Transcriptomic Analysis of Mild Hypothermia-dependent Alterations during Endothelial Reperfusion Injury

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Key Words

Mild hypothermia • Ischemia-Reperfusion • Endothelial cells • Microarray

Abstract

Background: Mild hypothermia (32–34°C) improves resistance to ischemia-reperfusion (I/R) injury. However, the mechanisms by which it affects human cellular function are not fully elucidated. To further test for hypothermic modulation of global biological processes, we used DNA microarray technique to detect the overall gene expression profile. **Methods:** Human umbilical endothelial cells (HUVECs) were incubated under control condition (37°C) or mild hypothermia (33°C) for 2 hours after stimulated ischemia. Detection of differentially expressed genes was performed with Affymetrix U133 plus 2.0 arrays and PARTEK software. We used DAVID and KEGG Pathways database to identify global trends in gene expression data. **Results:** Our analysis has identified numerous interesting genes and processes that are differentially presented in hypothermic group when compared with normothermic control. The cell cycle was the most prominent process; several genes involved in cell apoptosis and proliferation displayed

significantly differential expression; lower transcriptional level was observed for genes involved in chemokine and cell adhesion processes; genes associated with activity of transmembrane transporter and lipase were also under-expressed. **Conclusion:** Our data indicated that mild hypothermia altered endothelial expression pattern under the condition of I/R, preferably through varying the expression of genes associated with cell cycle, apoptosis, proliferation, and inflammatory response.

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Introduction

Reperfusion injury starts with the simple reoxygenation of tissues after ischemic insult causes hypoxia. Endothelial cells are known to be sensitive to hypoxia, and during the reperfusion, endothelium seems to be the primary starting ground for reperfusion injury [1], exhibiting multiple dysfunctions including loss of conjugation to the basement membrane, adherence of activated leukocytes, and overrelease of cytokines, all of which can impact significantly on overall outcome after global ischemia-reperfusion (I/R) insults [2].

Since the 1950s, induced hypothermia has been used

in surgery to resist ischemic or hypoxic insults, and up to now, mild-hypothermia-protection (32–34°C) has been widely accepted. Clinical application of mild hypothermia in I/R-mediated diseases, such as stroke, cardiac arrest, or organ transplantation has obtained favorable outcomes [3]. Although it has been suggested that a reduction of oxygen metabolism might not be the sole protective effect of mild hypothermia, the key mechanisms have not been clarified [4]. It is noteworthy that the degrees of induced hypothermia in the studies were comparable with what is required to induce changes in gene expression in mammalian cells in vitro, which raises the question of whether or not some of the beneficial effects of therapeutic hypothermia might be attributable to changes in gene expression.

We have demonstrated that mild hypothermia attenuated I/R-induced endothelial cell apoptosis via alterations in apoptotic pathways and JNK signaling [5]. Following our previous research, we used DNA microarrays to detect the differentially regulated gene expression in endothelial cells between hypothermic (33°C) and normothermic (37°C) reperfusion groups. The aim of this work was to provide new insights into mechanisms involved in protective effect of mild hypothermia.

Materials and Methods

Cell culture

Human umbilical endothelial cells (HUVECs) were cultured in endothelial cell medium (ECM) supplemented with endothelial cell growth supplement (ECGS), 5% fetal bovine serum (FBS) and penicillin/streptomycin (P/S) solution (all from Sciencell) in an incubator with 37°C, 5% CO₂ as described previously [5]. For experiments, cells of passage No. 4–6 were grown until confluence. The study was reviewed and approved by the institutional ethics review board of the Peking Union Medical College (Beijing, China).

Stimulated ischemia and reperfusion protocol

HUVECs were subjected to oxygen-glucose deprivation (OGD) injury as described previously [5]. Briefly, the OGD injury was initiated by placing cells in a tri-gas incubator with an atmosphere of 37°C, 5% CO₂, 94% N₂ and 1% O₂ and in the presence of OGD media for 5 hours. After that, the exposure medium was exchanged with oxygenated ECM, and the cells were placed in an incubator at 37°C (normothermic control, n=3) or 33°C (hypothermic group, n=3) to mimic the reperfusion condition for 2 hours.

