21st Century Breakthrough

Researching the Benefits of Mind-Body Practice by Investigating Genetic Expression

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Research on Mind-Body approaches is accelerating. One of a number of characteristics of Mind-Body practice is the purposeful elicitation of the Relaxation Response (RR). The various forms of practices which elicit the RR include Tai Chi, Qi Gong, Yoga, meditation, repetitive prayer, breathing exercises, progressive muscle relaxation, biofeedback, guided visualization, affirmation, etc. These methods tend to trigger physiological and perhaps energetic mechanisms that move the body into a state of deep rest. It appears that this can literally change how genes behave in response to stress.

Mind-Body practices that produce the Relaxation Response have been used by people across cultures for thousands of years to prevent and treat disease and generate states of mind that foster greater performance and intuitive insight. Recently, a number of studies have turned toward investigation of the effect that Mind-Body practice can have on genetic expression. In research on natural healing, functional maximization and holistic, complementary and integrative medicine there has been a growing trend away from simply studying disease mechanisms and outcomes, toward the study of the subtle factors that predispose individuals for sustainable wellbeing as well as for disease. Gene expression is an emerging arena wherein the total continuum – from wellbeing to disease – can be effectively investigated.

The RR is characterized by reduction in oxygen intake, increase in exhalation of nitric oxide, and lower psychological distress. It has been proposed as the

counterpoint to the "flight or fight" state (FF) – the stress response. FF and RR are like a Yin-Yang. Numerous studies have shown that both RR and the FF have distinct profiles of physiological and gene expression changes.

Recently - July, 2008 - a breakthrough study was completed exploring the extent to which Mind-Body practices that trigger the RR and have an influence on gene expression: ***Genomic Counter-Stress Changes Induced by the Relaxation Response***. Dr. Herbert Benson, who was the lead developer of the RR concept was among the researchers on this study.

Earlier -- February 2005 - a similar study was completed which focused specifically on gene expression in Qigong: ***Genomic Profiling of Neutrophil Transcripts in Asian Qigong Practitioners: A Pilot Study in Gene Regulation by Mind–Body Interaction.***

A less widely known study on a Yoga/Pranayama method – East Indian Qigong – also explored the effects of a RR method on gene expression: ***Gene expression profiling in practitioners of Sudarshan Kriya.***

The abstracts and links to PDFs of these studies are presented below. ***Genomic counter-stress changes induced by the relaxation response*** is included in full at the end of this article.

Due to the magnitude of the credibility of Dr. Herbert Benson, his role as the "father" of the relaxation response and the refined design of the study, the ***Genomic counter-stress changes induced by the relaxation response*** article has gotten a greater amount of press. The highest number of participants, however, was involved in the ***Gene expression profiling in practitioners of Sudarshan Kriya*** article. The Benson study, n=57 (RCT, 19 long term, 20 short term & 19 control), Qigong study, n=12 (6 Qigong and 6 control), Pranayama study, n=84 (42 Pranayama and 42 control).

In a number of press releases the authors of ***Genomic counter-stress changes induced by the relaxation response*** made a number of comments that are easily applicable to all three studies. They state that:

"This study provides the first compelling evidence that the RR [relaxation response] elicits specific gene expression changes in short-term and long- term practitioners."

Actually the other studies were earlier and they all suggest this.

The Genomic Counter-stress authors wrote that their findings suggest:
"Consistent and constitutive changes in gene expression resulting from RR may relate to long term physiological effects," and that "Our study may stimulate new investigations into applying transcriptional profiling for accurately measuring RR and stress related responses in multiple disease settings."

It is likely that these studies portend a “sea change” in research and will trigger an outpouring of similar research. Dr. Herbert Benson, professor emeritus of Harvard University and director emeritus of the Benson-Henry Institute and co-senior author of the study said:

"Now we've found how changing the activity of the mind can alter the way basic genetic instructions are implemented," said Benson.

Dr. Towia Libermann, director of the BIDMC Genomics Center and also co-senior author of the study added:

"This is the first comprehensive study of how the mind can affect gene expression, linking what has been looked on as a 'soft' science with the 'hard' science of genomics.” "It is also important because of its focus on gene

expression in healthy individuals, rather than in disease states," explained Libermann.

The authors said their study showed that the relaxation response changed the expression of genes involved with inflammation, programmed cell death and the handling of free radicals. Free radicals are normal byproducts of metabolism that the body neutralizes in order to stop damage to cells and tissues.

Co-lead author of the study Dr. Jeffery Dusek formerly of the Benson-Henry Institute and now with the Abbott Northwestern Hospital in Minneapolis said:

"Changes in the activation of these same genes have previously been seen in conditions such as post-traumatic stress disorder; but the relaxation-response-associated changes were the opposite of stress-associated changes and were much more pronounced in the long-term practitioners."

Dr. Benson reflected that people across different cultures have been using Mind-Body techniques for thousands of years. They found that it didn't particularly matter which techniques was used, whether it was Tai Chi, Qigong, meditation, Yoga, breathing, or repetitive praying, they all act through the same underlying mechanism.

"Now, we need to see if similar changes occur in patients who use the relaxation response to help treat stress-related disorders, and those studies are underway now".

Probably the most compelling statement from the article on the findings of the study was “It is becoming increasingly clear that psychosocial stress can manifest as system-wide perturbations of cellular processes, generally increasing oxidative stress and promoting a pro-inflammatory milieu. Stress associated changes in peripheral blood leukocyte expression of single genes have been

identified. More recently, chronic psychosocial stress has been associated with accelerated aging at the cellular level. Specifically, shortened telomeres, low telomerase activity, decreased anti-oxidant capacity and increased oxidative stress are correlated with increased psychosocial stress and with increased vulnerability to a variety of disease states."

