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D-Alanine, a Circadian Metabolite that Regulates Glucose Metabolism and Viral Infection

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D-Alanine, a rare D-amino acid, exhibits a clear circadian rhythm and is present in organs associated with glucose metabolism. Recent findings have revealed that D-alanine acts on the circadian rhythm, thereby regulating physiological processes related to circadian cycles that are essential for maintaining body homeostasis. The regulation of circadian rhythm by D-alanine is vital for correcting blood glucose levels in diabetic conditions. In viral infections, D-alanine serves as a sensitive biomarker that reflects the severity of the infection, as its level drastically decreases due to consumption. Supplementation with D-alanine is effective to alleviate the progression of viral infections, potentially through the maintenance of the circadian rhythm and its associated immune responses. In addition to its role as a circadian biomarker, D-alanine also functions as a circadian regulator and exerts a wide range of physiological effects. This review summarizes the physiological roles of D-alanine as a circadian metabolite.

1. Introduction

D-Alanine is a rare enantiomer (chiral body) of the more abundant L-alanine. The concentration of D-alanine in blood and urine exhibits a distinct circadian rhythm.[1,2] Additionally, D-alanine has also been detected in tissues associated with glucose metabolism.[1,3–6] Despite these findings, the function of D-alanine remains largely unknown.[7]

Recent studies have uncovered the key physiological functions of D-alanine in the body, including its role in the regulation of circadian rhythm.[2] Circadian rhythm has a link with a wide range of physiological processes,[8,9] and D-alanine has recently been identified as a significant contributor to these functions (Figure 1). This newly discovered function of D-alanine represents a new facet in the growing body of knowledge about D-amino acids.[10,11]

This review summarizes the recent evidence whereby D-alanine is a circadian metabolite that regulates the circadian rhythm.

2. Circadian Rhythm

Circadian rhythm is a natural 24-hour oscillation in our body which affects a variety of biological processes.[8] In the core machinery of circadian rhythm, a subset of clock genes beats the rhythm through a transcriptional feedback loop (Figure 1). In a simple model of this machinery, BMAL1 and CLOCK proteins form a heterodimer that binds to the promoter regions of the target genes. The target genes include those encoding PER and CRY proteins, which suppress the transcription of *BMAL1 and CLOCK* genes. Other targets of circadian transcription factors include genes encoding for a broad range of physiological functions, such as blood pressure and metabolism.

Circadian transcription factors regulate nearly half of all expressed genes and are responsible for the rhythmic physiological functions.[12] Consequently, the circadian transcriptional network is connected with a wide range of physiological processes. Nevertheless, this model is overly simplistic, and a significant number of circadian clock components remain unidentified.[8]

3. D-Alanine Serves as a Biomarker for the Circadian Rhythm

The initial association between D-alanine and circadian rhythm is its daily rhythm. This phenomenon has been demonstrated in experimental rodents raised under conventional light and dark (LD) cycle conditions.[1,2,13] The levels of D-alanine exhibit a distinct circadian rhythm in blood,[1,2] tissues such as pituitary gland and pancreas,[1] as well as urine.[2] Specifically, D-alanine levels are high when rodents are asleep in the light, and low when they are awake in the dark.[1,2] This rhythm of D-alanine, characterized by a pronounced sinusoidal variation, is notable among known metabolites. In contrast, melatonin, a well-known circadian metabolite, displays a circadian rhythm characterized by on-off patterns.[14] The circadian rhythms of hormones, including insulin and corticosterone, are significantly influenced by nutrient availability and hormonal interactions. Conversely, the circadian rhythm of D-alanine is distinctly observable, allowing the identification of daily peaks and troughs with relative ease.[1,2]