RNA isolation and Affymetrix GeneChip processing

Total RNA was isolated with TRIzol Reagent (Invitrogen) according to manufacturer's instructions and complemented

with an additional wash step of 70% ethanol to increase the purity of the RNA. For all RNA samples quantity and purity were determined with the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies), and RNA integrity was determined with the Bioanalyzer 2100 (Agilent Technologies). Five micrograms of total RNA was amplified with the One-Cycle cDNA synthesis Kit and GeneChip IVT labeling Kit. Fifteen micrograms biotin-labeled complementary RNA (cRNA) was fractionated and hybridized to Affymetrix GeneChip Human Genome U133 Plus 2.0 array according to the manufacturer's instructions. After 16 h of hybridization, the Gene Chips were washed and stained on a fluidics station 450 (Affymetrix) and scanned in a confocal scanner (Affymetrix GeneChip Scanner 3000) according to the Affymetrix GeneChip Expression Analysis Manual. On the U133 Plus 2.0 array, 54,614 probe sets analyze the expression of 47,000 transcripts representing 38,500 known genes.

Microarray data analysis

Raw signal intensities for each probe set in the .CEL files were analyzed using version 6.4 of the PARTEK GENOMICS SUITE software (Partek Inc.). The robust multiarray average (RMA) normalization method was used for RMA background correction, quantile normalization and medianpolish probe set summarization. Expression values were transformed to log₂ before statistical analysis. A sample intensity plot was calculated, showing that the data were normally distributed for all samples, as well as the homogeneity of variance (no outlier), which also indicated the great similarity of biological replicates. Genes that were expressed differentially between normothermic and hypothermic groups were detected with one-way analysis of variance (ANOVA), a statistical technique used to compare means of two or more samples implemented in PARTEK. The recovered P-values of the comparisons were then corrected using a step-up false discovery rate value of 5%. The resulting list of significantly differentially expressed genes was filtered to include only genes that demonstrated 1.5-fold or greater up- or down-regulation.

To search for enrichment of specific biological processes, the genes showing significantly differential expression between the two groups were classified into functional groups with DAVID (Database for Annotation, Visualization, and Integrated Discovery) according to Gene Ontology (GO). As input, the differentially regulated probe sets from the comparison were used. As output, significant processes (GOTERM) with $P < 0.05$ were generated. In addition, related biological processes were clustered with the functional annotation clustering algorithms of DAVID. For each clustered process, this results in an Enrichment Score, the -log value of the geometric mean of the member's P values.

In addition, pathway analysis for the comparison was conducted through the use of KEGG Pathways database (Kyoto Encyclopedia of Genes and Genomes), which is a bioinformatics resource for linking genomes to life and the environment. Details can be found at <http://www.genome.jp/kegg/>.

Real-time quantitative PCR analysis

In order to validate the microarray data, real-time quantita-

Table 1. List of primers used for Real-time qPCR.

Gene name	Genebank accession no.	Forward primer sequence	Reverse primer sequence
IL8	NM_000584	5'-CTC TCT TGG CAG CCT TCC TGA TT-3'	5'-AAC TTC TCC ACA ACC CTC TGC AC-3'
VCAM1	NM_001078	5'-ATG CAG GAA TTT CTG GAG GAT GC-3'	5'-GCA GCT TTG TGG ATG GAT TCA C-3'
SMAD4	NM_005359	5'-AAA ACG GCC ATC TTC AGC AC-3'	5'-AGG CCA GTA ATG TCC GGG A-3'
SKP2	NM_005983	5'-GCT GCT AAA GGT CTC TGG TGT-3'	5'-AGG CTT AGA TTC TGC AAC TTG-3'
CCL20	NM_001130046	5'-TTT GCT CCT GGC TGC TTT GAT GT-3'	5'-GTT TTG GAT TTG CGC ACA CAG AC-3'
CXCL3	NM_002090	5'-ATC CAA AGT GTG AAT GTA AGG TC-3'	5'-GCA GGA AGT GTC AAT GAT ACG-3'
DUSP6	NM_001946	5'-AAC AGG GTT CCA GCA CAG CAG-3'	5'-GGC CAG ACA CAT TCC AGC AA-3'
FGF18	AF075292	5'-ACT TGC CTG TGT TTA CAC TTC C-3'	5'-CCA GAA CCT TCT CGA TGA AC-3'

tive PCR (qPCR) analysis was performed using RNA obtained from normothermic group (n=3) and hypothermic group (n=3). Total RNA was purified from HUVECs with Trizol reagent (Invitrogen). Real-time PCR was performed using Bio-RAD iQ5 Multicolor Real-Time PCR Detection System with SYBR Green as fluorescent and ROX (Takara) as reference dyes as described previously [6]. The specific primers used were given in Table 1.

Statistics

Results from qPCR analysis were presented as means $\pm$ SEM. Differences between groups were evaluated for statistical significance using Student's t-test. In each case, significance was defined as $P < 0.05$.

