A PCR-BASED SCREEN TO IDENTIFY NATURAL COMPOUNDS WITH THE ABILITY TO INFLUENCE NRF2-MEDIATED TRANSCRIPTIONAL INDUCTION OF DETOXIFICATION/ANTIOXIDANT GENES

A Mastaloudis[1], JL Barger[2], TA Prolla[2,3], R Weindruch[2,3], SB Ferguson[1], SM Wood[1]

[1]Nu Skin Enterprises, Provo, Utah, USA; [2]LifeGen Technologies, Madison, Wisconsin, USA; [3]University of Wisconsin, Madison, Wisconsin, USA.

ABSTRACT

It is widely accepted that oxidative injury and inflammation are intimately involved in the aging process and the development of age-related diseases. To date, most anti-aging strategies have focused solely on the delivery of exogenous antioxidants to combat the negative effects of aging. A promising new strategy is to identify nutrients and plant extracts that can directly target intrinsic cytoprotective mechanisms including: 1) upregulation of genes involved in the detoxification of xenobiotic and xenobiotics metabolites, 2) upregulation of genes involved in the synthesis and regulation of intrinsic antioxidants and antioxidant enzymes and 3) modulation of genes involved in the regulation of inflammation. Therefore, the purpose of this study was to evaluate natural compounds for the ability to modulate a representative panel of genes for key age-related pathways: the Nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE)/Phase II detoxification and inflammatory pathways.

Nrf2 is a transcription factor that regulates the basal and inducible expression of a large battery of genes encoding for cytoprotective factors including those that defend against electrophilic stressors and oxidative insults. We selected a panel of 9 genes representative of the Nrf2 and inflammatory pathways based on a review of the literature. Mice (C57Bl/6), aged 8 weeks were fed an AIN 93M diet without (n = 8) or with one of 15 compounds (n = 8/group) for 3 months. In a second, exploratory study, we compared the effects of a subset of compounds in young mice (8 weeks) and old mice (24 months) with and without supplementation for 6 months. The compounds included a variety of nutrients or plant extracts; select examples include alpha-lipoic acid, CoQ10, quercetin, rosemary extract, broccoli seed extract and olive extract. For Study 1, liver tissues and for Study 2, gastrocnemius muscle tissues were collected and gene expression of the 9-gene panel was analyzed by RT-qPCR. Finally, nutrients/plant extracts were ranked based on the number of Nrf2 genes upregulated, number of inflammation-related genes downregulated and the robustness of the changes in gene expression. The most potent effects were seen with broccoli seed, alpha-lipoic acid, blood orange and olive extracts.

In summary, we have identified a robust panel of genes representative of the Nrf2 and inflammatory pathways that can be used as a rapid screening tool to evaluate the effects of specific nutrients on cellular detoxification, antioxidant status and inflammatory balance. This technique affords an opportunity to define the optimal blend of ingredients that can oppose gene expression changes in these key pathways that are directly related to human aging and age-related disease.

INTRODUCTION

- Aging is associated with the accumulation of cellular toxins and damage over time and impairments in cytoprotective and repair mechanisms.
- Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that regulates the basal and inducible expression of a large battery of genes encoding for cytoprotective factors including those that defend against electrophilic stressors and oxidative insults.
- The Nrf2/antioxidant response element (ARE) antioxidant protection and Phase II Detoxification pathways are impaired with aging due to age-related changes in gene expression. A key example is the reduction in glutathione (GSH) levels in all tissues with age due primarily to declines in GCL and glutathione synthase (GS) expression (1).
- Aging is also associated with inflammatory dysregulation and changes in expression of inflammatory related genes.
- The purpose of this study was to evaluate natural compounds for their ability to modulate a representative panel of genes for key age-related pathways: the Nrf2/ARE cytoprotective and inflammatory pathways.

