Methlyselenocysteine Resets the Rhythmic Expression of Circadian and Growth-Regulatory Genes Disrupted by Nitrosomethylurea In vivo

Ming Zhu Fang1, Xun Zhang1, and Helmut Zarbl1,2

Abstract

Epidemiologic and animal studies indicate that disruption of circadian rhythm increases breast cancer risk. Previously, we showed that methlyselenocysteine reduced the incidence of N-nitroso-N-methylurea (NMU)–induced mammary carcinomas in Fischer 344 rats by 63%. Methlyselenocysteine also increased the expression of Period 2 (Per2) and D-binding protein (DBP), providing evidence for a link between circadian rhythm and chemoprevention. Here, we report that NMU disrupted the expression of core circadian genes (Per1, Per2, Cry1, Cry2, and Rev-ErbAα) and circadian-controlled genes, including melatonin receptor 1α (MTNR1A), estrogen receptors (ERα and ERβ), and growth-regulatory genes (Trp53, p21, Gadd45α, and c-Myc) in mammary glands of Fischer 344 rats. By contrast, dietary methlyselenocysteine (3 ppm selenium) given for 30 days, significantly enhanced the circadian expression of these genes (except for Cry1 and Cry2). The largest effect was on the levels of the Per2, MTNR1A, and ERβ mRNAs, which showed 16.5-fold, 4.7-fold, and 9.5-fold increases in their rhythm-adjusted means, respectively, and 44.5-fold, 6.5-fold, and 9.7-fold increases in amplitude as compared with the control diet, respectively. Methlyselenocysteine also shifted the peak expression times of these genes to Zeitgeber time 12 (ZT12; lights off). Methlyselenocysteine also induced rhythmic expression of Trp53, p21, and Gadd45α mRNAs with peak levels at ZT12, when c-Myc expression was at its lowest level. However, methlyselenocysteine had no significant effect on the circadian expression of these genes in liver. These results suggest that dietary methlyselenocysteine counteracted the disruptive effect of NMU on circadian expression of genes essential to normal mammary cell growth and differentiation. Cancer Prev Res; 3(5): 640–52. ©2010 AACR.

Introduction

In mammals, circadian rhythm is controlled by the interaction of positive and negative biochemical feedback loops comprising a molecular oscillator. Heterodimers of brain and muscle arnt-like protein 1 (Bmal1, known as Arnt1 in rat) and Clock or neuronal PAS domain protein 2 (Npas2) activate transcription by binding to E-box elements present in the promoters of circadian genes, including Period (Per), Cryptochrome (Cry), Rev-ErbAα, retinoid-related orphan nuclear receptors (ROR), and numerous circadian-controlled genes (CCG). The Rev-ErbAα and ROR proteins, respectively, inhibit and activate Bmal1 transcription (1). Posttranscriptionally modified Per/Cry heterodimers, in turn, repress Clock/Bmal1 transcriptional activity, thus limiting their own transcription and the expression of CCGs (2). The Clock protein was shown to have histone acetyltransferase activity (3), suggesting that the activation of E-box–containing promoters is modulated at the level of the chromatin structure. The molecular oscillator functions in most peripheral tissues and maintains a periodicity of ~24 hours that persists in the absence of external cues (1, 2, 4, 5). To synchronize the periodicity among cells in peripheral organs in vivo and adjust the phase to changing environmental signals, the intrinsic molecular oscillator is entrained by external signals that includes melatonin and glucocorticoids (6). In vitro studies on fibroblasts indicate that the intracellular mediators of circadian gene expression include Ca2+, protein kinase C, protein kinase A, mitogen-activated protein kinase, and glucocorticoid receptor signaling pathways (6, 7). In mammals, the central pacemaker for the autonomus oscillators in peripheral organs is melatonin, a hormone whose secretion from the pineal...
gland is synchronized by light-induced signaling from the suprachiasmatic nucleus (8, 9). In this way, the suprachiasmatic nucleus coordinates the behavioral, physiologic, and biological functions of organisms and cells with light-dark cycles, food availability, and a variety of environmental signals.

Circadian rhythms also play a critical role in normal cell growth, differentiation, and cellular responses to genotoxic stressors (2). Disruption of normal circadian rhythm by environmental exposures is associated with an increased risk of several cancer types. For example, disruption of circadian rhythm by constant light exposure or pinealectomy increased the incidence of mammary adenocarcinoma in rodents (10, 11). Moreover, mice lacking Per2 show a significantly elevated incidence of carcinomas after exposure to genotoxic stress (1), whereas overexpression of either Per1 or Per2 in cancer cells inhibits their neoplastic cell growth and increases apoptotic rates (12–14). Clinical studies also reveal an association between the deregulation of Per genes and various human cancers (15–17). In addition, epidemiologic studies indicate that disruption of circadian rhythm by shift work (exposure to light at night) increases breast and prostate cancer risk (18), prompting the International Agency for Cancer Research to classify shift work as a probable human carcinogen (type 2A).