Results

Data analysis: general features

Under the stress of reperfusion injury, 499 probe sets were found to be differentially regulated between normothermic and hypothermic groups. Of these 499 probe sets, 103 were up-regulated and 396 down-regulated by mild hypothermia. We performed a hierarchical clustering on the complete set of data of present genes. The analysis showed a clear separation of the normothermic reperfusion controls and hypothermic reperfusion samples, which indicated that hypothermic group had their own characteristic gene expression profile. Fig. 1 shows that a clear separation of the two groups was still maintained, when a clustering was performed on a subset of genes that displayed significant expression changes at $P < 0.05$ and > 2 -fold.

qPCR validation of microarray analysis

To validate the microarray data, eight genes that were either up- or down-regulated in the microarray analysis were also determined by qPCR. These selected genes were IL8, VCAM1, CCL20, CXCL3, SMAD4, SKP2, DUSP6, and FGF18. Scatter plot analysis of the relative changes in expression as determined by qPCR and microarray, respectively, revealed a good correlation,

Fig. 1. Hierarchical clustering of the datasets shows significant separation between normothermic and hypothermic reperfusion groups. To appreciate the variance within each group, a hierarchical clustering of a subgroup of genes was performed (a subset of genes that displayed significant expression changes at $P < 0.05$ and > 2 -fold). Each column represents expression on one specific sample (N: normothermic reperfusion; H: hypothermic reperfusion); each row indicates expression level of a gene; green color: represents down-regulated expression; red color: up-regulated expression; deeper shade indicates larger difference. The dendrogram shows that the clustering (Euclidean distances) clearly separates hypothermic group (H1-H3) from normothermic control (N1-N3).

thereby confirming the validity of the microarray data set (Fig. 2).

Gene expression profiles

To assess the function of the differentially regulated probe sets, overrepresented GO classes were determined with DAVID. With this unbiased approach, biological processes (BP), molecular functions (MF), and cellular components (CC) affected by mild hypothermia were identified (data not shown). We should remember that the same gene may belong to different GO processes.

Fig. 2. Scatter plot of relative changes in gene expression as determined by microarray analysis and by real-time qPCR. Eight genes were analyzed: IL8, VCAM1, CCL20, CXCL3, SMAD4, SKP2, DUSP6, and FGF18. Each symbol represents the fold change of the respective gene in hypothermic reperfusion group over normothermic control.

Gene Symbol	Gene Name	Fold Ratio
RAP1A	rap1a, member of ras oncogene family	0.62
LATS2	lats, large tumor suppressor, homolog 2 (drosophila)	1.64
MN1	meningioma (disrupted in balanced translocation) 1	2.04
GADD45A	growth arrest and dna-damage-inducible, alpha	1.76
ARHGAP20	rho gtpase activating protein 20	0.65
TRIM13	ret finger protein 2	0.47
DST	dystonin	1.55
SESN2	sestrin 2	0.65
IL8	interleukin 8	0.55
HIC1	hypermethylated in cancer 1	2.23
EXT1	exostoses (multiple) 1	0.63
MOV10L1	mov10l1, moloney leukemia virus 10-like 1, homolog (mouse)	0.57
GAS1	growth arrest-specific 1	1.65
CCNI	cyclin i	0.63
ACTN4	actinin, alpha 4	0.63
CDC14A	cdc14 cell division cycle 14 homolog a (s. cerevisiae)	0.64
STAT1	signal transducer and activator of transcription 1, 91kda	0.58
SKP2	s-phase kinase-associated protein 2 (p45)	1.66
BMPR2	bone morphogenetic protein receptor, type ii (serine/threonine kinase)	0.62
SIK1	snf1-like kinase	2.69
RIF1	dkfzp434d193 protein	0.63
CLK1	cdc-like kinase 1	0.66
PPP3CA	protein phosphatase 3 (formerly 2b), catalytic subunit, alpha isoform (calcineurin a alpha)	0.56
PLCB1	phospholipase c, beta 1 (phosphoinositide-specific)	0.63
TOB2	transducer of erbb2, 2	0.59
DUSP4	dual specificity phosphatase 4	1.76
DUSP6	dual specificity phosphatase 6	1.86
NEDD9	neural precursor cell expressed, developmentally down-regulated 9	1.68
SMAD4	SMAD family member 4	0.64

Table 2. Fold change in hypothermic reperfusion group over normothermic control of genes involved in cell cycle.

Biological processes are summarized. The annotated clusters are listed according to their Enrichment scores (data not shown). We can see by far the most prominently enriched biological process in the differentially regulated genes was cell cycle, with the enrichment score of 4.3. Besides of the cell cycle, some other processes were also predominated (Enrichment score>0.9) under mild hypothermia. These biological processes were related to development, morphogenesis, regulation of kinase activity,

cell development and death, cell communication, cell proliferation and growth, response to stress and inflammation, and cytoskeleton organization.

