Nutrition, Metabolism & Cardiovascular Diseases (2014) **24**, 423–427

Available online at www.sciencedirect.com

Nutrition, Metabolism & Cardiovascular Diseases

journal homepage: www.elsevier.com/locate/nmcd

Effects of equol on gene expression in female cynomolgus monkey iliac arteries

K. Eyster [a,*], S. Appt [b], A. Chalpe [a], T. Register [b], T. Clarkson [b]

[a] Division of Basic Biomedical Sciences, Sanford School of Medicine of the University of South Dakota, 414 E. Clark St., Vermillion, SD 57069, USA
[b] Comparative Medicine Clinical Research Center, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA

Received 16 April 2013; received in revised form 19 August 2013; accepted 5 September 2013
Available online 1 November 2013

KEYWORDS
Equol;
Soy;
Gene expression;
DNA microarray;
Atherosclerosis;
Artery

Abstract *Background and aims:* To examine effects of equol, the soy phytoestrogen metabolite, on gene expression in the monkey iliac artery.
Methods and results: A high fat/high cholesterol diet was fed to eight ovariectomized cynomolgus monkeys for 6.5 years. After biopsy of the left iliac artery, the animals were randomized to two treatment groups for 8 months; the treatment groups were equol (23.7 mg/100 g diet, $n = 4$) and vehicle ($n = 4$). The right iliac artery was removed at necropsy. Gene expression in the iliac arteries in response to equol was determined by DNA microarray. Comparison of atherosclerotic lesions and plasma lipids at pre-versus post-equol treatment time points and in vehicle versus equol treatment groups did not identify any significant differences. Despite the lack of effect of equol on these parameters, 59 genes were down-regulated and 279 were up-regulated in response to equol. Comparison of these data to previous work identified 10 genes regulated in opposite directions by equol compared to presence of atherosclerosis plaque (*Menopause* 2011; 18:1087–1095) and 55 genes differentially expressed in the same direction in response to both equol and estradiol (Eyster et al., *Menopause* 2014;21:143–152.).
Conclusions: Similar responses of genes to both equol and estradiol may reflect the extent to which equol serves as a natural selective estrogen receptor modulator in the arteries. Opposite responses of 10 genes to equol versus the presence of atherosclerosis implicates those genes in the potential protective effects of equol in arteries.

Introduction

Equol is a metabolite of the soy isoflavone, daidzein, produced by the action of gastrointestinal bacteria [1]. Equol is a ligand for estrogen receptors (ER) with greater potency at ER than daidzein [1]. Many claims have been made regarding the cardiovascular health benefits of soy isoflavones, and by extension, equol [2]. Studies in laboratory animals support the atheroprotective effects of dietary soy components [3,4]. However, scientific evidence in human studies in support of these claims has been variable [2,5]. One contributor to the variability of soy isoflavones in humans is that as many as 30–50% of individuals are not significant producers of equol from daidzein because they lack the appropriate intestinal flora [6]. This heterogeneity leads to substantial variability in the circulating concentrations of equol and daidzein and difficulties in analysis and interpretation of results.

The effects of estrogen therapy on cardiovascular diseases are controversial. When the blood vessels are healthy, estrogen appears to protect them from the development of atherosclerotic plaque [7]. However, if atherosclerosis is well-established, estrogen appears to not be beneficial and may actually increase the risk of clinical events [8,9]. The exact mechanisms by which estrogen influences cardiovascular disease are not entirely clear, thus the evidence that daidzein, genistein, and equol can influence estrogen

* Corresponding author. Tel.: +1 605 677 5159; fax: +1 605 677 6381.
E-mail address: Kathleen.Eyster@usd.edu (K. Eyster).

0939-4753/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.numecd.2013.09.014

receptor activity does not clarify their mechanism of action in the cardiovascular system. However, these soy/soy-derived compounds have greater affinity for ERβ than for ERα [10]. Thus, these compounds may act as natural selective estrogen receptor modulators (SERMs) [11].

Similarities in the cardiovascular system of human and cynomolgus monkeys make this animal model an ideal system for the study of the effects of equol [12]. Cynomolgus monkeys develop atherosclerosis when placed on a North American-type atherogenic diet [3,12], and the effects of estrogen and soy isoflavones on the cardiovascular system and the development of atherosclerosis have been studied extensively in cynomolgus monkeys [3,12].