Both epidemiologic and animal studies have suggested that various forms of selenium, a dietary trace element, reduce cancer risk at multiple organ sites, including breast and prostate (19). Methylselenocysteine, an organic form produced by plants, mediates its chemopreventive effects at the early stages of carcinogenesis (20–22). Our recent studies were the first to suggest an association between circadian rhythm and chemoprevention. We showed that dietary methylselenocysteine, given for 30 days after exposure to a single carcinogenic dose of N-nitroso-N-methylurea (NMU), reduced the incidence of mammary carcinomas in Fischer 344 (F344) rats by 63% at 36 weeks. Gene expression profiles of normal mammary tissue indicated that 30 days of dietary supplementation with methylselenocysteine significantly increased the levels and rhythmic expression of the core circadian gene, Period 2 (Per2), and the circadian output gene, D-binding protein (DBP; ref. 23). In the present study, we extended these observations by demonstrating that a single carcinogenic dose of NMU significantly disrupted the rhythmic expression of most core circadian genes and CCGs during the early stages of carcinogenesis. By contrast, chemopreventive methylselenocysteine reset and enhanced the circadian gene expression in mammary tissues of NMU-treated rats. The circadian and CCGs affected by methylselenocysteine included core circadian genes (Per2 and Rev-ErbAα), estrogen and melatonin receptors, and DNA damage–responsive genes. We also found that the ablation and resetting of circadian expression of these genes by NMU and methylselenocysteine, respectively, were independent of serum melatonin levels.

Materials and Methods

Animal maintenance and diet preparation

All protocols were reviewed by and received the approval of the Institutional Animal Care and Use Committees. Animal experiments were done in Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facilities at the Fred Hutchinson Cancer Research Center (Seattle, WA) and the University of Medicine and Dentistry of New Jersey (Piscataway, NJ). Female F344 rats were from Harlan Laboratories (Indianapolis, IN). For acclimatization to a powdered ration from Harlan Tekland, animals were maintained on a powdered standard AIN-76A diet containing 0.1 ppm of selenium (sodium selenite). The methylselenocysteine-enriched diet was made by admixing i-Se-methylselenocysteine (Selenium Technologies) with the standard powdered AIN-76A diet to a final concentration of 3 ppm of selenium. Animals were housed under controlled climate conditions and a 12-hour light/12-hour dark cycle (7 a.m. on/7 p.m. off). Referring to 7 a.m. as Zeitgeber time (ZT) 0 indicates the time of lights on; and 7 p.m. is ZT12, indicating the time of lights off.

Animal treatment and sample collection

Forty-two female F344 rats between 50 and 55 days of age received a single i.p. injection of NMU (Ash Stevens, Inc.) at a dose of 50 mg/kg body weight. NMU was dissolved in acidified saline at a final concentration of 10 mg/mL just prior to injection. Rats were then randomized to two groups and maintained on either the standardized control diet or the methylselenocysteine-enriched diet for 30 days. An additional 21 age-matched female F344 rats injected with the vehicle and maintained on the standardized diet served as control group. Three rats of each group were sacrificed every 4 hours for 24 hours, beginning at 7 a.m. (ZT0). Blood samples were collected into heparinized-BD Vacutainers by cardiac punctures after CO2 asphyxiation. Plasma samples were separated by sequential centrifugation (at 1,300 rcf for 10 minutes first and then at 2,400 rcf for 15 minutes) and stored at −80°C. All mammary glands from each side of individual rats were carefully dissected, combined into a pool of left or right mammary glands, frozen on dry ice, and stored at −80°C. The liver of each rat was also dissected, frozen on dry ice, and stored at −80°C.

Quantitative real-time PCR

Total RNA was extracted from a small piece (~50 mg) of mammary tissue from the left side pool of mammary glands and liver of each rat using Tri Reagent (Sigma) and Qiagen RNeasy Mini Kit (Qiagen), and then digested with Qiagen RNase-free DNase. Relative mRNA expression levels were determined with real-time quantitative reverse transcription-PCR (RT-PCR) using specific primers (Supplementary Table S1), designed with Primer Express Software V 3.0 (Applied Biosystems), and SYBR as the reporter. For reverse transcription, 1 μg of RNA was
reverse-transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories). For real-time PCR, cDNA was amplified with the SYBR Universal PCR Master Mix (Applied Biosystems) in ABI Prism 7900 Sequencing Detector according to the instructions of the manufacturer. A no-template control was included in each assay. β-Actin was used as an endogenous control. The expression level of each gene in mammary tissue of the rat sacrificed at ZT0 was used as a calibrator in each group. Three independent samples were analyzed for each time point. The comparative Ct method was used to analyze the relative mRNA expression levels (24).

