ-1	PARK7	0.83	0.83	0.84
Prefoldin subunit 3	VBP1	0.95	0.66	0.83
Kinesin-1 heavy chain	KIF5B	0.97	0.80	0.83
Purine nucleoside phosphorylase	PNP	1.10	0.79	1.07
Peroxiredoxin-6	PRDX6	1.11	0.81	0.93
Tryptophan--tRNA ligase, cytoplasmic	WARS	0.84	0.86	0.71
Keratin, type II cytoskeletal 8	KRT8	0.87	0.86	0.66
Heterogeneous nuclear ribonucleoprotein K	HNRNPK	0.92	0.89	0.78
Myosin-9	MYH9	0.94	0.97	0.78
Na(+)/H(+) exchange regulatory cofactor NHE-RF1	SLC9A3R1	1.00	1.25	0.85
Ribosome-binding protein 1	RRBP1	1.09	1.29	1.06
Aconitate hydratase, mitochondrial	ACO2	1.04	0.93	1.33
Acetyl-CoA carboxylase 1	ACACA	1.07	0.98	1.30
Elongation factor Tu, mitochondrial	TUFM	1.14	1.03	1.42
Arginine--tRNA ligase, cytoplasmic	RARS	1.16	0.88	1.34
Keratin, type I cytoskeletal 18	KRT18	1.20	1.22	0.81
116 kDa U5 small nuclear ribonucleoprotein component	EFTUD2	1.21	1.04	1.09
Poly(U)-binding-splicing factor	PUF60	1.22	1.03	1.14
Fatty acid synthase	FASN	1.22	1.10	1.20
N-acetyl-D-glucosamine kinase	NAGK	1.26	1.08	1.19
Filamin-C	FLNC	1.33	1.13	1.08
Nucleosome assembly protein 1-like 4	NAP1L4	1.34	0.95	1.14
Calreticulin	CALR	1.56	0.87	1.10
Caldesmon	CALD1	1.24	1.32	0.99
Plectin	PLEC	1.27	1.22	1.19
Keratin, type II cytoskeletal 1	KRT1	1.40	0.77	1.34

TABLE 2. List of proteins expressed in HCMEC subjected to 17β-estradiol, 20% stretch and both stimuli based on gene ontology PloGO analysis. Only proteins that has similar regulations between estrogen only group (E) and estrogen + stretch (E + S) were selected. The red colour indicates the up-regulated proteins, the green colour indicates down-regulated protein and other indicates proteins that did not change.

Gene	Protein	Estrogen only (E)	Stretch only (S)	Stretch and estrogen (E + S)
	Cell Proliferation			
TACC1	Transforming acidic coiled-coil-containing protein 1	0.79	1.01	0.79
CDK1	Cyclin-dependent kinase 1	0.71	1.15	0.80
ATP5A1	ATP synthase subunit alpha, mitochondrial	1.23	0.98	1.23
CTNNB1	Catenin beta-1	1.24	1.06	1.24
TXNRD1	Thioredoxin reductase 1, cytoplasmic	1.55	1.18	1.44
	Cell Adhesion			
ACTN1	Alpha-actinin-1	1.36	1.16	1.33
ITGA3	Integrin alpha-3	1.50	1.18	1.43
CTNNB1	Catenin beta-1	1.24	1.06	1.24
PARVA	Alpha-parvin	0.76	0.91	0.72
TXNRD1	Thioredoxin reductase 1, cytoplasmic	1.55	1.18	1.44
	Structural Molecule Activity			
ACTR2	Actin-related protein 2	1.29	0.93	1.24