METHODS

STUDY 1. N = 8/GROUP:
Controls (C); 8 – 22 wks of age
Treated (T); 8 – 22 wks of age

STUDY 2. N = 5/GROUP:
Young Control (YC); 8 – 22 wks of age
Old Control (OC); 14 – 30 months of age
Old Treated (OT); 14 – 30 months of age

References

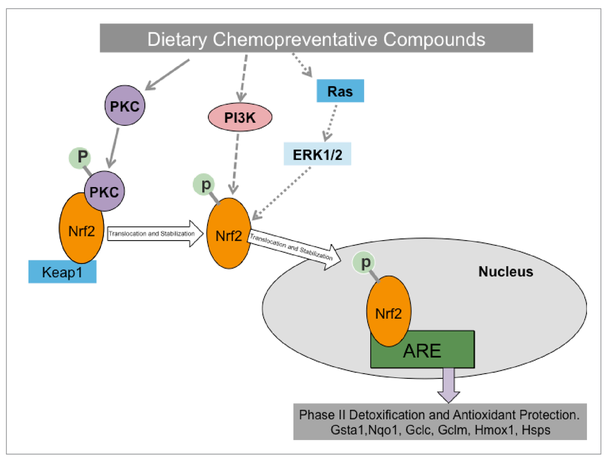
1. Lu Mol. Apsects. Med. 2009.
2. Barger et al. PLoSOne 2008.
3. Chen and Kong, FRBM:36; 2004
4. Suh et al. PNAS 2004.

METHODS

INGREDIENTS TESTED
Alpha lipoic acid (ALA)
Rosemary extract (RMY)
Red orange extract (ROC)
Pomegranate extract (POM)
Coenzyme Q10 (CoQ10)
Tart cherry extract (TCE)
Olive extract (OLV)
Grape seed extract (GSE)
Broccoli seed extract (BSE)
Curcumin (CUR)
Schizandra chinensis extract (SCE)
Quercetin (QUER)
Resveratrol (RSV)

RT-qPCR METHODOLOGY
Quantitative real-time PCR (RT-qPCR) analysis was performed on RNA isolated from entire livers and muscle. A detailed description has been published previously (2). Briefly, the magnitude of change was determined for each gene and compared to control. Study 1. C vs. T. Study 2. YC vs. OC and OC vs. OT. Two-tailed t-tests (assuming equal variance) were used to determine if the change in expression for individual genes was statistically significant. The magnitude of the change in expression is reported as "fold change" values which are log2-adjusted to fit normality assumptions for statistical analyses.

Figure 1. Overview of the signaling cascade regulating Phase II Detoxification and Antioxidant Protection adapted from Chen and Kong (3).



GENES SCREENED
GCLC. Encodes for the heavy catalytic subunit of glutamate-cysteine ligase (GCL), also known as gamma-glutamylcysteine synthetase, the first rate limiting enzyme of glutathione synthesis.
GCLM. Encodes for the light regulatory subunit of GCL, also known as gamma-glutamylcysteine synthetase, the first rate limiting enzyme
of glutathione synthesis.
GSR. Encodes glutathione reductase, a member of the class-I pyridine nucleotide-disulfide oxidoreductase family.
GSTA1. Encodes a glutathione S-tranferase belonging to the alpha class.
HMOX1. Encodes for the inducible isoform of heme oxygenase-1.
NQ01. A member of the NAD(P)H dehydrogenase (quinone) family and encodes a cytoplasmic 2-electron reductase.
UGT1A6. Encodes a UDP-glucuronosyltransferase, an enzyme of the glucuronidation pathway.
PTGS2 (COX2). Encodes for the inducible prostaglandin-endoperoxide synthase (PTGS2), also known as cyclooxygenase 2, the key enzyme in prostaglandin biosynthesis.
Nos2 (iNOS). Encodes for inducible nitric oxide synthase which is expressed in liver.

RESULTS

TABLE 1
Changes in gene expression in liver following short-term feeding of compounds. C vs. T

	Gclc	Gclm	Gsr	Gssta1	Hmox1	Nqo1	Ugt1a6a	Ptgs2	Nos2
Ctrl	1.01	1.01	1.01	1.06	1.02	1.04	1.01	1.39	1.11
ALA	1.18	1.31	1.33	3.41	-1.08	1.09	1.24	1.02	1.03
RMY	-1.11	1.28	-1.03	3.23	-1.22	1.17	1.10	-1.22	-1.03
ROC	1.09	1.60	1.47	4.86	1.04	1.25	1.10	1.05	1.01
POM	-1.04	1.12	1.14	2.94	1.16	1.19	1.03	1.50	1.37
CoQ	-1.17	1.26	1.03	2.38	-1.23	-1.21	1.01	-1.21	-1.01
TCE	-1.13	1.00	-1.12	2.53	-1.05	-1.02	1.05	1.69	2.98
OLV	-1.02	1.08	1.03	3.54	-1.13	1.22	1.19	1.11	1.14
GSE	-1.00	1.23	1.08	3.26	-1.03	1.47	1.09	1.79	1.45
BSE	-1.03	1.13	1.01	2.84	1.06	1.77	1.15	1.68	1.46
CUR	1.08	1.30	1.14	2.45	-1.11	-1.05	1.19	-1.17	1.47
SCE	-1.03	1.19	-1.13	1.93	2.41	1.46	1.04	2.17	2.00
QUER	-1.22	-1.18	-1.24	1.29	-1.24	-1.32	-1.04	1.92	1.14