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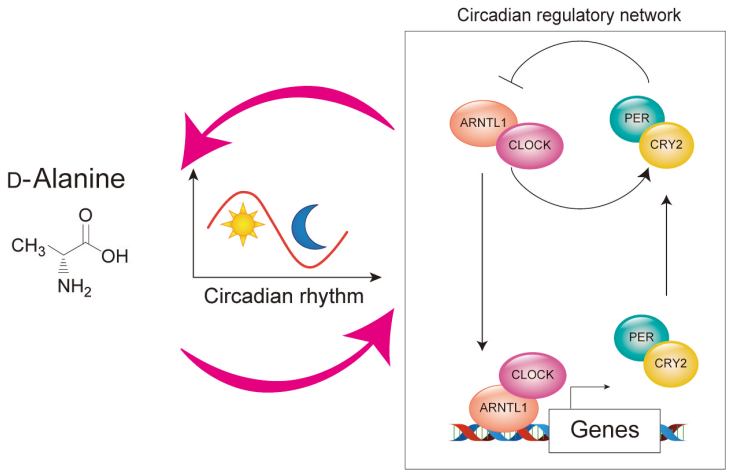

Figure 1. D-Alanine as a circadian metabolite. D-Alanine has a clear circadian rhythm and acts on the core circadian regulatory machinery. BMAL1 and CLOCK proteins form a heterodimer and bind the promoter regions of target genes. The target genes include those encoding the PER and CRY proteins, which suppress the transcription of *BMAL1 and CLOCK* genes in a negative feedback loop. This machinery controls the circadian rhythm of D-alanine.

The regulation of the D-alanine's circadian rhythm is influenced by daily activity, sleep patterns and kidney function. When the timing of activity is altered by restricting feeding to daytime hours, the circadian rhythm of D-alanine is completely inverted.[1] Sleep reduces the urinary excretion of D-alanine; consequently, sleep disturbances can lead to increased urinary excretion of D-alanine.[2] Therefore, sleep disturbances result in the excessive urinary excretion of D-alanine, thereby preventing the restoration of elevated blood levels.[2] Furthermore, D-Amino acid oxidase (DAO) may not play a major regulatory role in the circadian rhythm of D-alanine, as evidenced by the presence of this rhythm in mice lacking Dao activity.[13]

The relationship between circadian rhythm and D-alanine is particularly distinct, since the blood levels of L-alanine and other D- and L-amino acids examined thus far do not exhibit an evident circadian rhythm.[1,2] The pronounced circadian rhythm of D-alanine provides a new insight into the regulatory mechanisms governing circadian rhythms.

4. Dynamics and Distribution of D-Alanine

Where does D-alanine in the body come from? D-Alanine is not synthesized in mammalian cells. Therefore, the source of D-alanine is primarily exogenous in human. D-Alanine is abundant in fermented foods and fish from brackish water.[15–18] D-Alanine possesses a flavor profile that is perceived as sweeter compared to L-alanine.[19] Additionally, the gut microbiota plays a significant role in regulating the levels of D-alanine in blood.[13,20–23] To investigate the contribution of gut microbiota to D-alanine status, germ-free mice, which lack intestinal microbiota, were fed a rigorously controlled diet in which over 99% of amino acid was present as the L-isomer.[22] Analysis of blood samples from these germ-free mice revealed undetectable level of D-alanine. Therefore, it was considered that D-alanine in the body originates from the intestines, either contained in diet or produced by microbiota.

While it is challenging to distinguish whether dietary sources or gut microbiota contribute more to the D-alanine status, it is conceivable that the presence of either is sufficient to maintain physiological levels of D-alanine. An increase in dietary D-alanine elevates its blood level.[18,24] Blood D-alanine

Tomonori Kimura is dedicated to addressing medical challenges from a reverse translational perspective, conducting his research in the life sciences unbound by conventional paradigm. Particularly noteworthy is his groundbreaking work focusing on D-amino acids – scarcely found in living organisms – has far-reaching implications for our understanding of various pathologies. From kidney diseases, infectious diseases, neurological disorders, and rare diseases, his research sheds light on the mechanisms of disease across the medical spectrum, offering a novel lens through which to explore the complexity of life. This pioneering approach holds the potential to revolutionize medical diagnostics, while simultaneously heralding for innovative therapeutic interventions.

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level may increase by using bacterial species modified to increase D-alanine production or isolated species rich in D-alanine.[23,25] Conversely, dietary changes that reduce D-alanine intake or disruptions to the gut microbiota, such as those caused by infections,[26] can impact D-alanine blood levels.