These 3 studies strongly suggest that Mind-Body practices, especially those that trigger a sustained and accumulative RR effect – a counter stress effect – can prevent and ameliorate disease. This effect of Mind-Body practice on gene expression transforms the landscape of scientific exploration and launches an entirely new direction for the investigation for the emerging field of health maximization based integrative medicine.

Sea Change Studies -- The Abstracts

I. Genomic counter-stress changes induced by the relaxation response

Dusek JA, Otu HH, Wohlhueter AL, Bhasin M, Zerbini LF, Joseph MG, Benson H, Libermann TA. PLoS ONE – Online Journal of Medicine 2008 Jul 2;3(7):e2576

To review the article in full:
http://www.plosone.org/article/info:doi/10.1371/journal.pone.0002576
This is included at the end of this article.

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BACKGROUND: Mind-body practices that elicit the relaxation response (RR) have been used worldwide for millennia to prevent and treat disease. The RR is characterized by decreased oxygen consumption, increased exhaled nitric oxide, and reduced psychological distress. It is believed to be the counterpart of the stress response that exhibits a distinct pattern of physiology and transcriptional profile. We hypothesized that RR elicitation results in characteristic gene expression changes that can be used to measure physiological responses elicited by the RR in an unbiased fashion.

METHODS/PRINCIPAL FINDINGS: We assessed whole blood transcriptional profiles in 19 healthy, long-term practitioners of daily RR practice (group M), 19 healthy controls (group N(1)), and 20 N(1) individuals who completed 8 weeks of RR training (group N(2)). 2209 genes were differentially expressed in group M relative to group N(1) ($p<0.05$) and 1561 genes in group N(2) compared to group N(1) ($p<0.05$). Importantly, 433 ($p<10(-10)$) of 2209 and 1561 differentially expressed genes were shared among long-term (M) and short-term practitioners

(N(2)). Gene ontology and gene set enrichment analyses revealed significant alterations in cellular metabolism, oxidative phosphorylation, generation of reactive oxygen species and response to oxidative stress in long-term and short-term practitioners of daily RR practice that may counteract cellular damage related to chronic psychological stress. A significant number of genes and pathways were confirmed in an independent validation set containing 5 N(1) controls, 5 N(2) short-term and 6 M long-term practitioners.

CONCLUSIONS/SIGNIFICANCE: This study provides the first compelling evidence that the RR elicits specific gene expression changes in short-term and long-term practitioners. Our results suggest consistent and constitutive changes in gene expression resulting from RR may relate to long term physiological effects. Our study may stimulate new investigations into applying transcriptional profiling for accurately measuring RR and stress related responses in multiple disease settings.

II. Genomic profiling of neutrophil transcripts in Asian Qigong practitioners: a pilot study in gene regulation by mind-body interaction.

Li QZ, Li P, Garcia GE, Johnson RJ, Feng L. Journal of Alternative and Complement Medicine 2005 Feb;11(1):29-39

The full article can be reviewed at:
http://pkg.dajiyuan.com/pkg/2005-04-08/genomic%20profiling.pdf

Microarray Core, Center for Immunology, University of Texas Southwestern Medical Center, Dallas, TX, USA.

BACKGROUND AND OBJECTIVES: The great similarity of the genomes of humans and other species stimulated us to search for genes regulated by

elements associated with human uniqueness, such as the mind-body interaction. DNA microarray technology offers the advantage of analyzing thousands of genes simultaneously, with the potential to determine healthy phenotypic changes in gene expression. The aim of this study was to determine the genomic profile and function of neutrophils in Falun Gong (FLG, an ancient Chinese Qigong) practitioners, with healthy subjects as controls.

SUBJECTS AND DESIGN: Six (6) Asian FLG practitioners and 6 Asian normal healthy controls were recruited for our study. The practitioners have practiced FLG for at least 1 year (range, 1-5 years). The practice includes daily reading of FLG books and daily practice of exercises lasting 1-2 hours. Selected normal healthy controls did not perform Qigong, yoga, t'ai chi, or any other type of mind-body practice, and had not followed any conventional physical exercise program for at least 1 year. Neutrophils were isolated from fresh blood and assayed for gene expression, using microarrays and RNase protection assay (RPA), as well as for function (phagocytosis) and survival (apoptosis).

RESULTS: The changes in gene expression of FLG practitioners in contrast to normal healthy controls were characterized by enhanced immunity, downregulation of cellular metabolism, and alteration of apoptotic genes in favor of a rapid resolution of inflammation. The lifespan of normal neutrophils was prolonged, while the inflammatory neutrophils displayed accelerated cell death in FLG practitioners as determined by enzyme-linked immunosorbent assay. Correlating with enhanced immunity reflected by microarray data, neutrophil phagocytosis was significantly increased in Qigong practitioners. Some of the altered genes observed by microarray were confirmed by RPA.

CONCLUSION: Qigong practice may regulate immunity, metabolic rate, and cell death, possibly at the transcriptional level. Our pilot study provides the first evidence that Qigong practice may exert transcriptional regulation at a genomic level. New approaches are needed to study how genes are regulated by

elements associated with human uniqueness, such as consciousness, cognition, and spirituality.