**Western blot**

Total protein was extracted from a small piece (~200 mg) of mammary tissue from the left side pool of mammary glands of each animal using radioimmunoprecipitation assay buffer lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% sodium deoxycholate, 0.1% SDS] supplemented with a cocktail of protease inhibitor (1:100), phosphatase inhibitor I (1:100), and phosphatase inhibitor II (1:100; Sigma-Aldrich). Forty micrograms of a protein sample was separated on 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. After blocking, the membrane was incubated with primary antibody, anti-rat Per2 (G-19, 1:200; Santa Cruz Biotechnology), anti-rat ERβ (1:2,000; Abcam), or anti-rat melatonin receptor 1α (MTNR1A) antibody (R-18, 1:200; Santa Cruz Biotechnology) overnight at 4°C, followed by incubation with IR Dye 800 CW anti-goat or anti-rabbit IgG (LI-COR). Targeted protein signals with fluorescence were detected using Odyssey Infrared Imaging System (LI-COR). Whole tissue protein from rat brain was used as a positive control for Per2 and MTNR1A; rat ovary tissue protein was used for ERβ. The intensities of bands were determined using densitometry, and β-actin was used as an internal control for normalization.

**Immunohistochemistry**

A small piece (0.5 cm²) of frozen mammary tissue from the left side pool of mammary glands of each rat was embedded in optimal cutting temperature compound medium and stored at −80°C. Cryosections (15 μm) were fixed in 4% paraformaldehyde for 20 minutes. Immunohistochemical staining was done with anti-rat Per2 (1:50), ERβ (1:200), or MTNR1A (1:50) antibody used in Western blot, using avidin-biotin-peroxidase complex (ABC) staining systems (Santa Cruz Biotechnology; ref. 25). PBS containing 1.5% normal serum instead of primary antibody was used as a negative control in immunohistochemistry.

**Determination of plasma melatonin levels**

Plasma was separated from blood by centrifugation and stored at −80°C. Melatonin levels were determined using Melatonin ELISA Kit (GenWay Biotech) and SpectraMax V5 microplate reader (Molecular Devices).

**Sequence analyses of promoter regions**

The sequences of the promoter regions (10,000 bp 5’ upstream, exon 1, and 2,000 bp intron 1) of MTNR1A, ERβ, Per1, Per2, Cry1, Rev-ErbAα, and DBP genes were obtained from NCBI Map Viewer. The E-box motif (CAGCTG) of Bmal1 was identified within the promoter regions of these genes. The 66 bp sequences including the motif in the promoter regions of MTNR1A in different species were analyzed using Multiple Sequence Alignment algorithm of F. Corpet (http://bioinfo.genouf.toul.fr/multalin/).

**Statistical analyses**

Intergroup differences were evaluated using multivariate ANOVA. Statistical significance of circadian rhythmicity was documented by Cosinor analysis using Time Series Single Cosinor 6.3 (Expert Soft Tech; ref. 26). Period s = 24 hours or s = 12 hours was considered a priori. The rhythm characteristics estimated by this linear least squares method include mesor (rhythm-adjusted mean) and amplitude (a half of the difference between minimum and maximum of fitted cosine function). A rhythm was detected if the null hypothesis was rejected with P < 0.05. Significance of differences in protein expression levels between methylselenocysteine-enriched versus control diet group were analyzed with Student’s t test (mean ± SE, n = 3).

**Results**

**Effects of NMU and methylselenocysteine on the expression of circadian genes**

In our previous study, we showed that a chemopreventive regimen of dietary methylselenocysteine-enriched diet significantly increased the level and rhythm of Per2 mRNA in mammary gland, but not in the liver, of NMU-treated rats. To determine if NMU was directly responsible for the ablation of circadian gene expression, we used quantitative RT-PCR to generate circadian expression profiles of circadian and CCGs in mammary gland and liver tissues of rats treated with a carcinogenic dose of NMU or the vehicle. Our results showed that NMU significantly reduced the circadian expression of Per2, Cry1, Cry2, and Rev-ErbAα genes in mammary tissues (Fig. 1A), but had only a modest effect in liver tissues (Fig. 1B; Table 1). By contrast, the circadian expression of Per1 was significantly reduced by NMU in liver, but not in mammary gland.

The present study confirmed that in NMU-treated rats, methylselenocysteine induced the rhythmic expression of Per2, with a 16.5-fold increase of rhythm-adjusted mean and a 44.5-fold increase in amplitude of Per2 mRNA expression relative to the control diet (Fig. 1A; Table 1), and a 1.5-fold increase in rhythm-adjusted mean and 2.9-fold increase in amplitude as compared with the untreated control rats (Supplementary Table S2). Western blot analysis indicated that methylselenocysteine also induced a statistically significant increase in Per2 protein expression in mammary tissues as compared with the
control diet ($P < 0.05$). Because circadian expression profiles could differ among cell types, and the ratio of cell types could vary among the tissue specimens used for the analyses, we also compared Per2 expression levels by immunohistochemical staining. Results indicated that increased expression of Per2 in mammary tissue resulted primarily from increased expression in mammary epithelial cells (Fig. 2, left).