Statistically significantly upregulated compared to control; $p < 0.05$

Statistically significantly downregulated compared to control; $p < 0.05$

TABLE 2
Changes in gene expression in gastrocnemius following long-term feeding of compounds in YC vs. OC and OC vs. OT

	Gclc	Gclm	Gsr	Hmox1	Nqo1
YC	1.01	1.01	1.02	1.02	1.02
OC	-1.10	-1.02	-1.36	2.86	-1.06
ALA	1.58	1.00	2.10	-2.14	-1.04
POM	-1.11	1.56	-1.16	-3.06	-2.86
CoQ	1.41	1.01	1.41	-2.11	-1.21
CUR	1.40	-1.33	1.40	-2.74	-1.95
RSV	1.39	1.59	1.43	-1.55	-2.18

Statistically significantly upregulated compared to old control; $p < 0.05$

Statistically significantly downregulated compared to old control; $p < 0.05$

TABLE 3
Comparison of changes in gene expression in a subset of ingredients studied in both liver and gastrocnemius muscle

LIVER

	Gclc	Gclm	Gsr	Hmox1	Nqo1
Ctrl	1.01	1.01	1.01	1.02	1.04
ALA	1.18	1.31	1.33	-1.08	1.09
POM	-1.04	1.12	1.14	1.16	1.19
CoQ	-1.17	1.26	1.03	-1.23	-1.21
CUR	1.08	1.30	1.14	-1.11	-1.05

GASTROCNEMIUS

	Gclc	Gclm	Gsr	Hmox1	Nqo1
YC	1.01	1.01	1.02	1.02	1.02
OC	-1.10	-1.02	-1.36	2.86	-1.06
ALA	1.58	1.00	2.10	-2.14	-1.04
POM	-1.11	1.56	-1.16	-3.06	-2.86
CoQ	1.41	1.01	1.41	-2.11	-1.21
CUR	1.40	-1.33	1.40	-2.74	-1.95

Statistically significantly upregulated; $p < 0.05$

Statistically significantly downregulated; $p < 0.05$

SUMMARY & CONCLUSION

STUDY 1. SHORT TERM-TERM FEEDING IN YOUNG ANIMALS
- The greatest effects on gene expression were seen with ALA, ROC, BSE and OLV.
- SCE appeared to have no benefit and actually increased expression of the pro-inflammatory gene Ptgs2, suggesting a potential negative effect of this ingredient.

STUDY 2. LONG-TERM FEEDING IN YOUNG AND OLD ANIMALS
- Consistent with Study 1 results and the literature (4), ALA effectively opposed age-related declines in Gclc and Gsr expression.
- RSV effectively opposed age-related decreases in both catalytic subunits responsible for GSH synthesis, Gclm and Gclc.

GENE PANEL
- Differential results were observed in Gclc and Gclm expression in YT, OT and in the different tissues as reported previously (1).
- Gsta1 appears to be modulated by a number of compounds.
- Hmox1 was not influenced by any of the compounds.
- We recommend omitting both Gsta1 and Hmox1 from future screen panels.
- It is important to account for age and length of feeding in study design as well as to evaluate multiple tissues and multiple genes when screening ingredients.

In summary, we have identified a robust panel of genes representative of the Nrf2 and inflammatory pathways that can be used as a rapid screening tool to evaluate the effects of specific nutrients on age-related changes in cellular detoxification, antioxidant status and inflammatory balance. This technique affords an opportunity to rapidly identify compounds of interest and then to define the optimal blend of ingredients that may oppose genetic changes in these key pathways that are directly related to human aging and age-related disease.

Ongoing studies are utilizing genome wide gene expression profiling in brain, muscle, liver and other tissues using microarray technology to investigate potential effects of blends of natural ingredients in opposing the effects of aging.

Acknowledgments. The authors appreciate contributions from Glenn Cheney, Jessie Barrett, David Rockhill, Lih-Wen Ding, Bill Landreth, Jim Vann, PhD and Jihae Kwon for their technical expertise.