III. Gene expression profiling in practitioners of Sudarshan Kriya

Sharma H, Datta P, Singh A, Sen S, Bhardwaj NK, Kochupillai V, Singh N. Journal of Psychosomatic Research 2008 Feb;64(2):213-8

To review a more comprehensive version of the article:
http://www.jbtdrc.org/National%20Symposium%20-%202006/Proc%20pages/CMB/CMB1.pdf

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BACKGROUND: The rapid pace of life, eating habits, and environmental pollution have increased stress levels and its related disorders. Stress genes and a variety of regulatory pathways mediate the complex molecular response to stress. Oxidative stress is internal damage caused by reactive oxygen species. Increasing evidence suggests that chronic psychosocial stress may increase oxidative stress, which in turn may contribute to aging, and etiology of coronary diseases, cancer, arthritis, etc. Psychophysiological concomitants of meditation have been extensively researched, but there are very little data available on biochemical activity leading to relieving stress by causing a relaxation response by Sudarshan Kriya (SK). SK is a breathing technique that involves breathing in three different rhythms. It is preceded by Ujjayi Pranayam (long and deep breaths with constriction at the base of throat) and Bhastrika (fast and forceful breaths through nose along with arm movements).

METHODS: Forty-two SK practitioners and 42 normal healthy controls were recruited for our study. The practitioners had practiced SK for at least 1 year.

Selected normal healthy controls did not perform any conventional physical exercise or any formal stress management technique. Whole blood was used for glutathione peroxidase estimation and red blood cell lysate was used for superoxide dismutase activity assay and for glutathione estimation. White blood cells were isolated from fresh blood and assayed for gene expression using reverse transcriptase-polymerase chain reaction. The parameters studied are antioxidant enzymes, genes involved in oxidative stress, DNA damage, cell cycle control, aging, and apoptosis.

RESULTS: A better antioxidant status both at the enzyme activity and RNA level was seen in SK practitioners. This was accompanied by better stress regulation and better immune status due to prolonged life span of lymphocytes by up-regulation of antiapoptotic genes and prosurvival genes in these subjects.

CONCLUSIONS: Our pilot study provides the first evidence suggesting that SK practice may exert effects on immunity, aging, cell death, and stress regulation through transcriptional regulation.

The methods of the ***Genomic counter-stress changes induced by the relaxation response*** study are also somewhat exemplary and shed some light on how such research is done. The researchers recruited three groups of people. In the first group (called the M group) there were 19 long-term practitioners who had been practicing various ways of producing the relaxation response every day for a long time (for instance with daily yoga, repeated prayer, Tai Chi or meditation practice).

In the second group were another 19 people who they called the "healthy controls" (group N1), who were not daily practitioners, and the third group was like the healthy controls group, except these 20 people completed 8 weeks of relaxation response training (this group was N2).

The researchers assessed transcriptional profiles of the people in all three groups from blood samples.

They found the expressions of a total of 2,209 genes were significantly different between groups M and N1, and a total of 1,561 genes were similarly significantly different between groups N2 and N1.

More importantly, however, was the fact 433 of the genes were common to both sets of comparisons: the same ones were different between M and N1 and between M and N2, so even short term practice of the relaxation response appeared to produce changes in these 433 gene expressions.

Further analysis using techniques called gene ontology and gene set enrichment, showed that groups M and N1 (the long term and the short term practitioners of the relaxation response) exhibited similar physiological changes such as in "cell metabolism, oxidative phosphorylation, generation of reactive oxygen species and response to oxidative stress".

A second phase of the study involving 5 N1 healthy controls, 5 N2 short term practitioners, and 6 M long term practitioners, was done to validate a significant number of genes and pathways.

This is clearly a very well designed study, and extremely thorough. The findings of these three studies points the way to investigating gene expression profiles in well, at risk and diseased populations to demonstrate the benefits of Mind-Body practices which elicit the RR to both prevent and cure disease. Given the low cost of learning and practicing Mind-Body practice, it is clear that proliferation of these practices in school, at work and at home can potentiate a nationwide and culture wide response to eliminate the health cost crisis.

Details on the Dusek, Benson, Libermann study follow in full with an excellent references section at the conclusion.

Genomic Counter-Stress Changes Induced by the Relaxation Response

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For full text of research article

Abstract

Background

Mind-body practices that elicit the relaxation response (RR) have been used worldwide for millennia to prevent and treat disease. The RR is characterized by decreased oxygen consumption, increased exhaled nitric oxide, and reduced

psychological distress. It is believed to be the counterpart of the stress response that exhibits a distinct pattern of physiology and transcriptional profile. We hypothesized that RR elicitation results in characteristic gene expression changes that can be used to measure physiological responses elicited by the RR in an unbiased fashion.

Methods/Principal Findings

We assessed whole blood transcriptional profiles in 19 healthy, long-term practitioners of daily RR practice (group M), 19 healthy controls (group N_1), and 20 N_1 individuals who completed 8 weeks of RR training (group N_2). 2209 genes were differentially expressed in group M relative to group N_1 ($p<0.05$) and 1561 genes in group N_2 compared to group N_1 ($p<0.05$). Importantly, 433 ($p<10_{-10}$) of 2209 and 1561 differentially expressed genes were shared among long-term (M) and short-term practitioners (N_2). Gene ontology and gene set enrichment analyses revealed significant alterations in cellular metabolism, oxidative phosphorylation, generation of reactive oxygen species and response to oxidative stress in long-term and short-term practitioners of daily RR practice that may counteract cellular damage related to chronic psychological stress. A significant number of genes and pathways were confirmed in an independent validation set containing 5 N_1 controls, 5 N_2 short-term and 6 M long-term practitioners.