In addition to enhancing Per2 expression in NMU-treated rats, dietary methylselenocysteine seemed to reset the
Table 1. Circadian expression of circadian and circadian-controlled genes in methylselenocysteine-enriched versus control diet groups in NMU-treated rats

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mammary gland</th>
<th>Liver</th>
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<tbody>
<tr>
<td></td>
<td>Change of rhythm*</td>
<td>Change of level (fold change)†</td>
</tr>
<tr>
<td></td>
<td><strong>Control</strong></td>
<td><strong>Methylselenocysteine</strong></td>
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<tr>
<td>Per1</td>
<td>Nc</td>
<td>P-4</td>
</tr>
<tr>
<td>Per2</td>
<td>D</td>
<td>R/P-4</td>
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<tr>
<td>Cry1</td>
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<td>D</td>
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<tr>
<td>Cry2</td>
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<td>D</td>
</tr>
<tr>
<td>Rev-ErbAa</td>
<td>D</td>
<td>R</td>
</tr>
<tr>
<td>Arntl</td>
<td>Nc</td>
<td>Nc</td>
</tr>
<tr>
<td>Clock</td>
<td>Nc</td>
<td>Nc</td>
</tr>
<tr>
<td>Npas2</td>
<td>Nc</td>
<td>Nc</td>
</tr>
<tr>
<td>MTNR1A</td>
<td>D</td>
<td>R/P+4</td>
</tr>
<tr>
<td>ERα</td>
<td>D</td>
<td>R/P+4</td>
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<tr>
<td>ERβ</td>
<td>Nr/Nc</td>
<td>I</td>
</tr>
<tr>
<td>c-Myc</td>
<td>Nr/Nc</td>
<td>I</td>
</tr>
<tr>
<td>Trp53</td>
<td>D</td>
<td>R</td>
</tr>
<tr>
<td>P21</td>
<td>D</td>
<td>R</td>
</tr>
<tr>
<td>Gadd45α</td>
<td>D</td>
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*The rhythmicity was determined by Time Series Single Cosinor 6.3 software. The significance of the circadian rhythm was tested by a zero-amplitude test (P < 0.05). The rhythm of each gene per group was compared with that of a normal control rat. Changes of rhythms were described as D, significant disruption of rhythm (P < 0.05); R, significant restoration of rhythm (P < 0.05); I, induction of rhythm; P, phase resetting with advanced (−) or delayed (+) peak; N, no significant change (Nc) or no rhythm (Nr).

†Fold change = (methylselenocysteine diet − control diet) / control diet.

‡Peak times represent the times of the maximum values on the graphs of “NMU, MSC Diet” group in the figures, with light onset as phase reference.

§Mesor indicates rhythm-adjusted mean of relative expression.

∥Amplitude indicates the half of the difference between minimum and maximum of the fitted cosine function.
phase of Per2 expression with peak levels at ZT12 (right after the lights are turned off), which was advanced 4 hours from the peak at ZT16 in normal control rats. Methylselenocysteine also increased the rhythm-adjusted mean and amplitude of Rev-ErbAα mRNA expression by 2.99-fold and 3.55-fold, respectively (Fig. 1A; Table 1), exceeding those seen in normal mammary tissues of untreated rats (2.76-fold of rhythm-adjusted mean and 2.05-fold of amplitude; Supplementary Table S2). Methylselenocysteine increased the mRNA expression level and advanced the circadian phase for Per1 expression in both mammary and liver tissues, although the effect of NMU on this gene was somehow different between mammary glands and liver. Methylselenocysteine did not show any effect on Cry1 and Cry2 in either organ. Importantly, neither NMU treatment nor methylselenocysteine-enriched diets significantly altered the mRNA expression patterns of genes encoding the heterodimeric circadian transcription factors (Arntl, Npas2, and Clock; Supplementary Fig. S1).

Fig. 2. Determination of Per2 (left), MTNR1A (middle), and ERβ (right) protein expression levels in mammary tissues of rats sacrificed at ZT12. A, Western blot results with whole tissue protein. β-Actin was used as an internal control. B, quantitative analysis of protein expression levels. Mean expression levels were calculated from densitometric intensities of bands determined with Western blot and normalized to β-actin. *, P < 0.05, significant difference. C, representative immunohistochemical staining on frozen sections of mammary tissues (×200; bar, 0.1 mm). a-c, NMU, methylselenocysteine diet; d-f, NMU, control diet; g-i, representative epithelial cells with positive staining are indicated by arrows and magnified photomicrograph (×400).
Effects of NMU and methylselenocysteine on plasma melatonin and melatonin receptor expression