Genes associated with cell cycle

The biological process of cell cycle was the most overrepresented cluster between the normothermic and hypothermic reperfusion groups. In this process, twenty-nine genes were differentially regulated (Table 2). Of

Gene Symbol	Gene Name	Fold Ratio
SOCS3	suppressor of cytokine signaling 3	1.71
TNFRSF10D	tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	1.53
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	1.55
HSPA1A	heat shock 70kda protein 1a	0.62
TXNDC5	thioredoxin domain containing 5	0.61
TNFRSF18	tumor necrosis factor (ligand) superfamily, member 18	0.54
RASA1	ras p21 protein activator (gtpase activating protein) 1	0.59
SNCA	synuclein, alpha (non a4 component of amyloid precursor)	0.55
BIRC3	baculoviral iap repeat-containing 3	0.42
ERC1	rab6 interacting protein 2	0.62
ACTN4	actinin, alpha 4	0.63
SULF1	sulfatase 1	0.41
DDIT4	dna-damage-inducible transcript 4	0.66
NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, ep	0.66
ZBTB16	zinc finger and btb domain containing 16	2.55
GAS1	growth arrest-specific 1	1.65
DIDO1	death inducer-obliterator 1	0.63
CROP	acid-inducible phosphoprotein	0.58
STAT1	signal transducer and activator of transcription 1, 91kda	0.58
GADD45A	growth arrest and dna-damage-inducible, alpha	1.76
SNRK	snf related kinase	1.88
CXCR4	chemokine (c-x-c motif) receptor 4	1.98
PAWR	prkc, apoptosis, wt1, regulator	0.65
ATXN1	ataxin 1	1.82

Table 3. Fold change in hypothermic reperfusion group over normothermic control of genes involved in apoptosis.

them, thirteen genes were mediated in negative regulation of progression through cell cycle (the first thirteen genes in the Table 2), and five genes were involved in cell cycle arrest, including GAS1, GADD45A, DST, SESN2, and IL8. Surprisingly, we did not detect numerous cyclin genes displaying differential expression except CCN1. From the Table 2, we also noticed that SIK1, which encodes a protein kinase involved in cell cycle regulation, was up-regulated in hypothermic reperfusion group with a fold-change of 2.7; SKP2, the regulator of p21 and p27, increased with a ratio of 1.67; gene of dual specificity protein phosphatase (CDC14A), working in dephosphatation of CDH1, was down-regulated. The other important cell cycle regulator genes such as STAT1 (down-regulated) and SMAD4 (down-regulated) were also changed in hypothermic reperfusion group compared with normothermic control. In addition, it was noteworthy that significant changes occurred in DUSP4 and DUSP6, whose transcriptional levels were found to rise under mild hypothermia.

Genes involved in apoptosis

We identified twenty-four genes that were involved in the apoptosis biological process from these 499

differently expressed probe sets. The twenty-four genes were displayed in Table 3, including ten genes related to anti-apoptosis (the first ten genes in Table 3). It was valuable to notice that three anti-apoptosis genes, SOCS3, TNFRSF10D, and PIK3R1 were up-regulated by mild hypothermia. PIK3R1, is an upstream regulator of AKT1 which can phosphorylate BAD, inhibiting apoptosis in the IGF signaling pathway. However, we also observed NFKBIE, a molecular involved in inhibiting apoptosis, and BIRC3, mediated in caspase inactivation, were down-regulated. Some other important genes in apoptosis were also changed significantly, including PAWR (down-regulated), ATXN1 (up-regulated), HSPA1A (down-regulated), and TNFSF18 (down-regulated).

Genes associated with cell proliferation

Twenty-one genes were involved in cell proliferation (Table 4). We noticed that FGF18, a positive regulator of cell proliferation was down-regulated by mild hypothermia. However, we identified ten negative regulator genes which changes were confused (the first ten genes in Table 4). GAS1, IBTB16, BTG1, and KLF4 were up-regulated, but the other six genes including PAWR and TOB2 were in a lower mRNA level under