Conclusions/Significance

This study provides the first compelling evidence that the RR elicits specific gene expression changes in short-term and long-term practitioners. Our results suggest consistent and constitutive changes in gene expression resulting from RR may relate to long-term physiological effects. Our study may stimulate new investigations into applying transcriptional profiling for accurately measuring RR and stress related responses in multiple disease settings.

Introduction

The relaxation response (RR) has been defined as a mind-body intervention that offsets the physiological effects caused by stress [1], [2]. The RR has been reported to be useful therapeutically (often as an adjunct to medical treatment) in numerous conditions that are caused or exacerbated by stress [3]–[6].

Mind-body approaches that elicit the RR include: various forms of meditation, repetitive prayer, yoga, tai chi, breathing exercises, progressive muscle relaxation, biofeedback, guided imagery and Qi Gong [7]. One way that the RR can be elicited is when individuals repeat a word, sound, phrase, prayer or focus on their breathing with a disregard of intrusive everyday thoughts [2]. The non-pharmacological benefit of the RR on stress reduction and other physiological as well as pathological parameters has attracted significant interest in recent years to decipher the physiological effects of the RR. In addition to decreased oxygen consumption [8]–[10], other consistent physiologic changes observed in long-term practitioners of RR techniques include decreased carbon dioxide elimination, reduced blood pressure, heart and respiration rate [1], [2], [11], prominent low frequency heart rate oscillations [12] and alterations in cortical and subcortical brain regions [13], [14].

Despite these observations and the well-established clinical effects of RR-eliciting practices [15], [16], the mechanisms underlying the RR have not been identified. Similarly, the impact of the RR on gene expression and signaling pathways has not yet been explored in detail, although a transcriptional profiling study of Qi Gong [17] practitioners, another RR method, revealed apparent distinct gene expression differences between Qi Gong practitioners and age matched controls. It is likely that differences in gene expression may be an underlying factor in the physiologic and psychologic changes noted above. Toward that end, we conducted a study to explore the gene expression profile of healthy long-term practitioners versus healthy age and gender matched controls. As a further evaluation, we provided 8-weeks of RR training to the control

subjects and re-assessed their gene expression.

Results

Patient characteristics

This study includes both cross sectional and an 8-week prospective design. Healthy adults were enrolled, comprising 2 groups: individuals with a long-term RR practice (group M; n = 19) or those with no prior RR experience (novice; group N_1; n = 19). Group N_1 novices, furthermore, underwent 8-weeks of RR training (Group N_2; n = 20) for the prospective analysis. In the cross sectional study, we compare gene expression profiles (GEP) in whole blood between groups M and N_1, whereas in the prospective study GEP is compared for each individual novice subject before and after RR experience, matched individuals of groups N_1 versus N_2 respectively.

Gene expression changes associated with the RR

Transcriptional differences between the different groups and within individuals before and after the RR are assessed by microarray analysis using Affymetrix HG-U133 Plus 2.0 genechips (www.affymetrix.com). This technology is a well established and reliable method to assess global gene expression differences [18]. Comparing group M (subjects with long term RR practice) to group N_1 (subjects prior to RR training), we find statistically significant differential expression of 2209 genes; 1275 significantly up-regulated and 934 significantly down-regulated in M vs. N_1. Additionally, 1561 genes are differentially expressed in novices after RR experience, N_2 vs. N_1; 874 significantly up-regulated and 687 significantly down-regulated. Comparison of gene lists from M vs. N_1, N_2 vs. N_1 and M vs. N_2 with Venn diagrams reveals significant overlap (Fig. 1a). Significance of overlaps is calculated using hypergeometric distributional assumption [19] and p-values are adjusted using Bonferroni correction for multiple comparisons [20]. Heatmaps were generated from genes in the intersecting areas of the Venn diagrams (Fig. 1b). We find 316 up-regulated and

279 down-regulated genes are differentially expressed in group M compared to both group N_1 and N_2; these changes in GEP are only observed in long-term RR practitioners. Similarly, 260 genes are up-regulated and 168 genes are down-regulated in both groups M and N_2 compared to N_1; they represent GEP changes characteristic of RR practice over at least 8 weeks.

Figure 1. Gene Ontology Analysis.

Analysis of differentially expressed genes: a) Venn diagrams: * indicates significant overlaps ($p<10^6$); b) Heatmaps of the 595 differentially regulated genes in both M vs. N_1 and M vs. N_2 (left) and the 418 differentially regulated genes in both M vs. N_1 and N_2 vs. N_1; c) Heatmap of 15 genes in the intersection of all three groups (gene symbols listed on the right). In heatmaps, rows represent genes and columns represent samples from N_1, N_2, and M groups. Genes are clustered using row-normalized signals and mapped to the $[-1,1]$ interval (shown in scales beneath each heatmap). Red and green represent high and low expression values, respectively.

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Heatmaps generated using these genes exhibit consistent GEP changes across the three groups with a few samples in each group resembling the GEP of another group. To determine if any demographic characteristics (e.g. age, ethnicity, etc.) influences this observation, we clustered each group separately using the same set of genes. For each cluster analysis, we calculated the significance of observing a characteristic among the samples in the subgroups formed. We found that number of times M subjects reported eliciting the RR per week was significantly associated with the subgroups formed when M samples were clustered using genes differentially expressed in long term RR practitioners only. Specifically, there were 316 up-regulated and 279 down-regulated genes differentially expressed in group M compared to both group N_1 and N_2; (Fig. 1b). All remaining cluster analyses revealed no such significant influence of demographic characteristics (see online supplementary data).