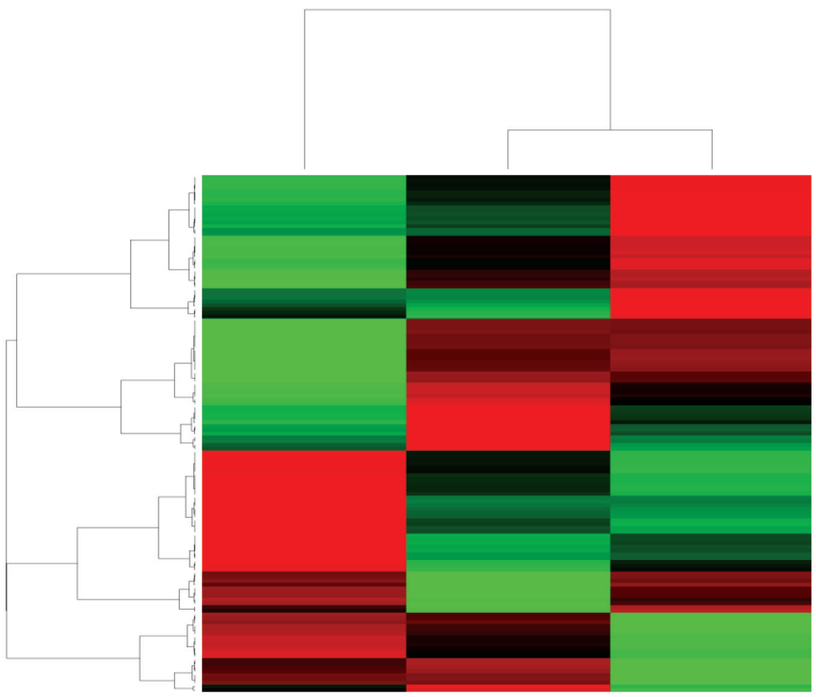


FIGURE 1. A heatmap of all proteins identified as significantly different ($p \leq 0.05$) with cut-off ratio < 0.82 and > 1.2 against control. The proteins were assigned based on ratios shown to be changing from control with up-regulated proteins represented by red colour while down-regulated proteins represented by green colour. Black shading indicates the highest ratio in up-regulated proteins or the lowest ratio for down-regulated proteins

17β-estradiol is the most potent form of estrogen and it has been documented to exert direct protective effects on vascular endothelial cells (Heldring et al. 2007). The protective effects of estrogen such as vasodilation, enhancing nitric oxide production, reduced ROS exposure, anti-inflammatory and antioxidant effects, coupled with increased mitochondrial efficiency for energy production are particularly prominent in premenopausal women who are exposed to a physiological level of this hormone (Duckles & Krause 2007; Razmara et al. 2008; Strehlow et al. 2003). However, as women enter menopause, cessation of menstruation occurs resulting from the loss of ovarian follicular activity (Gruber et al. 2002). The protective effects gradually decline coupled with increased risk of cerebrovascular and cardiovascular disease (Kannel et al. 1976). Since hypertension is the main risk factor for cerebrovascular disease in post-menopausal women, blood vessels of these women are exposed to high hemodynamic pressure involving tensile stretch. The 20% stretch was chosen as it has been documented to be pathological at peripheral arteries and the only established reference for the pathological stretch intensity on artery (Anwar et al. 2012).

ENDOTHELIAL CELL RESPONSE TO 17B-ESTRADIOL ONLY

Comparisons across three different groups against controls showed different protein trends as the proteins abundance emerges under specific conditions. For instance, some proteins did not show changes in S but were found to be up-regulated (trend I, Table 1 & Figure 2) or down-regulated (trend II, Table 1 & Figure 2) in E and E + S. These findings suggest that these proteins were only activated by 17β-estradiol regardless of stretch stimulation. For instance, TXNRD1 was upregulated approximately 1.5 fold in trend I. TXNRD1 is an enzyme that is involved in thioredoxin reduction, which is a key protein for the antioxidant system in reacting against oxidative stress (Lu & Holmgren 2014). Cyclic strain had been shown to generate increased fluxes of the superoxide anion free radical in human umbilical vein endothelial cells (HUVEC) and exposure of 17β-estradiol successfully inhibits the expression of adhesion molecules, chemokines and down regulates ROS production (Wagner 2001). Thus, this may indicate that 17β-estradiol exposure contributes to the protective effects on HCMEC by increasing antioxidant expression to counteract the ROS produced during stretch (Birukov 2009; Chapman et al. 2005).