To investigate the mechanisms by which NMU and methylselenocysteine alter the expression levels and phases of circadian genes, we first examined their effects on the entrainment of circadian rhythm. We compared plasma melatonin levels of control rats over a 24-hour period to those of NMU-treated rats with or without a methylselenocysteine-enriched diet. All three groups showed normal circadian patterns of plasma melatonin, with nighttime levels attaining peak concentrations (~111.7 pg/mL) at ZT20, and the minimum daytime levels (~59.9 pg/mL) occurring at ZT4 (Fig. 3A). These results excluded the possibility that either compound altered the production or stability of the hormone. Because the melatonin-mediated responses in peripheral organs are mediated through the MTNR1A (27, 28), we next asked if NMU and/or methylselenocysteine affected the expression level of MTNR1A in mammary tissue. The results showed that MTNR1A expression is normally under circadian control in rat mammary gland with peak expression level at ZT8 (Fig. 3B). A single carcinogenic dose of NMU completely disrupted MTNR1A expression in the mammary gland. By contrast, methylselenocysteine restored the levels of the MTNR1A mRNA expression to the normal levels of untreated control rats, increasing in rhythm-adjusted mean (4.67-fold) and amplitude (6.5-fold) in mammary gland (Fig. 3B) relative to those in NMU-treated rats on control diet (Table 1; Supplementary Table S2). Dietary methylselenocysteine also shifted the phase of peak MTNR1A mRNA expression by 4 hours from ZT8 to ZT12 (Fig. 3B). The methylselenocysteine-enriched diet also significantly increased expression levels of MTNR1A protein in mammary tissues of NMU-treated rats compared with the control diet (P < 0.05). Immunohistochemistry confirmed that the differences observed by Western blot were primarily due to the increased MTNR1A protein expression in mammary epithelial cells (Fig. 2, middle) in methylselenocysteine versus control diet group. By contrast, the rhythmic MTNR1A expression in normal liver was much lower, and neither NMU nor methylselenocysteine had an appreciable effect on its expression levels in liver (Fig. 3C).

Many circadian genes and CCGs are transcriptionally regulated by the binding of Bmal1/Clock heterodimeric transcription factors to the E-box binding elements in their promoter regions. To examine the possibility that the rhythmic expression of the melatonin receptor is under Bmal1/Clock-mediated transcriptional control, we analyzed the sequence of the promoter region of the rat MTNR1A gene and compared its conservation across species. DNA sequence comparisons identified two well-conserved E-box binding motifs (CACGTG) of Bmal1 in the promoter regions of human (at −2926 and −2509), mouse (at −4505 and −237), and rat (at −8949 and −7047) MTNR1A genes (Fig. 3D, a). We also found numerous copies of the E-box binding motifs of Bmal1 in the promoter regions of ERβ and various circadian genes in rat, as reported previously in mouse (Fig. 3D, b; ref. 29). These findings indicated that the MTNR1A might be a CCG, the expression of which could be entrained by exogenous signals that alter Bmal1 and Clock levels or activity.

Methylselenocysteine induced circadian rhythm of ERβ and reset the phase of ERα expression

Recent studies indicated that both MTNR1A-mediated melatonin signaling and Per2 regulate the expression levels of estrogen receptors (ER) in vitro (29–32). We therefore asked if NMU and methylselenocysteine altered the circadian expression profiles of both ERα and ERβ genes in mammary and liver tissues. We observed that the expression levels of ERβ mRNA in both tissues were normally low, with a relatively weak circadian rhythm. Nonetheless, a carcinogenic dose of NMU reduced the expression of ERβ mRNA in both tissues. More importantly, dietary methylselenocysteine dramatically induced circadian expression of ERβ mRNA in mammary gland (Fig. 4A), but not in liver (Fig. 4B). In mammary glands of NMU-treated rats, the methylselenocysteine-enriched diet induced a 9.48-fold increase in the rhythm-adjusted mean level and a 9.71-fold increase in amplitude of ERβ mRNA expression, as compared with the control diet (Table 1). Western blot results indicated that dietary methylselenocysteine only resulted in a marginal, statistically insignificant increase of ERβ protein expression relative to the control diet. However, immunohistochemical staining showed that ERβ protein expression was dramatically enriched in the mammary epithelial cells of rats on methylselenocysteine-enriched diets as compared with rats on a control diet. ERβ protein was also expressed in stromal cells of mammary glands in both groups (Fig. 2, right). Although the effect was less dramatic, methylselenocysteine also restored NMU-disrupted circadian expression of ERα mRNA in mammary tissues and shifted its peak expression to ZT12 (Fig. 4C).

Methylselenocysteine reset the circadian expression of growth-regulatory genes

Our analysis further indicated that NMU significantly reduced the circadian expression of c-Myc, Trp53, p21, and Gadd45α genes in mammmary tissues. As was the case for Per2, MTNR1A, and ERβ, dietary methylselenocysteine also reset the phase and increased the circadian expression of these growth-regulatory genes in the mammary gland, but not in the liver (Fig. 5; Table 1). The expression levels of the Trp53, p21, and Gadd45α were at their peak levels at ZT12, immediately after the light was turned off, whereas the peak expression of the c-Myc proto-oncogene was at its lowest level (Fig. 5). These results indicate that NMU disrupted, whereas a methylselenocysteine-enriched diet reset, the circadian expression of these genes involved in cellular responses to genotoxic stress and cell cycle control.