Finally, the intersection of all 3 areas (M vs. N_1, N_2 vs. N_1 and M vs. N_2) identifies genes with expression behavior that is monotonically changed between N_1 to N_2 to M (Fig. 1c). These results clearly demonstrate that short term as well as long term RR practice lead to distinct and consistent gene expression changes in hematopoietic cells.

Signaling pathways modulated by the RR

We performed Expression Analysis Systematic Explorer (EASE) analysis [21] using M vs. N_1, and N_2 vs. N_1 data-sets, to identify Gene Ontology (GO) categories where specific genes in these data occur more often than would be expected by random distribution of genes. These findings (and those of the validation data-set below) are summarized in Table 1, where select over-represented GO categories are listed along with specific genes differentially expressed in our data-sets. These categories include oxidative phosphorylation, ubiquitin-dependent protein catabolism, nuclear messenger RNA (mRNA) splicing, ribosomes, metabolic processes, regulation of apoptosis, NF-κB pathways, cysteine-type endo-peptidase activity and antigen processing. Most are significant in both long-term (M vs. N_1) and short-term (N_2 vs. N_1) practitioners of daily RR practice (see Table).

Table 1. Gene Ontology Categories

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Even though our analyses of differentially expressed genes and GO categories associated with RR practice meet widely accepted criteria for statistical significance, we were concerned about the relatively small fold changes that were observed (see Supplementary Methods). To address this issue we employed Gene Set Enrichment Analysis (GSEA). GSEA has proven to be useful for capturing subtle expression changes in complex gene signatures based on predefined gene sets or pathways [22]. As described above, we examined expression data for 2 comparisons, M vs. N_1 and N_2 vs. N_1. The selected

pathways or gene sets that are significantly enriched (False Discovery Rate (FDR.)<50%, nominal p-value (NPV)< = 0.02) are shown in Figure 2, with gene sets for N_2 vs. N_1 and M vs. N_1 in Fig 2A and Fig 2B respectively.

Figure 2. GSEA Analysis.

The analysis has been performed for >1200 predefined datasets using GSEA 2.0 software. Signal values for each gene are obtained by collapsing the probe values using max_probe algorithm. Representative datasets, significantly enriched (FDR.<50%, or NPV< = 0.01) between any two groups and corresponding heatmaps (depicting relative gene expression changes of core enrichment) are shown in a) N_2 vs. N_1 and b) M vs. N_1. Datasets that are enriched in both the original and validation analyses are marked with *. c) Heatmaps of ribosomal proteins and ubiquitin mediated proteolysis illustrate transitional trends in gene expression across the N_1, N_2 and M groups.

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GSEA analysis of N_2 vs. N_1 showed highly significant enrichment in gene sets related to various cellular stressors/stress responses and metabolism. To a pronounced degree these observations complement the results of GO analysis presented in the Table, also depicting significant alterations in cellular response to stress, oxidative and primary metabolism. The transition effect of the RR from novice to short term (8 weeks) to long term RR practitioners has been denoted through a colorgram of ribosomal proteins and ubiquitin mediated proteolysis gene sets (Fig. 2C). Whereas expression of ribosomal genes is significantly upregulated in RR practitioners at 8 weeks and more pronounced in long term practitioners, ubiquitin mediated proteolysis gene expression in general shows an opposite trend. Closer inspection of the colorgrams for ribosomal proteins and Ub proteolysis gene sets shows some variation in the GEP in each subgroup (N_1, N_2 M). The GEP of a few N_1 or N_2 subjects resembles the GEP of M subjects and

vice versa. To elucidate the association between GEP and subject characteristics (Race, Age, etc), we performed clustering of each subgroup separately (N_1, M) using the enriched gene sets (Ribosomal and Ub Proteolysis). This analysis identified a subcluster in the N_2 subgroup that has significant over-representation of Asian subjects (P value <0.05) when the clustering was performed using the ribosomal protein gene set. This observation needs further validation on a larger dataset as the current study contains only five Asian subjects. No other characteristic exhibited significant association with the ribosomal protein gene set. No significant association between the GEP profiles and subject characteristic was found when clustering was performed using the Ub proteolysis gene set. This analysis provides further insight into the stress response related genes that are influenced by RR practice.

Independent validation set analysis

As a validation of our results, we repeated the experimental and analysis procedures defined in the "Methods" section on a new set of samples consisting of 5 N_1, 5 N_2 and 6 M subjects. We found 1846 and 2390 probe sets differentially expressed between M vs. N_1, and N_2 vs. N_1 groups. The validation data-set showed a significant ($p<10^{-5}$) number of genes in common with the original analysis of 58 samples. We also found that 70–75% of all GO categories from the original analysis were retained in the validation set (p ~0), and 30–65% of significantly over-represented GO categories were shared. Of note, biologically relevant GO categories such as oxidative phosphorylation, regulation of apoptosis, and antigen presentation, come up as significantly over-represented in both the original and validation analyses. Results of the validation set and comparison analyses can be found in the online supplementary data. In addition, validation GSEA analysis on N_2 vs. N_1 subjects shows enrichment of ribosomal proteins and platelet expressed gene sets and enrichment of ribosomal proteins, oxidative phosphorylation and electron transport chain gene sets in M vs. N_1 subjects (Fig. 2A and 2B). The similarities between the original and validation

results from GSEA analysis argues against random chance accounting for the observed enrichment of these gene sets.

Discussion

Results from our study indicate that there are distinct differences in the GEPs between individuals with many years of RR practice (group M) and those without such experience (group N_1). Furthermore we find significant GEP changes within the same individuals before (N_1) and after 8 weeks of RR training (N_2). Finally, the changes in GEP found in M vs. N_1, and those of N_2 vs. N_1, are to a great degree similar when assessed by analysis of differentially expressed genes, GO analysis and GSEA.