D-Alanine of either origins is rapidly absorbed from the digestive tract like L-alanine,[24] and subsequently enters the bloodstream[24] through transporters.[27] Although the complete picture of D-amino acid transporters remains largely unknown, they are presumed to exhibit a spectrum of characteristics, from high chiral specificity to broad recognition of various amino acids. Intestinal D-alanine transporters demonstrate high efficiency presumably because of the comparable absorbance of L- and D-alanine.[24] While some transporters for D-serine have been identified,[11,28–31] little is known about those specific for D-alanine.[24]

D-Alanine is then delivered to tissues, mainly glucose metabolism-related tissues, such as pituitary gland and pancreas.[3,5,6,21,32] D-Alanine in the blood is also delivered to the kidney, where about 30% is excreted in the urine. This excretion rate is noteworthy considering that kidney receives 20% of the cardiac output. The rest of 70% of D-alanine is reabsorbed in the kidney through chiral-selective transporters;[11,31,33–37] a portion of this is metabolized in the kidney,[2] while the remainder is returned to the bloodstream.

Consequently, D-alanine is rapidly cleared from the blood. The distribution volume of D-alanine exceeds that of the blood volume; however, it is significantly less than the total body weight.[24] Despite its limited distribution, D-alanine appears to be sequestered in specific pools within the body.[24]

5. D-Alanine Activates Transcription Factors Involved in the Regulation of Circadian Rhythm

Does D-alanine delivered to tissues have functions? This question was pursued in the kidney, where a significant uptake of D-alanine occurs.[2] Following treatment, D-alanine rapidly induces the expression of genes associated with diverse array of biological processes, including development, metabolism, immunology, cancer, infection, cellular proliferation, and circadian rhythms. A deep learning analysis has identified the key genes that play central roles in the observed expression profile. Notably, these include gluconeogenic genes, *G6PC3*, and the circadian transcription factor, *CRY2*.

Since CRY2 maintains the core machinery of the circadian rhythm,[8] it was hypothesized that D-alanine could influence circadian responses. This hypothesis was tested by treating D-alanine to mice maintained in constant darkness (DD).[2] Under DD conditions, the circadian cycle shifts forward by 10 minutes a day. Treatment of D-alanine prevented this shift and normalized the circadian rhythm in DD. In addition to its role in transcriptional regulation, D-alanine may modulate circadian rhythms as a neurotransmitter.[38] D-Alanine possesses the ability to interact with the core components of circadian signaling, thereby sustaining circadian rhythms under abnormal conditions.

6. Modes of D-Alanine's Action on Systemic Circadian Rhythm and Metabolism

This section summarizes how D-alanine transmits signals of systemic circadian rhythm and metabolism (Figure 2). The circadian rhythm is primarily regulated by the suprachiasmatic nucleus in the brain.[8] This central clock coordinates with peripheral clocks present in various organs.

D-Alanine exerts its effects on both central and peripheral circadian rhythms. Evidence of D-alanine on the central rhythm includes behavioral modification, as aforementioned in mice maintained in DD.[2] Without light cues, mice exhibit a shorter than 24 hour rhythm, but administering D-alanine in drinking water prevents this shortening, indicating that D-alanine influences the brain's rhythm. It is also plausible that this brain rhythm interacts with the internal pool of D-alanine, controlling its release according to circadian rhythms.

D-Alanine also acts on peripheral organs, inducing physiological processes related to the circadian rhythms. In the kidney, D-alanine directly stimulates gluconeogenesis.[2] This effect has been observed in *ex vivo* cultured kidney cells, indicating a direct action of D-alanine on the kidney. This action exhibits a circadian rhythm, with increased sensitivity to D-alanine during periods of low blood D-alanine concentration. Additionally, D-alanine administration in mice increases the expression of