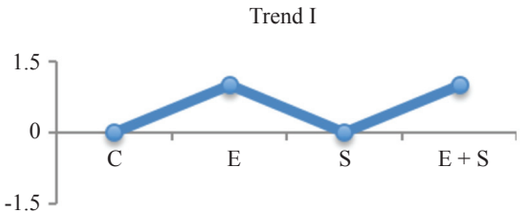
ENDOTHELIAL CELL RESPONSE TO PATHOLOGICAL MECHANICAL STRETCH ONLY

A few sets of proteins were identified as being up-regulated (trend III, Table 1 & Figure 2) or down-regulated (trend IV, Table 1 & Figure 2) due to stretch only. For example, transcription factor II B (GTF2B) is a general transcription factor which is involved in the formation of the RNA polymerase II preinitiation complex and aids in stimulating transcription initiation (Lewis et al. 2004). Mechanical stretch known to activate transcription factors such as nuclear factor κB (NF-κB) that is involved in inflammation (Inoh et al. 2002). Meanwhile for trend IV (Table 1 & Figure 2), stretch suppressed anti-apoptotic related proteins such as 14-3-3 protein epsilon (YWHAE) by 0.8 fold. YWHAE has been found to inhibit pro-apoptotic BAD from activating apoptosis. This supports the concept that pathological stretch is potentially responsible for apoptosis as the down-regulation of these proteins renders the cell increasingly prone to apoptosis during stretch.

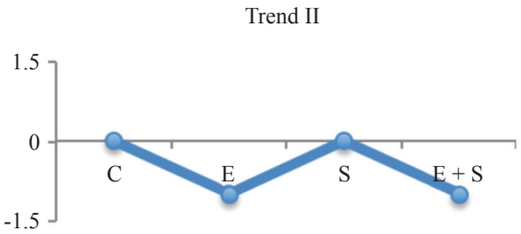
NEUTRALIZING EFFECTS OF 17B-ESTRADIOL ON PATHOLOGICAL MECHANICAL STRETCH

17β-estradiol combined with stretch showed different protein regulation as shown in trend V and VI (Table 1 & Figure 2). For these trends, the protein regulation trends were either up or downregulated by estrogen or stretch exposure respectively and do not show any changes in expression during exposure to estrogen and stretch. Thus, the neutralization term been used to represent these trends. For instance, D-dopachrome decarboxylase (DDT), an enzyme involved in melanin synthesis, points to a neutralization effect of 17β-estradiol when combined with stretch as the proteins were differentially regulated in E and S. Currently, there is no literature to support DDT expression in relation to estrogen or stretch, thus its function is currently not clearly understood. Meanwhile, a similar neutralizing effect was also observed for thrombospondin-1 (THBS1), a known anti-angiogenic protein (Lawler 2002). Mechanical stretch is known to stimulate angiogenesis and down-regulation of THBS1 will not inhibit this process. However, exposure of 17β-estradiol and stretch returned the expression level to the baseline. Interestingly, the protein FAM49B was found to be down-regulated in E (0.73 fold) whilst up-regulated in S (1.22 fold) (Table 1 & Figure 2). The precise function of this protein has yet to be elucidated but is associated with immune dysregulation (Gilli et al. 2011).

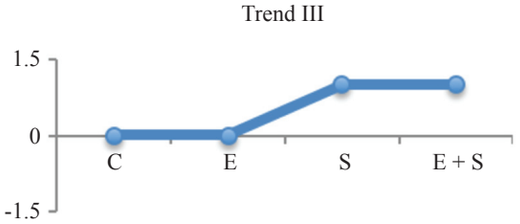
HCMECs express estrogen receptors (ER) that consist of ERα, ERβ and G protein estrogen receptors across the cell membrane, cytoplasm and nucleus (Tu & Jufri 2013). Genomic (involves gene activation for transcription and protein synthesis) or non-genomic signaling (does not involve gene activation) will be activated after the binding of 17β-estradiol to these receptors that determines cellular responses and phenotypic change in response to 17β-estradiol stimulation (Meyer & Barton 2009). Gene ontology analysis suggest that 17β-estradiol exposure stimulates cell proliferation, structural protein activity and cell adhesion. Exposure of 17β-estradiol has been found to preserve membrane integrity and focal adhesion localization of actin (Razandi et al. 2000). In this study, we reported that 17β-estradiol was found to upregulate ACTR2 expression that builds cross-linked actin networks



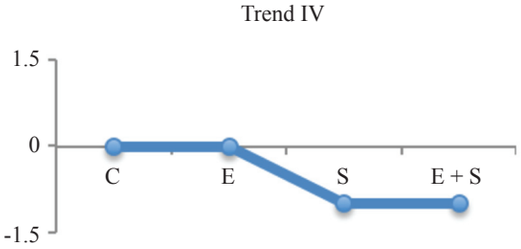
IPO5, RPN2, ATP5A, CTNNB1, DDX5, ACTR2, SWAP70, ACTN1, ISOC1, KRT10, GPI, PLD3, ITGA3, TXNRD1, ATP5A1, TKT, TAGLN