It is becoming increasingly clear that psychosocial stress can manifest as system-wide perturbations of cellular processes, generally increasing oxidative stress and promoting a pro-inflammatory milieu [23]–[25]. Stress associated changes in peripheral blood leukocyte expression of single genes have been identified [26]–[28]. More recently, chronic psychosocial stress has been associated with accelerated aging at the cellular level. Specifically, shortened telomeres, low telomerase activity, decreased anti-oxidant capacity and increased oxidative stress are correlated with increased psychosocial stress [29] and with increased vulnerability to a variety of disease states [30]. Stress-related changes in GEP have been demonstrated by microarray analysis in healthy subjects, including up-regulation of several cytokines/chemokines and their receptors [31], and in individuals suffering from post-traumatic stress disorder, including inflammation, apoptosis and stress response [32] as well as metabolism and RNA processing pathways [33]. The pro-inflammatory transcription factor NF-kappa B (NF-κB) which is activated by psychosocial stress has been identified as a potential link between stress and oxidative cellular activation [34].

The RR is clinically effective for ameliorating symptoms in a variety of stress-related disorders including cardiovascular, autoimmune and other inflammatory

conditions and pain [15]. We hypothesize that RR elicitation is associated with systemic gene expression changes in molecular and biochemical pathways involved in cellular metabolism, oxidative phosphorylation/generation of reactive oxygen species and response to oxidative stress and that these changes to some degree serve to ameliorate the negative impact of stress. Genome-wide evaluation of PBL GEP is a reasonable approach to survey the transcriptional changes that are involved in elicitation of the RR. The GEP of RR practitioners presented here reveals altered gene expression in specific functional groups which suggest a greater capacity to respond to oxidative stress and the associated cellular damage. Genes including COX7B, UQCRB and CASP2 change in opposite direction from that in the stress response [31], [32].

Our findings are relatively consistent with those found in a study of Qi Gong [17], a practice that elicits the RR. In their study of 6 Qi Gong practitioners and 6 aged matched controls, practitioners had down-regulation of ubiquitin, proteasome, ribosomal protein and stress response genes and mixed up- and down-regulation of genes involved in apoptosis and immune function. We find a similar pattern of GO categories that are significantly over-represented in GO or enriched in GSEA in our cross sectional comparison, M vs. N_1. However, in our data-set ribosomal proteins were up-regulated.

Overall, similar genomic pattern changes occurred in practitioners of a specific mind body technique (Qi Gong) as well as in our long-term practitioners who utilized different RR practices including Vipassana, mantra, mindfulness or transcendental meditation, breath focus, Kripalu or Kundalini Yoga, and repetitive prayer. This indicates there is a common RR state regardless of the techniques used to elicit it.

Our study is the first to prospectively evaluate GEP changes in individuals before and after a short-term (8 week) RR training which consequently enables an appreciation of the parallel GEP changes that occur with short- and/or long-term RR practice. Replications in larger cohorts are warranted. Future investigations

could better define the therapeutic value and required duration of RR training to counter stress-related disorders.

Materials and Methods

Participants

Nineteen healthy practitioners of various RR eliciting techniques (including several types of meditation, Yoga, and repetitive prayer) participated (M group; n = 19). Years of practice averaged 9.4 years (5.0 sd) and ranged from 4 to 20 years. Twenty individuals without any prior RR eliciting experience served as controls (N group; n = 20).. As shown in Table 2, the M and N groups are matched with respect to age, gender, race, height, weight, and marital status, which do not exhibit significant difference between the groups ($p>0.05$, t- and chi-square test).

Table 2. Demographics

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Protocol

The study protocol was approved by the Committee on Clinical Investigations at the Beth Israel Deaconess Medical Center (BIDMC), Boston MA. All subjects provided written informed consent and the study was conducted in the General Clinical Research Center (GCRC) of the BIDMC. After providing written informed consent, participants were screened by a physician and had blood drawn to ensure good health. All participants completed a testing session in the GCRC. N_1 (novice) subjects had 8-weeks of RR training, listened to a 20-minute RR-eliciting CD daily and returned to the GCRC for a repeat testing session (hereafter classified as the N_2 group).

Relaxation-Response Training

N subjects received 8 weeks of RR training. Training included information about reducing daily stress, and a 20-minute elicitation of the RR [35]. Subjects randomized to the RR group received 8 weekly individual RR-training sessions from an experienced clinician as per our manualized research protocol [35]. The first session provided an educational overview of the stress response and the RR, instructions on how to elicit the RR, and a 20-minute guided RR experience. Sessions 2 through 8 consisted of a review of the subject's home practice card for compliance and a 20-minute guided RR experience.

During the RR elicitation in the weekly session, the subject was guided through a RR sequence including: diaphragmatic breathing, body scan, as well as mantra and mindfulness meditation, while subjects passively ignored intrusive thoughts. The specific CD guided the subject through the same sequence and has our clinical research studies and clinical practice for more than 15 years [35]. Subjects were asked to listen to the RR-eliciting CD once a day for 20 minutes at home.

To measure compliance, participants' daily home practice logs were reviewed each week and at the end of the 8 week training. These logs indicate that N subjects listened for an average of 17.5 minutes per day (3.7 sd) over 8-weeks.