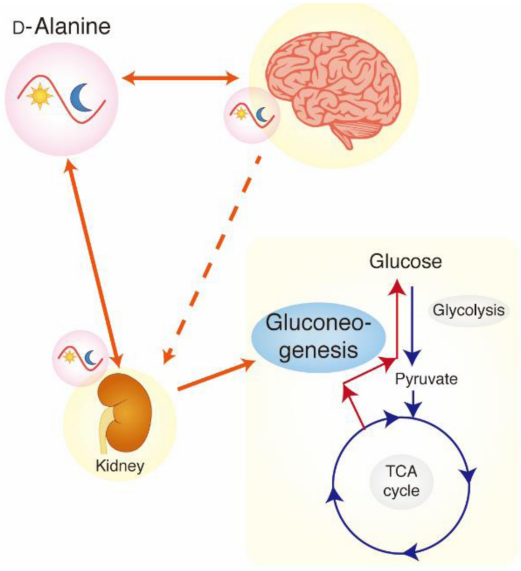

Figure 2. Modes of D-alanine's action on systemic circadian rhythm. D-Alanine acts on the brain to modulate circadian rhythms, conveying these rhythms to peripheral organs and inducing behavioral changes. Notably, D-alanine exhibits its own circadian rhythm within the hypothalamus. In the kidney, D-alanine directly stimulates gluconeogenesis, a circadian-related metabolic pathway that generates glucose from intermediates of the tricarboxylic acid (TCA) cycle. Conversely, both the brain and kidney contribute to the regulation of D-alanine's intrinsic rhythm.

circadian rhythm-related genes in the kidney, leading to the induction of genes involved in gluconeogenesis. It is important to note that the brain's circadian rhythm influences overall metabolism, likely impacting kidney gluconeogenesis as well.

Thus, D-alanine impacts circadian rhythms in both central and peripheral organs, though the exact nature of these interactions remains unclear. For instance, while D-alanine's influence on behavioral rhythms strongly suggests a central action, the possibility of indirect effects through its impact on energy homeostasis cannot be disregarded. As observed in high-fat diet models, systemic metabolism can influence behavioral patterns.[26] Similarly, alterations in the metabolism of target organs, such as the kidney, likely feedback to the central clock. Nevertheless, the presence of rhythmic D-alanine fluctuations in the hypothalamus and its influence on kidney metabolism via circadian-related genes strongly suggest both central and peripheral actions, warranting further investigation.

Moreover, blood D-alanine level is regulated by both the brain and kidney. The brain, as the master regulator of circadian rhythms, is presumed to play a crucial role in shaping the circadian rhythm of blood D-alanine levels. The presence of a circadian rhythm in D-alanine levels within the hypothalamus supports this concept. Although the body pool of D-alanine is small, a potential centrally-mediated release of D-alanine in accordance with circadian rhythms. On the other hand, the kidney contributes to D-alanine's circadian rhythm by regulating its urinary excretion. Notably, while D-alanine levels in mice typically rise during sleep, sleep deprivation impairs urinary D-alanine excretion, leading to decreased blood D-alanine levels.

The intricate interplay between D-alanine and the circadian rhythms of the brain and peripheral organs involves numerous direct and indirect regulatory mechanisms, making it challenging to fully elucidate this relationship. However, this complexity underscores the close association and interdependence between D-alanine and the circadian system.

Both D-serine and D-alanine act as co-agonists at the *N*-methyl-D-aspartate receptor (NMDAR) and influence its signaling pathways, with D-alanine presumed more potent than D-serine.[38] While the phenomenon of circadian rhythm is associated with NMDAR-related signaling, the specific functions of D-alanine compared to D-serine remain unknown. In mice administered with D-serine and D-alanine, D-alanine induced significant circadian rhythmicity in the kidney,[2] whereas no such effect was observed with D-serine.[39] Thus, it is plausible that the effect of D-alanine on circadian rhythm, at least in part, occur independently of NMDAR signaling.

7. D-Alanine Activates Gluconeogenesis With Chiral-Selectivity

D-Alanine also stimulates the gluconeogenic pathway.[2] As previously noted, a deep learning method also identified gluconeogenetic genes as central genes in signals from D-alanine. Gluconeogenesis is a metabolic process through which glucose is synthesized from various metabolites, including tricarboxylic acid (TCA) cycle intermediates and amino acids.[40,41] This process occurs exclusively in the kidney and liver, both of which play crucial roles in glucose production.

The activity of gluconeogenesis was assessed *in vivo* or through *ex vivo* culture of tissues, since the immortalized cell lines lack the complete enzymatic machinery for gluconeogenesis.[2] *ex vivo* analyses confirmed the gluconeogenetic activity of D-alanine in kidney. In the kidney, D-alanine enhances the expression of *G6PC* gene and activates G6PC, thereby facilitating gluconeogenesis.