Discussion

Epidemiologic and animal studies indicate that disruption of the circadian rhythm is associated with an increased risk of breast cancer (18). In the present study, we showed that exposure of pubescent female F344 rats to a carcinogenic
Fig. 3. Effects of NMU and methylselenocysteine on the plasma melatonin concentrations and *MTNR1A* mRNA expression levels in the mammary gland and liver. A, plasma melatonin levels were measured by ELISA in plasma samples of rats in different groups as described in the legend of Fig. 1; B and C, relative mRNA expression levels were determined using quantitative real-time RT-PCR with total RNA samples from mammary gland (MG; B) and liver tissues (C) of rats as described previously; D, conservation of Bmal1 E-box elements (framed) in the promoter regions of rat *MTNR1A* and circadian genes. The E-boxes within the 10 kb of 5′ upstream, exon 1, and 2 kb of intron 1 region were analyzed. r, rat; m, mouse; h, human. a, alignment of the proximal *MTNR1A* promoter regions from rat, mouse, and human; b, sequence comparison of the E-boxes found in *MTNR1A* promoter with those in the promoter regions of the indicated circadian genes in rat.
dose of NMU significantly reduced the rhythmic expression of most core circadian genes (especially Per2 and Rev-ErbAα) and numerous CCGs, including hormone receptor genes (MTNR1A and ERs) and DNA damage-responsive and growth-regulatory genes (Trp53, p21, Gadd45α, and c-Myc) in the mammary glands. The effect of NMU on circadian gene expression was much less pronounced in the liver, which is not a major target organ in the NMU-induced rat mammary tumor model. Our preliminary results further indicated that NMU exposure has little effect on circadian gene expression in mammary glands of the resistant Copenhagen strain (data not shown). Together, these findings link NMU-induced disruption of circadian gene expression, which altered the DNA damage response and growth control pathways, to the mechanism of mammary carcinogenesis. It is interesting to note that expression of the melatonin receptor MTNR1A, which could be a CCG in mammary tissue, displayed little rhythmicity in the liver. This finding is consistent with the observation that food availability, rather than the photoperiod, is the major regulator of circadian rhythm in liver (33). This tissue-specific regulation of circadian rhythm explains how the liver is able to maintain rhythmic expression of Per2 in the absence of rhythmic MTNR1A expression. Moreover, the fact that disruption of circadian rhythm by NMU during mammary carcinogenesis might be mediated via reduced MTNR1A expression explains, in part, the tissue-specificity of NMU in this animal tumor model.

Our results also indicated that a chemopreventive regimen of methylselenocysteine counteracted the effect of NMU by enhancing and resetting the circadian expression of these genes during the early stages of carcinogenesis. To investigate how NMU and methylselenocysteine altered the regulation of the circadian rhythm, we first examined their effects on the entrainment of circadian gene expression in peripheral tissues. In mammals, entrainment is primarily mediated by light entering the eye. By modulating the number and the synchronicity of postsynaptic signals reaching the pineal gland responding to the light, the suprachiasmatic nucleus regulates the synthesis and secretion of melatonin into the circulation as a function of light/dark cycles (28). Serum melatonin is rapidly metabolized with a half-life of less than 20 minutes (27), permitting rapid adjustment of circulating levels of melatonin to the amount of light entering the eyes. Circulating melatonin binds to MTNR1A on the surface of peripheral cells, producing a long-lasting sensitization to adenyl cyclase that induces the synchronization of the circadian gene expression in peripheral cells with the central rhythm (34, 35). Normally, there is an inverse relationship between serum melatonin and melatonin receptor levels in peripheral organs, indicative of a regulatory feedback loop (36). However, we found that neither NMU nor methylselenocysteine had an effect on the circulating melatonin levels, indicating that neither compound altered melatonin secretion from the pineal gland, or its metabolism in serum. These findings suggest that the effects of NMU and methylselenocysteine were mediated at the level of melatonin signaling in peripheral cells. To test this hypothesis, we first confirmed that rhythmic expression of MTNR1A in the mammary tissues of normal rats is normally linked to the photoperiod. We next showed that a single carcinogenic dose of NMU ablated, whereas methylselenocysteine restored, the circadian expression of MTNR1A in mammary tissue. Methylselenocysteine-mediated restoration on melatonin receptor signaling might reset the rhythmic expression of circadian and CCGs in peripheral tissues, which lack an innate capacity for self-recovery of circadian rhythm. Significantly, methylselenocysteine not only restored MTNR1A levels in mammary tissues of NMU-treated rats to normal levels as seen in untreated control rats, but also induced a shift of peak expression time of the MTNR1A gene from ZT8 to ZT12 (lights off), responding ways, to the mechanism of mammary carcinogenesis. It is interesting to note that expression of the melatonin receptor MTNR1A, which could be a CCG in mammary tissue, displayed little rhythmicity in the liver. This finding is consistent with the observation that food availability, rather than the photoperiod, is the major regulator of circadian rhythm in liver (33). This tissue-specific regulation of circadian rhythm explains how the liver is able to maintain rhythmic expression of Per2 in the absence of rhythmic MTNR1A expression. Moreover, the fact that disruption of circadian rhythm by NMU during mammary carcinogenesis might be mediated via reduced MTNR1A expression explains, in part, the tissue-specificity of NMU in this animal tumor model.