The current study was undertaken to examine global gene expression patterns in the iliac arteries of cynomolgus monkeys in response to treatment with a synthetic racemic mixture of S- and R-equol in order to identify potential equol specific effects in the absence of other isoflavone components and to avoid problems associated with variable equol conversion rates. The extent of atherosclerosis in the iliac arteries has been shown to be directly correlated with that of the coronary arteries in macaques [3]; therefore, iliac arteries were used as proxies for the coronary artery in this study. Moreover, the use of the iliac arteries permitted a longitudinal assessment of pre-versus post-equol treatment.

Methods

Animals and study design

Eight adult female cynomolgus macaques (*Macaca fascicularis*), age 20 years or older, were used for this study. The animals were surgically menopausal with ovariectomy 4–6 years prior to the study. They had been housed in stable social groups of 3–4 animals each and had consumed various semi-purified high fat/high cholesterol diets for 6.5 years upon entering this study. The diet used for this study was formulated to mimic a typical North American diet and contained 0.20 mg cholesterol/Calorie of diet, 29.4% fat, and 19.8% protein from animal sources (casein/lactalbumin). The monkeys received approximately 120 kcal/kg body weight of the diet once daily for 8 months.

The left common iliac artery was biopsied to obtain pretreatment arterial tissue as described [4,13]. Equol was added to the diet as a supplement at 23.7 mg equol/100 g of diet ($n = 4$); control animals received the same diet without equol ($n = 4$). The equol supplement contained a 96.0% pure racemic mixture of S- and R-equol enantiomers in a 1:1 ratio and was provided by Solae, a division of Dupont (St. Louis, MO, USA). The dose of equol was designed to mimic the amount of isoflavones consumed by women in several clinical trials [2] and then scaled to account for metabolic differences between women and monkeys. The dietary supplement was the only source of equol in the diet; the diet contained no soy isoflavones. After 8 months on the North American diet with equol or control treatment, the monkeys were euthanized and the post-treatment common iliac artery was collected and processed as described [14]. Briefly, the iliac artery was opened longitudinally, laid flat, and divided into 3 equal segments. One segment was fixed in paraformaldehyde, embedded in paraffin, sectioned, and stained with Verhoeff-van Gieson's stain for histologic assessment of atherosclerotic plaque intimal area. As previously described [3,14,15], digital images were captured from each arterial section and morphometric measurements were made to assess the extent (defined as cross-sectional area in mm^2) of iliac artery plaque [3,15]. A second segment of iliac artery was placed in RNAlater (Sigma R-0901) and stored at −70° until used for extraction of total RNA. The third section was frozen and archived. At the initiation of the study (baseline) and after 3, 6, and 8 months of treatment, blood samples were obtained for the measurement of lipids and lipoproteins as described [4,14].

All animal procedures were carried out at Wake Forest University whose facilities and laboratory animal program are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. All procedures using animals conformed to State and Federal laws and were conducted in compliance with standards of the U.S. Department of Health and Human Services, and guidelines established by the Wake Forest University Animal Care and Use Committee (ACUC).

Analysis of gene expression

Total RNA was isolated from segments of iliac artery using a method designed to maximize RNA extraction from small tissue samples [13,16]. Arterial segments ranging in size from 2.94 to 13.64 mg were minced in 600 μl TRI reagent (Molecular Research Center, Cincinnati, OH) and homogenized in a 2 ml tube with a 7 mm probe on a Polytron homogenizer (Kinematica, Luzern, CH). The probe was then rinsed in 400 μl of fresh TRI reagent to recover residual sample from the probe. The 2 aliquots of TRI reagent were pooled. Bromochloropropane (200 μl) and 3 M sodium acetate (60 μl) were added and the samples were centrifuged (5 min at 8000 g) to effect phase separation. The aqueous layer containing RNA was then purified on a silica gel membrane spin column (RNeasy, Qiagen, Valencia, CA) per company instructions [16]. Gene expression signatures were analyzed using CodeLink Whole Human Genome Bioarrays (Applied Microarrays, Tempe, AZ) to compare pretreatment versus posttreatment gene expression for both equol and vehicle treated groups as described [13]. Differential expression of SET domain, bifurcated 2 (SETDB2) was identified by DNA microarray and confirmed by two-step real time reverse transcription-polymerase chain reaction (RT-PCR) as described [13]. Primers and probe were obtained from Applied Biosystems (Life Technologies; Hs00230475_m1). Data from real time PCR reactions were analyzed by qBase software as described [13].

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SE), the experimental number (n) was 4 per group,