Gene Symbol	Gene Name	Fold Ratio
BTG1	b-cell translocation gene 1, anti-proliferative	1.89
ZBTB16	zinc finger and btb domain containing 16	1.87
GAS1	growth arrest-specific 1	1.65
KLF4	kruppel-like factor 4 (gut)	2.47
CXCL1	chemokine (c-x-c motif) ligand 1 (melanoma growth stimulating activity, alpha)	0.54
IL8	interleukin 8	0.55
TOB2	transducer of erbb2, 2	0.57
PAWR	prkc, apoptosis, wt1, regulator	0.65
CD274	cd274 antigen	0.58
SMAD4	smad, mothers against dpp homolog 4 (drosophila)	0.64
ACVR2A	activin a receptor, type iia	0.62
CLK1	cdc-like kinase 1	0.66
CDC14A	cdc14 cell division cycle 14 homolog a (s. cerevisiae)	0.64
ZFP36L2	zinc finger protein 36, c3h type-like 2	1.51
SKP2	s-phase kinase-associated protein 2 (p45)	1.66
NRP1	neuropilin 1	1.51
TBC1D8	tbc1 domain family, member 8 (with gram domain)	0.54
CXCR4	chemokine (c-x-c motif) receptor 4	1.98
FGF18	fibroblast growth factor 18	0.61
MOV10L1	mov10l1, moloney leukemia virus 10-like 1, homolog (mouse)	0.57
BMPR2	bone morphogenetic protein receptor, type ii (serine/threonine kinase)	0.62

Table 4. Fold change in hypothermic reperfusion group over normothermic control of genes involved in cell proliferation.

mild hypothermia. In addition, it was interesting to observe that BMPR2 (bone morphogenetic protein receptor, type α), was decreased under mild hypothermia, which was a new target to further test for the influences of mild hypothermia on TGF β -BMP pathway.

Chemokines and cytokine-cytokine receptor interaction

Response to stress and inflammation was also a prominently over-expressed process. In this cluster, seven genes were involved in chemokine activity (Table 5). Among them, six chemokine genes were significantly down-regulated by hypothermic reperfusion including CXCL1, CXCL3, CXCL6, IL8, CCL20, and CMTM8. Using the KEGG Pathway Analysis, we also found that many genes altered by mild hypothermia were related to cytokines or cytokine receptors, including the family of chemokines, TNF, and TGF β . The significant interaction between cytokines and cytokine receptors was presented in Fig. 3.

Cell-cell adhesion

Cell adhesion was not an enriched term in the biological process, but the cell adhesion, especially

endothelial adhesion molecules were of great importance during I/R injury. We observed that 10 genes were related to cell adhesion, and interestingly, all of them showed a lower expression level under hypothermic reperfusion (Table 6). Two endothelial adhesion molecules, VCAM1 (vascular cell adhesion molecule 1) and ALCAM (activated leukocyte cell adhesion molecule), were noticed for the down-regulation with the ratio of 3.2 and 1.5, respectively.

Genes associated with transmembrane transporter and lipase activity

Although the precise cellular mechanisms induced by hypothermia are unclear, it is noteworthy that hypothermia can reduce oxygen consumption and metabolic rate. In the metabolic processes, we found that two molecular function terms, transmembrane transporter and lipase activity, were much affected (data not shown). We observed that the differentially expressed genes were involved in carboxylic acid, organic acid, amino acid, or ion transmembrane transport and phosphoglycerides hydrolysis metabolism, and interestingly, all of them were down-regulated. However, no significant change occurred in enzyme genes associated with ATP metabolism.

Gene Symbol	Gene Name	Fold Ratio
ITGAV	integrin, alpha v (vitronectin receptor, alpha polypeptide, antigen cd51)	0.64
FREM3	fras1 related extracellular matrix 3	0.6
ALCAM	activated leukocyte cell adhesion molecule	0.65
RND1	rho family gtpase 1	0.57
RASA1	ras p21 protein activator (gtpase activating protein) 1	0.59
FLRT2	fibronectin leucine rich transmembrane protein 2	0.62
PVRL3	poliovirus receptor-related 3	0.63
ZNF862	sco-spondin homolog (bos taurus)	0.62
CD274	cd274 antigen	0.58
VCAM1	vascular cell adhesion molecule 1	0.31

Table 6. Fold change in hypothermic reperfusion group over normothermic control of genes involved in cell adhesion.

the gene expression profile of hypothermic protection during I/R insult. These results, however, do not yet allow drawing precise conclusion about mechanism of hypothermic protection. It is interesting to discuss the changes that were put in evidence in the present microarray analysis.

The cell cycle was the most prominent process in the comparison between normothermic and hypothermic reperfusion groups. Twenty-nine genes were significantly affected by reperfusion under mild hypothermia (Table 2). Among them, SIK1 was observed. Recently, a study suggested a possible function for SIK1 (also known as SNF1LK) in G2/M regulation [11], but there was no report about its role in hypothermic effects, and in our study, we presented it as a hypothermia-sensitive gene. Moreover, it was interesting to notice that SKP2 was induced in reperfusion under mild hypothermia. As we know, P21 and P27 were important negative regulator of cell cycle, and SKP2 expression can promote their ubiquitin-mediated degradation [12], which to some degree, suggested that mild hypothermia might mitigate cell cycle arrest during I/R. The lower level of SMAD4 in hypothermic group was significant, because it inhibited cell cycle and induced apoptosis [13], which also supported the hypothermic resistance to I/R-induced cell cycle arrest.