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Fig. 4. Effects of NMU and methylselenocysteine on the expression of ERs in rat mammary gland and liver. Relative mRNA expression levels of ERβ (A, MG, mammary gland; B, liver) and ERα (C) were determined using quantitative real-time RT-PCR as described in the legend of Fig. 1.
to the light change. This phase shift resulted in a closer temporal alignment of peak serum melatonin levels (ZT20) with peak cellular melatonin receptor levels (ZT12). If the observed realignment of ligand concentrations and receptor expression levels results in increased MTNR1A-mediated melatonin signaling, the methylselenocysteine-induced phase shift could contribute to the enhanced expression of many core circadian genes and CCGs relative to those seen in normal control rats.

However, because our results showed that expression of MTNR1A is itself under circadian control, the effects of NMU and methylselenocysteine on MTNR1A expression could also be an indirect consequence of altered circadian rhythm. For example, methylselenocysteine could restore circadian gene expression by increasing the activity of the Clock/Bmal1 heterodimeric transcription factors. The Clock protein, which has histone acetyltransferase activity, could regulate Clock/Bmal1-dependent transcriptional activity directly by acetylating the Bmal1 protein or indirectly by acetylating histone proteins associated with promoter regions of core circadian genes (3). Other studies have shown that histone acetyltransferase activity of...
Clock protein can be counteracted by Sirt1, a NAD+-dependent histone deacetylase, thereby linking cellular energy balance to Clock/Bmal1-regulated transcription of circadian genes and CCGs (37). Moreover, the inhibition of Sirt1-associated histone deacetylase activity with splitomicin or nicotinamide increased histone 3 acetylation levels, and further increased the circadian oscillation of circadian gene expression (37). Based on these findings, we hypothesize that NMU and methylselenocysteine regulate the circadian expression of circadian genes and CCGs at the epigenetic level during carcinogenesis and chemoprevention. This hypothesis is supported by several lines of evidence. Inorganic selenium, which has antioxidant activity, alters NADP/NADPH and NAD/NADH-coupled redox cycling (21). Methylselenocysteine could therefore alter the redox state of these cofactors to inhibit the histone deacetylase activity of Sirt1 (37, 38), further enhancing the rhythmic expression of circadian genes. Consistent with this possibility, our previous studies suggested that NMU might mediate part of its carcinogenic effects through epigenetic mechanisms that alter DNA conformation (39, 40). In addition, the majority of the circadian genes deregulated in human cancers harbor increased DNA methylation and/or deacetylated histones in their promoter regions (15–17). Recent studies also showed that selenite can inhibit DNA hypermethylation and histone deacetylation in prostate cancer cells in vitro (41). More importantly, our preliminary in vitro experiments also showed that methylselenocysteine increases histone acetylation in mammary epithelial cells (data not shown).

The present study also provides insight into the mechanisms by which resetting of circadian gene expression by methylselenocysteine can contribute to chemoprevention. Previous studies showed that dietary methylselenocysteine supplements did not prevent the initiation of carcinogenesis, but suppressed the outgrowth of premalignant lesions (21). Other studies showed that ovariectomy after carcinogen exposure prevents mammary carcinogenesis in pubescent female rats, implicating estrogens in the promotion of initiated cells (42). Significantly, the core circadian protein Per2 binds to and increases the degradation of Erα protein and also indirectly regulates the transcription of Erβ (29, 31). These findings suggest that disruption of circadian gene expression could enhance, whereas restoration of circadian rhythm could inhibit, estrogen-mediated promotion of mammary carcinogenesis. Erα and Erβ have

