Spermidine resets circadian clock phase in NIH3T3 cells

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ABSTRACT

Irregular light-dark cycles desynchronize the circadian clock via hormonal and neuronal pathways and increase the risk of various diseases. This study demonstrated that a single pulse of spermidine—a polyamine—strongly induced circadian phase advances in the presence or absence of dexamethasone (a synthetic glucocorticoid) in NIH3T3 cells transfected with the Bmal1 promoter-driven luciferase reporter gene. The spermidine-induced phase advances were 2–3 fold greater than were the dexamethasone-induced shifts. The phase resetting effect of spermidine occurred in a dose- and time-dependent manner and was not blocked by RU486, an antagonist of glucocorticoid receptors. Spermidine treatment modulated the expression of clock genes within 60 min, which was sooner than changes in the expression of autophagy-related genes. These findings suggested that spermidine is a potent modulator of the circadian phase, acting through glucocorticoid receptor-independent pathways, and may be useful for treating diseases related to circadian desynchrony.
corticoids also activate the transcription factor HNF4alpha, which targets downstream rhythmic genes (Reddy et al. 2007; Cheon et al. 2013). Because both initiation and cessation of glucocorticoid signaling occur with $t_{1/2} \sim 5$–10 min (Freeman and Yamamoto 2001), a 15 min dexamethasone stimulation is sufficient to reset the circadian clock in fibroblasts (Nagoshi et al. 2004).

Polymamines such as putrescine, spermidine, and spermine are ubiquitous polycationic mediators of cell growth and apoptosis. In most mouse tissues, levels of spermidine are higher than those of other polymamines, with higher levels in the pancreas and thymus than other tissues, while spermine is the most highly concentrated polyamine in the kidney and heart (Nishimura et al. 2006). Polymamines have modulatory roles in glucocorticoid receptor activation and circadian clock. For example, they are implicated in glucocorticoid-induced apoptosis (Bjelaković et al. 2010), and polyamine-modulated factor 1—a cofactor of transcriptional regulation of polyamine-metabolic enzymes—represses glucocorticoid receptor activity (Shoji et al. 2007). In addition, spermidine is a possible mediator of the glucocorticoid effect on milk protein synthesis in the mouse mammary epithelium in vitro (Oka and Perry 1974). Polyamine synthesis in the liver is directly regulated by the circadian clock; in turn, dietary supplementation with spermidine reversed age-dependent changes in the circadian period in mice (Zwighaft et al. 2015). The same study demonstrated that spermidine promotes the interaction between PER2 and CRY1 proteins in NIH3T3 cells, and the effect was stronger than that observed with putrescine or spermine (Zwighaft et al. 2015). Autophagy, a highly conserved cellular catabolic and energy-producing process for bulk degradation, may be one of the pathways linking polyamines and the circadian clock. Spermidine is an inducer of autophagy (Pietrocola et al. 2015) that is rhythmically activated in a clock-dependent manner (Ma et al. 2012).

Chrononutritional approaches have succeeded in identifying nutrients that modulate the circadian period and clock gene expression in animal and cellular models (Oike 2017). Most studies in cellular models have focused on period modulation during chronic treatment, and several studies have examined the phase shift after transient stimulation (Narishige et al. 2014; Kim et al. 2016). However, the effect of the combination of nutrients with circadian signaling hormones, such as glucocorticoids, has not been addressed. As circadian disruption in organisms occurs through irregular light-dark or feeding-fasting cycles that are conveyed to cells via signaling hormones, effects of the combination are important to understand functional food factors that target circadian disruption. Thus, the current study focused on polyamines as candidates that modulate dexamethasone-induced circadian phase shift in cellular models. We further analyzed acute changes in the expression of clock genes, autophagy-related genes, and a gene involved in spermidine synthesis after spermidine stimulation to assess the mechanisms underlying the modulatory effect on the cellular clock. We used NIH3T3 fibroblast cells with the Bmal1 promoter-driven luciferase reporter gene (Bmal1-Luc) as a cellular model, which enabled real-time monitoring of the cellular clock.