Attenuating stress-induced apoptosis is a major aspect of hypothermic protection [14-15]. Although our results showed that some genes related to anti-apoptosis exhibited lower expression levels in hypothermic group, including TNFSF18, BIRC3, and NFKBIE, the changes of other important genes did make sense. We observed a high level of TNFRSF10D expression in our hypothermic data. Tumor necrosis factor receptor superfamily member

10D is a member of the TNF-receptor superfamily and is encoded by the TNFRSF10D gene. This receptor has been shown to play an inhibitory role in TRAIL-induced cell apoptosis [16], therefore, its high level in hypothermic groups might interest investigators to perform further studies. PAWR is a human gene coding for a tumor-suppressor protein that induces apoptosis in cancer cells [17], and its expression was inhibited under mild hypothermia. The IGF signaling pathway has been accepted to promote cell survival in many cell types [18-19]. PIK3R1, an upstream activator of AKT1 which can phosphorylate BAD, inhibiting apoptosis in the IGF signaling pathway, was shown to be up-regulated under hypothermic reperfusion. Following these presentation, we suggested that these genes could be selected as novel targets to fully investigate the mechanisms involved in hypothermic resistance to apoptosis. Furthermore, many studies referred hypothermic protection against stress-induced apoptosis to kinase signal pathway. Our microarray profile presented the regulation of kinase activity as an enriched cluster (data not shown), which also argued its important role in hypothermic protection.

Mild hypothermia suppresses stress-induced apoptosis, but it did inhibit cell proliferation in some studies [20-21]. Several genes associated with regulation of cell proliferation were identified in the comparison between normothermic and hypothermic reperfusion groups. We noticed several negative proliferation regulator genes in Table 4 including GAS1, KLF4, PAWR, and TOB2. GAS1 and KLF4 exhibited higher expression levels in hypothermic group, whereas PAWR and TOB2 were down-regulated. In addition, fibroblast growth factor 18 (FGF18), an activator of cell proliferation [22], appeared

in a lower expression level. Some studies reported that TGF β -BMP pathway was involved in regulation of cell proliferation [23-24]. Thus, it was interesting to note that BMPR2, bone morphogenetic protein receptor, type II (serine/threonine kinase), was decreased when reperfusion under mild hypothermia. As we can see, from our current evidences, it was hard to draw a definite conclusion about the effect of mild hypothermia on cell proliferation. Therefore, thorough investigations were needed to discover the precise relationship between mild hypothermia and proliferation of cells.

Mild hypothermic protection is associated with remarkable suppression of the inflammatory response, such as reduced inflammatory cell infiltration, and reduction of a variety of inflammatory cytokines. In our study, we were pleasantly surprised by noting that, mild hypothermia produced a decrease in the level of expression of a number of chemokine mRNA, including CXCL1, CXCL3, CXCL6, IL8, CCL20, and CMTM8. A recent study indicated that acute hypothermia (30°C) up-regulates the expression level of a functionally diverse array of splenic cytokines and chemokines, such as IL1, IL6, CXCL2, CXCL10, CCL2, and CCL20 [25]. Additionally, mild hypothermia did not attenuate the increases in cerebrospinal fluid cytokine levels after severe traumatic brain injury versus normothermia [26], but significantly reduced serum levels of IL1 β , TNF α and IL6 during acute pancreatitis [27]. In our research, the data tended to show a pattern of decreased expression of chemokines in hypothermic reperfusion, which provided strong evidence to elucidate the anti-inflammatory effect of mild hypothermia. Furthermore, it appeared that reperfusion under mild hypothermia could affect the expression of a number of molecules known to be involved in cytokine-cytokine receptor interaction (Fig. 3), which covered the families of chemokines, TNF, and TGF β .

Endothelial cell surface expression of adhesion molecules is considered to be a crucial step in the systemic inflammatory response to I/R insult. Moreover, leukocyte trafficking from the blood to target organ is essential for the initiation of inflammation, which is dependent on intercellular cell adhesion (ICAM1) and vascular cell adhesion (VCAM1) expressed by the endothelial cells. Activated leukocyte cell adhesion molecule (ALCAM) known as a novel endothelial adhesion molecule was also mediated in leukocyte infiltration [28]. Previous studies reported that mild hypothermia attenuated neutrophil infiltration in the rat neocortex after focal I/R injury [29], and neutrophil adherence to activated endothelium mediated by E-selectin was reversibly inhibited by

hypothermia [30]. In our study, we detected ten genes associated with cell adhesion, and interestingly, all of them showed a lower expression level under hypothermic reperfusion (Table 6). Among them, two endothelial adhesion molecule genes, VCAM1 and ALCAM were of great significance. However, the other endothelial adhesion molecules were not detected for differential expression. Thus, our microarray data supported the notion that mild hypothermia might inhibit inflammatory response through reduced expression of VCAM1 and ALCAM.