Fig. 6. Hypothetical model for the interaction of NMU and methylselenocysteine with the circadian control network during the early stages of carcinogenesis. The proposed model combines the changes in gene expression observed in the present study with known regulating networks (blue arrows), comprising and modulating the activity of the molecular oscillator in peripheral mammalian cells (5). The rhythmic expression of the melatonin receptor (MTNR1A), itself a CCG that plays a crucial role in the entrainment and synchronization of peripheral circadian rhythm with a central pacemaker, was abolished by NMU and reset by methylselenocysteine without altering the expression of Bmal1 and Clock. These data are consistent with the possibility that NMU and methylselenocysteine regulate rhythmic expressions of circadian and CCGs by modifying the activity of Clock/Bmal1. Altered Bmal1/Clock activity would also modulate the expression of Erβ and other growth-regulatory genes. Hypothetically positive or negative inputs from NMU and methylselenocysteine are indicated by dotted red or green lines, respectively. E, estrogen; ERE, estrogen-response element; M, melatonin; CK, casein kinase; PKA, protein kinase A; CREB, cAMP-response element binding protein; HAT, histone acetyltransferase; HDAC, histone deacetylase.
opposing effects on estrogen-mediated cell growth of mammary epithelial cells, whereas overexpression of Per2 inhibited breast cancer cell growth (43, 44). Consistent with these in vitro study results, we found that methylselenocysteine dramatically enhanced circadian expression of Per2 and ERβ at mRNA and protein levels in mammary epithelial cells in vivo. Although we observed a less dramatic effect of methylselenocysteine on ERα, the large increase in ERβ levels would elevate the ERα/ERβ ratio in mammary cells. It has been shown that when coexpressed in breast cancer cells, ERβ antagonizes the expression of ERα-mediated expression of growth-regulatory genes, including c-Myc and cyclin D1. Our in vivo studies also found that methylselenocysteine-enriched diets inhibited the expression of c-Myc in mammary tissues in vivo, with the lowest level of c-Myc expression occurring at ZT12, whereas Per2 and ERβ expression levels were at their highest levels. Taken together, these findings suggest that enhanced circadian expression of ERβ protein might suppress the promotion phase of mammary carcinogenesis by inhibiting estrogen-induced cell growth.

Another mechanism by which methylselenocysteine-mediated effects on circadian rhythm could contribute to the suppression of mammary carcinogenesis is through altered responses to genotoxic stressors (45, 46). Disrupting normal circadian rhythm through shift work, exposure to chemical carcinogens, or genetic manipulation would therefore be expected to negatively affect the ability of cells to respond to and repair DNA damage. Consistent with such a mechanism, our data showed that disruption of circadian rhythm by NMU inhibited the rhythmic expression of DNA damage-responsive and growth-regulatory genes (Trp53, p21, Gadd45α, and c-Myc). By contrast, a dietary regimen of methylselenocysteine reset and enhanced the rhythmic expression of core circadian genes, Per2 and Rev-Erbαα, and CCGs, including these DNA damage-responsive genes. Interestingly, the peak expression levels of the Trp53, p21, and Gadd45α genes corresponded temporally with the lowest level of c-Myc gene expression, suggesting that enhanced circadian gene expression by methylselenocysteine also plays a role in coordinating cellular growth with DNA damage responses.

Together, our findings suggest that disruption and resetting of circadian rhythm by NMU and chemopreventive methylselenocysteine, respectively, are linked to the mechanisms of mammary tumor promotion (Fig. 6). Disruption of circadian rhythm by shift work, exposure to light at night, and blindness has also been associated with increased rates of breast, prostate, and colon cancers (18). Our findings thus have potential implications for mitigating the increased risk of breast and prostate cancers associated with shift work (47). Numerous studies have suggested that normal dietary levels of methylselenocysteine could reduce the risk of several cancers without excess tissue accumulation or toxicity (19). Methylselenocysteine is a naturally occurring amino acid synthesized in plants grown in selenium-containing soil. The highest levels are found in Brazil nuts (up to 200 μg each), garlic, wild leeks, onions, and broccoli. Lower levels are found in some meats and seafood. Our chemoprevention studies in rats indicated that dietary methylselenocysteine at 3 ppm of selenium for up to 8 months yielded no apparent toxicity (23). This level of dietary supplementation with methylselenocysteine corresponds to a daily dose of ~200 to 300 μg in humans, which is well below the recommended maximal daily intake level of selenium (400 μg/d) established by the Institute of Medicine (48). Significant toxicity in humans is typically seen only after prolonged intake exceeding 800 μg/d (49). The average dietary intake of selenium in Americans and Europeans is ~100 μg daily, with many populations around the world routinely ingesting 400 to 600 μg of selenium daily without apparent adverse effects (50). Multiple clinical trials with 200 and 400 μg/d of inorganic selenium for months to years have shown no toxicity (51, 52). Another study reported no obvious toxicity with doses from 1,600 to 3,200 μg/d given for 12 months (53). We therefore expect that a dietary supplement of ~200 μg of methylselenocysteine per day, a level currently found in many multivitamin preparations, would be effective in resetting circadian rhythm in humans. This prediction is currently being tested in shift workers in preparation for a prospective intervention trial to evaluate the efficacy of methylselenocysteine in mitigating the increased risk of cancer associated with the disruption of circadian rhythm by shift work.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Michael Gallo for helpful discussion and Jennifer Williams for critical reading of the manuscript.

Grant Support

NIH grants U19ES011387, P30ES05022, and P30ES007033; funds from the Environmental and Occupation Health Sciences Institute, a joint institute of the University of Medicine and Dentistry of New Jersey and Rutgers, the State University of New Jersey.

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Received 08/16/2009; revised 02/02/2010; accepted 02/15/2010; published OnlineFirst 04/27/2010.

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Ming Zhu Fang, Xun Zhang and Helmut Zarbl


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