Microarray Analysis

Following previously described protocols, the transcriptional profile of samples were probed using Affymetrix HG-U 133 Plus 2.0 chips representing over 47,000 transcripts and variants using more than 54,000 probesets. Scanned image output files were visually examined for major chip defects and hybridization artifacts and then analyzed with Affymetrix GeneChip Microarray Analysis Suite 5.0 (MAS5) software (Affymetrix). The image from each chip was scaled such that the 2% trimmed mean intensity value for all arrays was adjusted to target

intensity and reported as a non-negative quantity. Chips used for subsequent analysis consisted of 19 M, 19 N_1 and 20 N_2 samples (one chip from the N_1 group had insufficient signal values). A hierarchical clustering technique was used to construct an Unweighted Pair Group Method with Arithmetic-mean (UPGMA) tree using Pearson's correlation as the metric of similarity [36]. The expression data matrix was row-normalized for each gene prior to the application of average linkage clustering. When comparing 2 groups of samples to identify genes enriched in a given group, we used combination of three criteria. We considered genes with significantly different expression across the two groups using t-test ($p<0.05$) that further remained significant at a 5% false discovery rate (FDR.) using permutation testing with 1,000 permutations [37], [38]. In order to finalize a set of genes significantly up-regulated in a given group compared to another group, among the genes that passed the aforementioned steps, we filtered the ones that are “present” in at least half of the samples in the enriched group using Affymetrix' MAS5 Presence/Absence (P/A) calls. We used a paired t-test when comparing samples in groups N_1 and N_2.

Data deposition: All data sets have been deposited in the Gene Expression Omnibus, www.ncbi.nlm.nih.gov/geo (accession nos. GSE10041 and GSM253663-253734).

Gene Ontology and Gene Set Enrichment Analyses

Differentially expressed genes between the 3 groups (N_2 vs. N_1, M vs. N_1 and M vs. N_2) were separately analyzed using EASE to identify biologically relevant categories that are over-represented in the input set [21]. EASE analyses tested each list against all genes on the chip and overrepresentation describes a group of genes belonging to a certain GO category that appear more often in the given input list than expected to occur if the distribution were random. GO categories that had EASE scores of 0.05 or lower were selected as significantly over-represented. Gene Set Enrichment Analysis (GSEA 2.0 package http://www.broad.mit.edu/gsea/) was used to determine whether an *a priori*

defined set of genes showed statistically significant, concordant differences between 2 groups (N_2 vs. N_1, and M vs. N_1) in the context of known biological pathways. We tested expression values of all the genes in the relevant sample groups against 1687 gene sets obtained from the MSigDB2.0 for enrichment belonging to various metabolic pathways, chromosomal locations and functional sets (gene sets related to cancer/cancer cells are not included). The enriched gene sets have nominal p-value (NPV) less than 1% and False Discovery Rate (FDR.) <50% after 100 random permutations. These criteria ensure that there is minimal chance of identifying false positives.

References

1. Wallace RK, Benson H, Wilson AF (1971) A wakeful hypometabolic physiologic state. Am J Physiol 221: 795–799. **FIND THIS ARTICLE ONLINE**
2. Benson H, Beary JF, Carol MP (1974) The relaxation response. Psychiatry 37: 37–46. **FIND THIS ARTICLE ONLINE**
3. Sternberg EM (1997) Emotions and disease: from balance of humors to balance of molecules. Nat Med 3: 264–267. **FIND THIS ARTICLE ONLINE**
4. Benson H, Kotch JB, Crassweller KD (1978) Stress and hypertension: interrelations and management. Cardiovasc Clin 9: 113–124. **FIND THIS ARTICLE ONLINE**
5. Nakao M, Myers P, Fricchione G, Zuttermeister PC, Barsky AJ, et al. (2001) Somatization and symptom reduction through a behavioral medicine intervention in a mind/body medicine clinic. Behav Med 26: 169–176. **FIND THIS ARTICLE ONLINE**
6. Benson H, Goodale IL (1981) The relaxation response: your inborn capacity to counteract the harmful effects of stress. J Fla Med Assoc 68: 265–267. **FIND THIS ARTICLE ONLINE**
7. Benson H (1983) The relaxation response: Its subjective and objective

historical precedents and physiology. TINS 6: 281–284. FIND THIS ARTICLE ONLINE

8. Benson H, Steinert RF, Greenwood MM, Klemchuk HM, Peterson NH (1975) Continuous measurement of O2 consumption and CO2 elimination during a wakeful hypometabolic state. J Human Stress 1: 37–44. FIND THIS ARTICLE ONLINE
9. Kesterson J, Clinch NF (1989) Metabolic rate, respiratory exchange ratio, and apneas during meditation. Am J Physiol 256: R632–638. FIND THIS ARTICLE ONLINE
10. Warrenburg S, Pagano RR, Woods M, Hlastala M (1980) A comparison of somatic relaxation and EEG activity in classical progressive relaxation and transcendental meditation. J Behav Med 3: 73–93. FIND THIS ARTICLE ONLINE
11. Beary JF, Benson H (1974) A simple psychophysiologic technique which elicits the hypometabolic changes of the relaxation response. Psychosom Med 36: 115–120. FIND THIS ARTICLE ONLINE
12. Peng CK, Henry IC, Mietus JE, Hausdorff JM, Khalsa G, et al. (2004) Heart rate dynamics during three forms of meditation. Int J Cardiol 95: 19–27. FIND THIS ARTICLE ONLINE
13. Lazar SW, Bush G, Gollub RL, Fricchione GL, Khalsa G, et al. (2000) Functional brain mapping of the relaxation response and meditation. Neuroreport 11: 1581–1585. FIND THIS ARTICLE ONLINE
14. Jacobs GD, Benson H, Friedman R (1996) Topographic EEG mapping of the relaxation response. Biofeedback Self Regul 21: 121–129. FIND THIS ARTICLE ONLINE
15. Astin JA, Shapiro SL, Eisenberg DM, Forys KL (2003) Mind-body medicine: state of the science, implications for practice. J Am Board Fam Pract 16: 131–147. FIND THIS ARTICLE ONLINE
16. Esch T, Fricchione GL, Stefano GB (2003) The therapeutic use of the relaxation response in stress-related diseases. Med Sci Monit 9: RA23–