The opinions about the role of energy metabolism in hypothermic effects are controversial. Some supported that mild hypothermia attenuated ATP depletion [31], however, the other studies disagree with this conclusion [32], therefore, a report reasoned that hypothermia's effect on energy depletion was unlikely to play a significant role in its protective mechanisms [14]. Our results showed that no enzyme associated with ATP metabolism had different expression, such as ATP synthase, but it was noteworthy to observe that the expression of fourteen transmembrane transporter genes changed in reperfusion under mild hypothermia, all of which were significantly down-regulated, and interestingly, four lipase genes showed the same change (data not shown). These, virtually supported that mild hypothermia might reduce the rate of material metabolism, predominantly through suppression of substance transmembrane transport.

In conclusion, this microarray investigation yielded a large number of genes related to important biological processes and functions, the expression of which was significantly changed within mild hypothermia. Cell cycle, apoptosis, and cell proliferation appeared to be involved in the most prominent over-expressed processes. Chemokine and cell adhesion were among the most significantly under-expressed inflammatory processes, the same as transmembrane transporter and lipase activity. All of these results are suggested to play prominent roles in mild hypothermia-induced effects. Some views for the possible functional consequences of these changes are discussed. Furthermore, some altered gene expression may be novel points to elucidate the protective mechanisms of mild hypothermia.