NIH3T3 cells stably transfected with the Bmal1-Luc reporter vector were cultured at a density of $5 \times 10^5$ cells per well in a growth medium consisting of Dulbecco’s modified Eagle’s medium (DMEM; Wako, Osaka, Japan) with 4,500 mg/L high glucose, 10% fetal bovine serum (FBS; Corning, NY, USA), and 100 U/mL penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified 5% CO$_2$ incubator. For the real-time monitoring of Bmal1 promoter activity and to evaluate the rhythmicity of clock gene expression, the growth medium was replaced with a synchronization medium consisting of DMEM, 1% penicillin-streptomycin, and 100 nM dexamethasone (Sigma-Aldrich) and incubated for 2 h as an initial synchronization. The bioluminescence in the cells was monitored using a dish-type luminometer (AB2550 Kronos; ATTO, Tokyo, Japan) with a recording medium consisting of DMEM, 0.1 mM D-luciferin potassium salt (Nacalai Tesque, Kyoto, Japan), 10% FBS, and 100 U/mL penicillin-streptomycin at 37°C in a humidified 5% CO$_2$ incubator. Bioluminescence was monitored for 1 min every 9 min.

For transient stimulation with each substance (single 15 min pulse), the NIH3T3-Bmal1-Luc cells were incubated in DMEM medium consisting of 100 U/mL penicillin-streptomycin and 100 μM of spermine or spermidine (Sigma-Aldrich), in the presence or absence of 100 nM dexamethasone. For dexamethasone stimulation, 0.001% ethanol was used as the control stimulation, while water was used as the control stimulation for spermine and spermidine. For spermidine treatment, the effects of other doses (10, 50, 100, and 500 μM) or co-incubation with 100 nM RU486 (Sigma-Aldrich), an antagonist of glucocorticoid receptor, were also analyzed. After 15 min of pulse stimulation, the cells were washed with phosphate-buffered saline (PBS, Wako)
and returned to the recording medium after each treatment, because changing the fresh culture medium may have an effect on the circadian rhythm of the cells. The stimulation was started 40 h after the initial synchronization (hours post synchronization, hps), in which the phase advance of the rhythm is expected (Nagoshi et al. 2004). For spermidine treatment, the stimulation was also given 52 hps to monitor the phase delay of the rhythm. For the chronic treatment with spermine or spermidine, the cells were incubated in DMEM containing 100 U/mL penicillin-streptomycin, 100 μM spermine or spermidine, and 0.1 mM D-luciferin potassium salt. The treatment was started immediately after initial synchronization.

To analyze the acute effect of spermidine on the expression of clock genes, autophagy-related genes, and a gene involved in spermidine synthesis, the cells were incubated in DMEM containing 100 U/mL penicillin-streptomycin, 100 μM spermine or spermidine, and 0.1 mM D-luciferin potassium salt. The treatment was started immediately after initial synchronization.

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Spermidine resets the cellular clock phase in NIH3T3 cells. (A–C) Bmal1-Luc bioluminescence rhythms in NIH3T3 cells that were stimulated with 15 min pulse of solvent (0.0001% ethanol, Control), dexamethasone (Dex, 100 nM) alone, spermidine (100 μM) alone, or dexamethasone with spermine or spermidine (both 100 μM). Triangles indicate the time of pulsing, 40 hps (A and B), or 52 hps (C). Dotted lines indicate the peak times in the first and second cycles (peak 1 and peak 2, respectively) after stimulation. Phase shifts in Peak 1 and Peak 2 were separately calculated by the differences in peak times between treatment and control and are shown in the graphs below. Values are presented as mean ± SEM (Bonferroni’s multiple comparison test, *P < 0.05, **P < 0.01, ***P < 0.001). (D) Effect of different doses of spermidine (15 min pulse at 40 hps) on phase shifts (Bonferroni’s multiple comparison test, ***P < 0.001). Phase shifts were evaluated by peak times in the first cycle after stimulation. (E) Effects of RU486 on phase shifts induced by dexamethasone or spermidine (Bonferroni’s multiple comparison test, *P < 0.05). (F) Effect of chronic treatment with solvent (0.0001% ethanol, control), spermine (100 μM), or spermidine (100 μM) on Bmal1-Luc bioluminescent rhythms.
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12 h period at the beginning, and then the rhythms were eliminated (Fig. 1F). Chronic treatment with spermidine resulted in a loss of bioluminescent rhythms, and we confirmed that the cells had died at 12 hps (Fig. 1F).

After a single pulse of spermidine, the expression of Per1 was upregulated at 60 and 120 min after stimulation ($P = 0.0202$ and $0.0003$, respectively), while expression of Per2 and Rev-erbα was downregulated (Per2: 60 min, $P = 0.0348$; Rev-erbα: 60 min, $P < 0.0001$; 120 min, $P = 0.015$) (Fig. 2A–C). Spermidine increased the expression of Binp3, but not LC3b, at 120 min after stimulation ($P = 0.0012$) (Fig. 2D, E). The expression of Amd1, a gene involved in spermidine synthesis, was downregulated by spermidine at 60 and 120 min after stimulation ($P = 0.0161$ and $0.0083$, respectively) (Fig. 2F).