Interestingly, alanine has a chiral-selectivity on the activation of gluconeogenesis in tissues (Figure 3). D-Alanine preferentially promotes gluconeogenesis in the kidney, while L-alanine exerts its strong effects in the liver. As discussed in the following section, this difference likely arises from downstream signaling events rather than differences in their interactions with DAO. This clear chiral specificity exemplifies the body's ability to recognize and differentially respond to chirality.

8. Metabolism of D-Alanine

D-Amino acids are recognized as substrate for oxidizaion by DAO,[42] an enzyme that predominantly exists in the kidney.[43] Utilizing a purified enzyme and an *ex vivo* culture system, the product of this enzymatic reaction was identified as pyruvate.[2] The formation of pyruvate from D-alanine is dependent on DAO, as pyruvate is not produced from D-alanine in the kidney of *Dao*-deficient mice.[2]

Pyruvate lies on an important metabolic crossroad. Pyruvate is a product of glycolysis and a substrate for both TCA cycle and gluconeogenesis. D-Alanine can stimulate gluconeogenesis through two modes of actions: the upregulation of gluconeogenic signals and its role as a substrate.[2] Between two mechanisms, the former is important for several reasons. The concentration of D-alanine in the body is trace and insufficient to elevate blood glucose levels, even if all D-alanine is converted

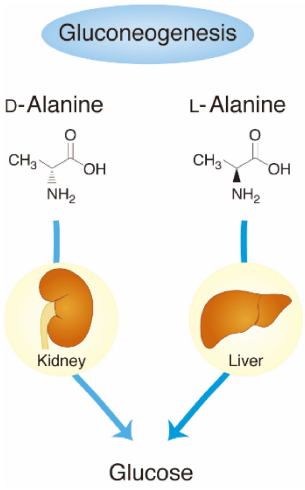

Figure 3. Chiral-preference of alanine on gluconeogenesis in tissues. D-Alanine preferentially promotes gluconeogenesis in the kidney, while L-alanine exerts its strong effects in the liver.

to glucose. However, even a small quantity of D-alanine is sufficient to activate gluconeogenesis in the kidney. Additionally, D-alanine can promote gluconeogenesis under conditions of *DAO*-deficient. Thus, D-alanine primarily functions as a mediator of gluconeogenesis.

9. D-Alanine Activates Gluconeogenesis Through Circadian Transcriptional Regulation

Then, how does D-alanine mediate the signals on gluconeogenesis? Given its strong association between gluconeogenesis and D-alanine, its role of circadian signals has been examined.[2] Gluconeogenesis exhibits a distinct circadian rhythm, while the effects of D-alanine on kidney gluconeogenesis also has a daily rhythm. Kidney is more sensitive to D-alanine when the blood level of D-alanine level is low during the night.

The role of the circadian transcriptional system was examined under these conditions.[2] D-Alanine induces the enrichment of ARNTL at the region of G6PC3 promoter when gluconeogenesis is active. D-Alanine activates gluconeogenesis through circadian transcriptional regulation (Figure 4).

10. D-Alanine in Glucose Metabolism

Gluconeogenesis is a metabolic pathway that maintains blood glucose levels during fasting. In glucose metabolism, gluconeogenesis shares its importance with other hormones like insulin. Consequently, the effects of D-alanine on glucose metabolism in body were investigated.

Treatment with D-alanine elevates both blood glucose and insulin levels.[2] This pattern necessitates distinguishing between two possibilities: whether gluconeogenesis elevation by D-alanine leads to a reactive increase in insulin, or if D-alanine induces systemic insulin resistance. To discern between these possibilities, an insulin clamp test was conducted, confirming that insulin sensitivity was maintained.[2] The observed increase in insulin levels following D-alanine administration, therefore, is attributed to a glucose-stimulated response in the absence of impaired glucose tolerance.

Nevertheless, there is a possibility that the elevation in insulin secretion following systemic D-alanine administration be a partial direct influence. If D-alanine solely stimulated insulin release, a decrease in blood glucose levels would be expected. However, previous studies have reported the presence of D-alanine in the mouse pancreas, prompting investigations into its potential role in insulin secretion.[6,32] There is no consensus on this yet. Classically, D-alanine was considered not to affect calcium potential of pancreatic beta cells, a surrogate for insulin secretion,[53,54,55] yet recent studies suggest D-alanine might modulate calcium potentials,[32] emphasizing the need for further investigation.