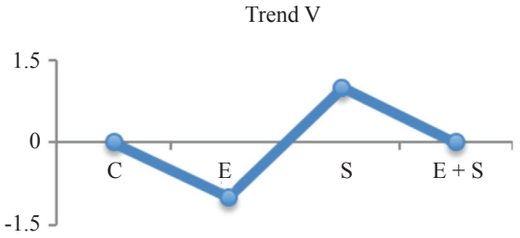
DNM2, CDK1, HNRNPA3, HDLBP, PARVA, TACC1, ARHGAP17, DYNC1I2, HNRNPM



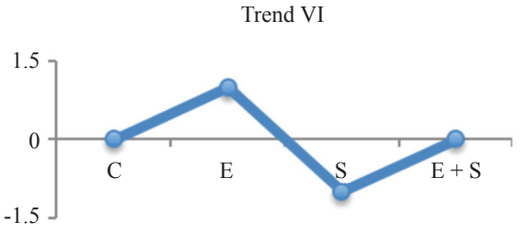
BICD2, TM9SF4, PDHA1, ALDH1A2, NUP93, SRRM2, GTF2B



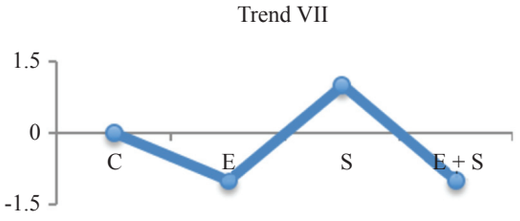
STAT1, SHMT2, XRCC6, DYNC1H1, YWHAE, ANP32B, CS, RAN



DDT



THBS1, ATP5B



FAM49B

FIGURE 2. The proteins were classified into several trends against control. Trend I: protein upregulated in E and E + S; trend II: proteins downregulated in E and E + S; trend III: proteins upregulated in S and E + S; trend IV: proteins downregulated in S and E + S; trend V: protein downregulated in E and upregulated in S, trend VI: proteins upregulated in E and downregulated in S; trend VII: proteins downregulated in E, upregulated in S and downregulated in E + S.

which contributes to stress fibers formation (Hotulainen & Lappalainen 2006). The alignment of fibers at a particular angle can prevent the cell tension that the cell is subjected to during deformation (Takemasa et al. 1998). In addition, this study suggests that 17β-estradiol also seems to promote cell-to-cell adhesion by up-regulation of proteins appearing to be compromised during pathological stretch. 17β-estradiol up-regulated alpha actinin-1 (ACTN1) and catenin beta-1 (CTNNB1) that are tightly regulated with cadherin that serves to link these proteins to the cytoskeleton (Knudsen et al. 1995). However, the cells did not significantly change expression of these proteins when they were subjected to stretch. This may indicate that chronic pathological stretch has the capacity to compromise cell-cell adhesion integrity.

Apolipoprotein E (APOE; Table 1) was found to be the most upregulated and has largest change of fold in E and E + S groups. APOE is a type of lipid transport protein to

remove excess cholesterol from the blood and has been documented to provide protection similar to estrogen as it has antioxidant and anti-inflammatory effects (Horsburgh et al. 2002). The 17β-estradiol has been proven to upregulate APOE at transcriptional and posttranscriptional level and this study recapitulates this finding at proteome level (Srivastava et al. 1997; Stone et al. 1997). In addition, heme oxygenase (HMOX1) was shown to have the largest changes after APOE in all groups. HMOX1 is a known cytoprotective stress-response enzyme, that plays a critical role in the prevention of vascular inflammation and has protective effects against atherogenesis (Marcantoni et al. 2012a). It is normally expressed at low levels in most tissue, but different stimuli (e.g., oxidative stress) will increase the expression (Araujo et al. 2012). Indeed, a previous study demonstrated that 17β-estradiol exposure was found to increase HMOX1 level in HUVECs (Marcantoni et al. 2012b). In this study, the upregulated of HMOX1 may be due to concurrent stimuli of tensile stretch and the 17β -estradiol exposures.

CONCLUSION

This study suggests that 17β-estradiol can alter HCMEC proteome during pathological stretching, as it can change the human proteome expression, when compared to pathological stretching only. Further investigation of these proteins may reveal additional functions of 17β-estradiol in relation to vascular endothelial cells abnormality.

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