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References

- Carden DL, Granger DN: Pathophysiology of ischaemia-reperfusion injury. *J Pathol* 2000;190:255-266.
- Armstrong SC: Protein kinase activation and myocardial ischemia/reperfusion injury. *Cardiovasc Res* 2004;61:427-436.
- Bernard SA, Gray TW, Buist MD, Jones BM, Silvester W, Gutteridge G, Smith K: Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. *N Engl J Med* 2002;346:557-563.
- Safar PJ, Kochanek PM: Therapeutic hypothermia after cardiac arrest. *N Engl J Med* 2002;346:612-613.
- Yang D, Guo S, Zhang T, Li H: Hypothermia attenuates ischemia/reperfusion-induced endothelial cell apoptosis via alterations in apoptotic pathways and JNK signaling. *FEBS Lett* 2009;583:2500-2506.
- Fan Y, Xie P, Zhang T, Zhang H, Gu D, She M, Li H: Regulation of the stability and transcriptional activity of NFATc4 by ubiquitination. *FEBS Lett* 2008;582:4008-4014.
- Kerrigan CL, Stotland MA: Ischemia reperfusion injury: a review. *Microsurgery* 1993;14:165-175.
- Miyazawa T, Tamura A, Fukui S, Hossmann KA: Effect of mild hypothermia on focal cerebral ischemia. Review of experimental studies. *Neurol Res* 2003;25:457-464.
- Kawai N, Okauchi M, Morisaki K, Nagao S: Effects of delayed intraischemic and postischemic hypothermia on a focal model of transient cerebral ischemia in rats. *Stroke* 2000;31:1982-1989.
- Yanamoto H, Nagata I, Niitsu Y, Zhang Z, Xue JH, Sakai N, Kikuchi H: Prolonged mild hypothermia therapy protects the brain against permanent focal ischemia. *Stroke* 2001;32:232-239.
- Stephenson A, Huang GY, Nguyen NT, Reuter S, McBride JL, Ruiz JC: snf1lk encodes a protein kinase that may function in cell cycle regulation. *Genomics* 2004;83:1105-1115.
- Gonzalez-Nicolini V, Fux C, Fussenegger M: A novel mammalian cell-based approach for the discovery of anticancer drugs with reduced cytotoxicity on non-dividing cells. *Invest New Drugs* 2004;22:253-262.
- Pardali K, Kowanetz M, Heldin CH, Moustakas A: Smad pathway-specific transcriptional regulation of the cell cycle inhibitor p21(WAF1/Cip1). *J Cell Physiol* 2005;204:260-272.
- Zhao H, Steinberg GK, Sapolsky RM: General versus specific actions of mild-moderate hypothermia in attenuating cerebral ischemic damage. *J Cereb Blood Flow Metab* 2007;27:1879-1894.
- Gressens P, Dingley J, Plaisant F, Porter H, Schwendimann L, Verney C, Tooley J, Thoresen M: Analysis of neuronal, glial, endothelial, axonal and apoptotic markers following moderate therapeutic hypothermia and anesthesia in the developing piglet brain. *Brain Pathol* 2008;18:10-20.
- Pan G, Ni J, Yu G, Wei YF, Dixit VM: TRUNDD, a new member of the TRAIL receptor family that antagonizes TRAIL signalling. *FEBS Lett* 1998;424:41-45.
- Zhao Y, Burikhanov R, Qiu S, Lele SM, Jennings CD, Bondada S, Spear B, Rangnekar VM: Cancer resistance in transgenic mice expressing the SAC module of Par-4. *Cancer Res* 2007;67:9276-9285.
- Wang PH: Roads to survival: insulin-like growth factor-1 signaling pathways in cardiac muscle. *Circ Res* 2001;88:552-554.
- Vincent AM, Feldman EL: Control of cell survival by IGF signaling pathways. *Growth Horm IGF Res* 2002;12:193-197.
- Lee KS, Lim BV, Jang MH, Shin MC, Lee TH, Kim YP, Shin HS, Cho SY, Kim H, Shin MS, Kim EH, Kim CJ: Hypothermia inhibits cell proliferation and nitric oxide synthase expression in rats. *Neurosci Lett* 2002;329:53-56.
- Kanagawa T, Fukuda H, Tsubouchi H, Komoto Y, Hayashi S, Fukui O, Shimoya K, Murata Y: A decrease of cell proliferation by hypothermia in the hippocampus of the neonatal rat. *Brain Res* 2006;1111:36-40.
- Hu MC, Qiu WR, Wang YP, Hill D, Ring BD, Scully S, Bolon B, DeRose M, Luethy R, Simonet WS, Arakawa T, Danilenko DM: FGF-18, a novel member of the fibroblast growth factor family, stimulates hepatic and intestinal proliferation. *Mol Cell Biol* 1998;18:6063-6074.
- Itoh F, Itoh S, Goumans MJ, Valdimarsdottir G, Iso T, Dotto GP, Hamamori Y, Kedes L, Kato M, ten Dijke P: Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. *EMBO J* 2004;23:541-551.
- Scharpfenecker M, van Dinther M, Liu Z, van Bezooijen RL, Zhao Q, Pukac L, Lowik CW, ten Dijke P: BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. *J Cell Sci* 2007;120:964-972.
- Ganta CK, Helwig BG, Blecha F, Ganta RR, Cober R, Parimi S, Musch TI, Fels RJ, Kenney MJ: Hypothermia-enhanced splenic cytokine gene expression is independent of the sympathetic nervous system. *Am J Physiol Regul Integr Comp Physiol* 2006;291:R558-R565.
- Buttram SD, Wisniewski SR, Jackson EK, Adelson PD, Feldman K, Bayir H, Berger RP, Clark RS, Kochanek PM: Multiplex assessment of cytokine and chemokine levels in cerebrospinal fluid following severe pediatric traumatic brain injury: effects of moderate hypothermia. *J Neurotrauma* 2007;24:1707-1717.
- Matsuoka K, Ueno T, Morita K, Kawano H, Yamaguchi K, Maekawa T, Tangoku A, Oka M: Effects of moderate hypothermia on proinflammatory cytokine production in a rat model of caerulein-induced pancreatitis. *Pancreas* 2003;26:e12-e17.
- Cayrol R, Wosik K, Berard JL, Dodelet-Devillers A, Ifergan I, Kebir H, Haqqani AS, Kreymborg K, Krug S, Moumdjian R, Bouthillier A, Becher B, Arbour N, David S, Stanimirovic D, Prat A: Activated leukocyte cell adhesion molecule promotes leukocyte trafficking into the central nervous system. *Nat Immunol* 2008;9:137-145.
- Toyoda T, Suzuki S, Kassell NF, Lee KS: Intraischemic hypothermia attenuates neutrophil infiltration in the rat neocortex after focal ischemia-reperfusion injury. *Neurosurgery* 1996;39:1200-1205.
- Haddix TL, Pohlman TH, Noel RF, Sato TT, Boyle EM, Jr., Verrier ED: Hypothermia inhibits human E-selectin transcription. *J Surg Res* 1996;64:176-183.
- Ibayashi S, Takano K, Ooboshi H, Kitazono T, Sadoshima S, Fujishima M: Effect of selective brain hypothermia on regional cerebral blood flow and tissue metabolism using brain thermo-regulator in spontaneously hypertensive rats. *Neurochem Res* 2000;25:369-375.
- Kimura T, Sako K, Tanaka K, Kusakabe M, Tanaka T, Nakada T: Effect of mild hypothermia on energy state recovery following transient forebrain ischemia in the gerbil. *Exp Brain Res* 2002;145:83-90.