Based on the current evidence, the observed increase in systemic blood glucose and the enhanced gluconeogenic capacity observed *ex vivo* suggest that D-alanine's primary influence on systemic glucose regulation lies in its impact on gluconeogenesis.[2]

11. D-Alanine in Diabetes

An aberrant circadian rhythm is associated with the pathogenesis of diabetes. Gluconeogenesis, a physiological process regulated by circadian rhythm, is activated under diabetic conditions.[44,45] Conversely, D-alanine can modulate circadian rhythm and gluconeogenesis. Therefore, the role of D-alanine in glucose metabolism and diabetic conditions was investigated.[46]

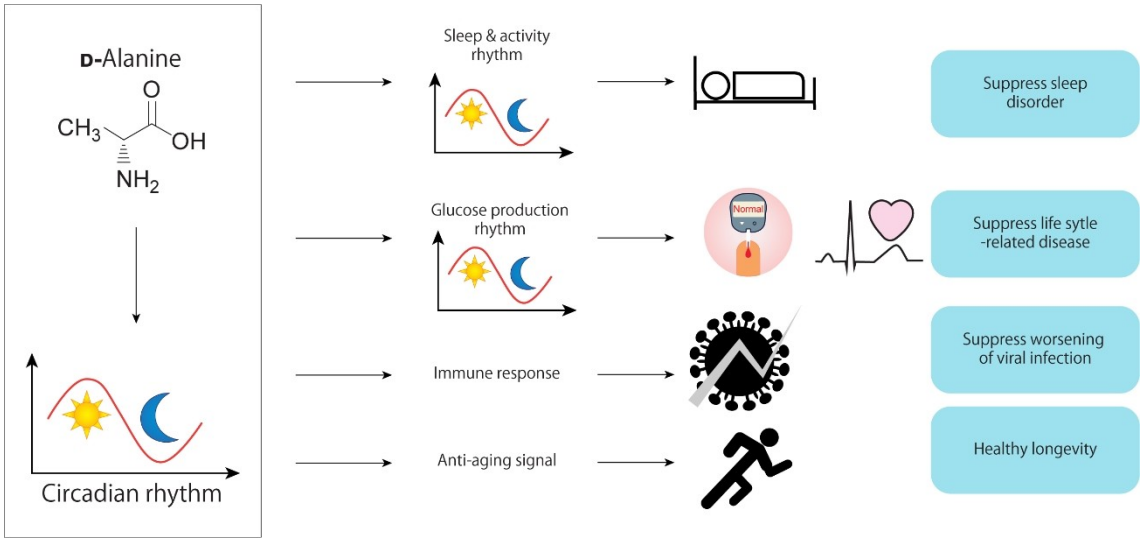

Figure 4. D-Alanine acts on the circadian rhythm and maintains related physical conditions for health. Through the regulation of circadian rhythm, D-alanine maintains sleep and activity rhythm, glucose metabolism, immune response, as well as anti-aging signals. Adopted from https://resou.osaka-u.ac.jp/en/research/2024/20240124_1.

Then, what is the effect of D-alanine in diabetes? First, the circadian rhythm of D-alanine is disrupted in diabetic conditions.[46] This finding was confirmed in studies using two diabetic mouse models: *db*/*db* mice and mice fed high-fat diet. These mouse models exhibit elevated urinary excretion of D-alanine, which occurred without a recognizable circadian rhythm. Consequently, the blood levels of D-alanine were found to be significantly reduced in diabetic conditions. Furthermore, excessive urinary excretion of D-alanine has also been observed in patients with diabetes.

The circadian rhythm of gluconeogenesis is disrupted in diabetic conditions.[46] Despite this disruption, D-alanine retains the capacity to upregulate gluconeogenesis in mouse models.

Upon initial examination, D-alanine appeared to exert opposing effects in the context of diabetes. The regulation of circadian rhythm by D-alanine is crucial for ameliorating diabetic conditions, since this rhythm is disrupted in patients with diabetes. Conversely, D-alanine has the potential to elevate blood glucose level through gluconeogenesis.