34. FIND THIS ARTICLE ONLINE
17. Li QZ, Li P, Garcia GE, Johnson RJ, Feng L (2005) Genomic profiling of neutrophil transcripts in Asian Qigong practitioners: a pilot study in gene regulation by mind-body interaction. J Altern Complement Med 11: 29–39. FIND THIS ARTICLE ONLINE
18. Lee NH, Saeed AI (2007) Microarrays: an overview. Methods Mol Biol 353: 265–300. FIND THIS ARTICLE ONLINE
19. Ivanova NB, Dimos JT, Schaniel C, Hackney JA, Moore KA, et al. (2002) A stem cell molecular signature. Science 298: 601–604. FIND THIS ARTICLE ONLINE
20. Schaffer J (1995) Multiple hypothesis testing. Ann Rev Psych 46: 561–584. FIND THIS ARTICLE ONLINE
21. Hosack DA, Dennis G Jr., Sherman BT, Lane HC, Lempicki RA (2003) Identifying biological themes within lists of genes with EASE. Genome Biol 4: R70. FIND THIS ARTICLE ONLINE
22. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, et al. (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 102: 15545–15550. FIND THIS ARTICLE ONLINE
23. Irie M, Asami S, Nagata S, Miyata M, Kasai H (2002) Psychological mediation of a type of oxidative DNA damage, 8-hyDr.oxydeoxyguanosine, in peripheral blood leukocytes of non-smoking and non-Dr.inking workers. Psychother Psychosom 71: 90–96. FIND THIS ARTICLE ONLINE
24. Yamaguchi T, Shioji I, Sugimoto A, Yamaoka M (2002) Psychological stress increases bilirubin metabolites in human urine. Biochem Biophys Res Commun 293: 517–520. FIND THIS ARTICLE ONLINE
25. Zheng KC, Ariizumi M (2007) Modulations of immune functions and oxidative status induced by noise stress. J Occup Health 49: 32–38. FIND THIS ARTICLE ONLINE

26. Glaser R, Kennedy S, Lafuse WP, Bonneau RH, Speicher C, et al. (1990) Psychological stress-induced modulation of interleukin 2 receptor gene expression and interleukin 2 production in peripheral blood leukocytes. Arch Gen Psychiatry 47: 707–712. FIND THIS ARTICLE ONLINE
27. Glaser R, Lafuse WP, Bonneau RH, Atkinson C, Kiecolt-Glaser JK (1993) Stress-associated modulation of proto-oncogene expression in human peripheral blood leukocytes. Behav Neurosci 107: 525–529. FIND THIS ARTICLE ONLINE
28. Platt JE, He X, Tang D, Slater J, Goldstein M (1995) C-fos expression in vivo in human lymphocytes in response to stress. Prog Neuropsychopharmacol Biol Psychiatry 19: 65–74. FIND THIS ARTICLE ONLINE
29. Epel ES, Blackburn EH, Lin J, Dhabhar FS, Adler NE, et al. (2004) Accelerated telomere shortening in response to life stress. Proc Natl Acad Sci U S A 101: 17312–17315. FIND THIS ARTICLE ONLINE
30. Epel ES, Lin J, Wilhelm FH, Wolkowitz OM, Cawthon R, et al. (2006) Cell aging in relation to stress arousal and cardiovascular disease risk factors. Psychoneuroendocrinology 31: 277–287. FIND THIS ARTICLE ONLINE
31. Morita K, Saito T, Ohta M, Ohmori T, Kawai K, et al. (2005) Expression analysis of psychological stress-associated genes in peripheral blood leukocytes. Neurosci Lett 381: 57–62. FIND THIS ARTICLE ONLINE
32. Zieker J, Zieker D, Jatzko A, Dietzsch J, Nieselt K, et al. (2007) Differential gene expression in peripheral blood of patients suffering from post-traumatic stress disorder. Mol Psychiatry 12: 116–118. FIND THIS ARTICLE ONLINE
33. Segman RH, Shefi N, Goltser-Dubner T, Friedman N, Kaminski N, et al. (2005) Peripheral blood mononuclear cell gene expression profiles identify emergent post-traumatic stress disorder among trauma survivors. Mol Psychiatry 10: 500–513, 425. FIND THIS ARTICLE ONLINE
34. Bierhaus A, Wolf J, AnDr.assy M, Rohleder N, Humpert PM, et al. (2003)

A mechanism converting psychosocial stress into mononuclear cell activation. Proc Natl Acad Sci U S A 100: 1920–1925. **FIND THIS ARTICLE ONLINE**

35. Dusek JA, Chang BH, Zaki J, Lazar S, Deykin A, et al. (2006) Association between oxygen consumption and nitric oxide production during the relaxation response. Med Sci Monit 12: CR1–10. **FIND THIS ARTICLE ONLINE**
36. Sneath PHA (1973) Numerical taxonomy; the principles and practice of numerical classification (W. H. Freeman, San Francisco, CA).
37. Li C, Wong WH (2001) Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc Natl Acad Sci USA 98: 31–36. **FIND THIS ARTICLE ONLINE**
38. Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 98: 5116–5121. **FIND THIS ARTICLE ONLINE**

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