Our data demonstrated that a single pulse of spermidine can reset the circadian phases of Bmal1-Luc bioluminescent rhythms of NIH3T3 cells in the presence or absence of dexamethasone. The spermidine-induced phase advances were 2–3 fold greater than dexamethasone-induced shifts, suggesting that spermidine is a potent modulator of the cellular circadian clock phase. Conversely, spermidine-induced phase delay was transient, and the presence of spermidine did not disturb dexamethasone-induced phase delay. These data suggested that the phase resetting effect of spermidine depends on the circadian time of pulsing. The time-dependency of the phase shift response was also observed in dexamethasone-treated NIH3T3 cells (Nagoshi et al. 2004) and insulin-treated rat hepatocytes (Yamajuku et al. 2012). The relationship between pulse timing and phase shift responses (i.e., phase advance or delay) depends on the nature of the treatment and the cells. A strong effect on phase advances and a modest effect on phase delays suggest a limited time window for spermidine action on the circadian clock in NIH3T3 cells. Because polyamines are implicated in glucocorticoid-induced apoptosis (Bjelaković et al. 2010) and polyamine-modulated factor 1 represses glucocorticoid receptor activity (Shoji et al. 2007), we speculate that spermidine resets the circadian clock phase via modulation of glucocorticoid receptor activation. However, to our surprise, spermidine-induced phase shifts were not blocked by RU486, a glucocorticoid receptor antagonist. These data suggested that spermidine reset the cellular circadian clock phase in a glucocorticoid receptor-independent manner.
Polyamines participate in the regulation of various key cellular processes, including gene transcription/translation and modulation of protein-protein and protein-DNA interactions. A previous study reported that intracellular polyamine levels affect circadian periods in NIH3T3 cells, and spermidine supplementation in polyamine-depleted cells could restore this period (Zwighaft et al. 2015). Therefore, we speculated that a single pulse of spermidine rapidly altered clock gene expression to induce a strong phase shift. Indeed, spermidine treatment induced Per1 expression and decreased Per2 and Rev-erba expression within 60 min. As REV-ERBα is a repressor of Bmal1 transcription (Pretitner et al. 2002), the downregulated Rev-erba expression is consistent with our Bmal1-Luc bioluminescent data that showed earlier increases in Bmal1 promoter activity after spermidine treatment. To address the mechanisms underlying spermidine-induced regulation of clock genes, we analyzed the expression of genes involved in autophagy regulation, which is one of the physiological roles of spermidine (Pietrocola et al. 2015). However, spermidine-induced changes in Bnip3 expression were delayed by 120 min after stimulation. The time lag between the expression of clock genes and autophagy genes suggested that the transcription of clock genes is regulated by other pathways. A recent study suggested that intracellular polyamines regulate the translation of BMAL1 and REV-ERBα proteins by enhancing ribosomal shunting (Sakamoto et al. 2021). Another study showed that spermidine promotes PER2:CRY1 interaction, possibly by inducing a conformational change in PER2 (Zwighaft et al. 2015). It has also been reported that spermidine inhibits acetyltransferase EP300 (Pietrocola et al. 2015), which regulates gene transcription via chromatin remodeling. These mechanisms may be involved in clock gene expression after exogenous spermidine treatment.

A previous study described that chronic supplementation of NIH3T3 cells with spermidine did not significantly alter intracellular polyamine levels or the circadian period (Zwighaft et al. 2015). Although we did not measure intracellular spermidine levels, a single pulse of spermidine may have modified intracellular levels, as suggested by the strong suppression of Amd1, a gene involved in spermidine synthesis. The enzymatic activity of AMD1 is inversely related to the intracellular polyamine content, and spermidine reduces serum-induced increases in Amd1 mRNA levels (Shantz et al. 1992). These might be associated with the tightly regulated intracellular polyamine levels by de novo synthesis/catabolism and inter-conversion (Casero and Marton 2007).

In summary, this study demonstrated that spermidine resets the circadian clock phase in a dose- and time-dependent manner in NIH3T3 cells. The mediating pathway is likely to be independent from the glucocorticoid receptor-activated pathways or spermidine-induced autophagy pathways. Alteration of clock gene expression after spermidine treatment suggests the involvement of transcriptional regulation of molecular clockwork through unknown mechanisms, which needs to be investigated in future studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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