The effects of D-alanine were evaluated in diabetic mice. While a single administration of D-alanine resulted in an increase in blood glucose level, long-term treatment was confirmed to decrease the level. These seemingly contradictory effects suggest that D-alanine may have the potential to modulate blood glucose profiles under the tested conditions.

Currently, *in vivo* glucose elevation and *ex vivo* gluconeogenetic capacity imply D-alanine's critical involvement in systemic glucose management through gluconeogenesis. Understanding how alterations in gut microbiota associated with diabetic conditions influence D-alanine's actions is complex. Factors like lifestyle and its-related diseases like diabetes, as well as antibiotic use and viral infections, can disrupt the gut microbial composition.[26] However, changes in gut microbiota alone cannot fully explain D-alanine's dynamics in diabetes. For instance, both diabetic mouse models and human patients exhibit increased urinary D-alanine excretion and a relative decrease in blood D-alanine levels.[46,47] These observations indicate alterations in D-alanine metabolism and kinetics within the body due to the diabetic state, emphasizing the need for further investigation through interventional clinical trials.

12. D-Alanine in Viral Infections

The presence of D-amino acids is fundamental to the essence of life, prompting investigators to understand the significance of D-alanine in various disease contexts, including viral infections. Recently, the Covid-19 pandemic has underscored the urgency of understanding viral pathogenesis,[48] while the influenza virus still remains a potential threat to future pandemics.[49] Despite extensive research efforts, the disease severity and effective treatment options for severe Covid-19 remain limited. Consequently, the role of D-amino acids in the viral infections has bene investigated.[50,51]

The body of evidence indicates that viral infections affect the dynamics of D-amino acids within the body.[50,51] Severe cases of Covid-19 and influenza virus infections significantly decrease the levels of D-amino acids, including D-alanine, in the blood of model mice. Similar observations were made in human patients. While the levels of D-amino acids remain stable in healthy control subjects, the levels of D-amino acids were also reduced in Covid-19 patients.

The effects of supplementation with D-amino acids were next explored.[50] For this purpose, D-alanine was selected because of prominent reduction upon infections. Treatment of D-alanine mitigated the loss of body weight in influenza virus-infected mice. Treatment of D-alanine also improved the survival of Covid-19 mice.

D-Alanine is considered to be consumed upon severe viral infections for the protection of the body, and therefore, supplementation of D-alanine is effective against viral infections.[50] One of the key physiological outcomes of circadian rhythm is the immune response,[2,52] and D-alanine may regulate viral infections through circadian-immune axis (Figure 4).

13. Conclusion: D-Alanine as a Circadian Metabolite

This review summarizes the close connection between D-alanine and circadian rhythm. D-Alanine has a clear circadian rhythm and also acts on the circadian rhythm. Even the trace amount of D-alanine in the body has the key physiological functions that are related with circadian rhythm (Figure 4). By acting on the rhythm while beating the steady rhythm, D-alanine modulates a broad range of circadian phenomena. D-Alanine bridges the circadian rhythm with a wide range of biological processes. D-Alanine, a circadian metabolite, opens up possibilities for research in a fairly new scientific field. D-Alanine, of the circadian, by the circadian, for the circadian.

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Conflict of Interests

The authors declare no conflict of interest.

Keywords: D-Alanine · D-Amino acids · Circadian rhythm · Glucose metabolism · Viral infection

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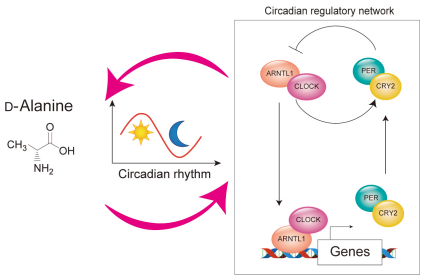
REVIEW

D-Alanine is a circadian metabolite essential for life. Beyond exhibiting a pronounced circadian rhythm in its concentration within the body, D-alanine regulates the circadian rhythm itself by influencing the circadian transcriptional network, thereby modulating related physiological processes such as glucose homeostasis. D-Alanine, of the circadian, by the circadian, for the circadian.



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D-Alanine, a Circadian Metabolite that Regulates Glucose Metabolism and Viral Infection