ABSTRACT

Xiaojia Wang. CIRCADIAN ACTIVATION OF MAST CELLS MEDIATES THE NOCTURNAL RESPONSE IN ALLERGIC ASTHMA (under the direction of Michael R. Van Scott, Ph.D.) Department of Physiology, July 23, 2010

Circadian rhythm is a conserved physiological feature of most organisms. Nocturnal symptoms are a common feature of allergic asthma in humans. Patients with nocturnal asthma show circadian variation of their pulmonary function and inflammation, but nocturnal asthmatic responses have not been documented in animal models. In this study we tested the hypotheses that house dust mite (HDM) allergen-sensitive nonhuman primates (NHP) manifest nocturnal symptoms, and whether intrinsic circadian activity in mast cells might underlie nocturnal asthma response. Dynamic compliance (C_{dyn}) and lung resistance (R_L) measured as indicators of bronchoconstriction in anesthetized NHP, at rest and following exposure to HDM allergen, methacholine, and albuterol, were highly correlated with 3 respiratory inductive plethysmography (RIP) parameters: Phase Angle of the rib cage and abdomen waveforms (PhAng), Baseline Effort Phase Relation (eBPRL) and Effort Phase Relation (ePhRL). 21 allergic subjects were challenged with HDM early in the morning, and eBPRL and ePhRL were monitored for 20 hours after provocation. 15 of the allergic subjects exhibited gradual increases in eBPRL and ePhRL between midnight and 6 AM, with peak activity at 4 AM. The results demonstrate that animals exhibiting acute responses to allergen exposure during the day also exhibit nocturnal airway obstruction, possibly resulting from bronchoconstriction. Mast cells are central effector cells in asthma, and were subsequently investigated for intrinsic circadian activity that could

underlie nocturnal asthma responses. Bone marrow derived mast cells (BMMCs) were exposed to high concentrations of serum (serum shocked) to synchronize gene expression. Following serum shock, circadian genes (*mPer2*, *Bmal1*, *Rev-erbα*, and *Dbp*) exhibit oscillations for up to 72 hours. Likewise, the high affinity IgE receptor in BMMCs (FcεRIα) exhibited circadian expression. The *fcer1a* gene and FcεRIα protein following serum shock exhibited mean periods of 18.9 and 28.6 hours, respectively. Synchronized BMMCs stimulated with IgE/Ag at different circadian time display circadian rhythms in IL-13 mRNA. Taken together, synchronized BMMCs suggest an underlying circadian mechanism of nocturnal asthma *in vitro*.

CIRCADIAN ACTIVATION OF MAST CELLS MEDIATES THE NOCTURNAL RESPONSE IN ALLERGIC ASTHMA

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To My Husband and My Parents

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AB	Abdomen
AHR	Airway hyperresponsiveness
APCs	Antigen-presenting cells
AUC	Area Under the Curve
BAL	Bronchoalveolar lavage
BMMCs	Bone marrow derived mast cells
CCGs	Clock controlled genes
C_{dyn}	Dynamic compliance
dAB	Direction of abdominal movement
DCs	Dendritic cells
Df	*Dermatophagoides farina*
Dp	*Dermatophagoides pteronyssinus*
dRC	Direction of rib cage movement
DRN	Dorsal raphe nucleus
EARs	Early-phase asthmatic responses
eBPRL	Baseline Effort Phase Relation
ePhRL	Effort Phase Relation
FEV_1	Forced expiratory volume in 1 second
FRC	Functional residual capacity
GHT	Geniculohypothalamic
GM-CSF	Granulocyte macrophage-colony stimulating factor
HDM	House dust mite
IL	Interleukin

ITAM	Immunoreceptor tyrosine-based activation motif
LARs	Late-phase asthmatic responses
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein-1α
MRN	Median raphe nucleus
NAR	Nocturnal asthmatic response
NHP	Nonhuman primates
NK	Natural killer
PBMCs	Peripheral blood mononuclear cells
PC_{50}	Provocative concentration of methacholine leading to a 50% maximal response
PE	Phycoerythrin
PEFR	Peak expiratory flow rate
PhAng	Phase Angle of the rib and abdomen waveforms
PVN	Paraventricular nucleus
RC	Rib cage
RHT	Retinohypothalamic tract
RIP	Respiratory inductive plethysmography
R_L	Lung resistance
RR	Respiratory rate
SCN	Suprachiasmatic nucleus
TAA	Thoracoabdominal asynchrony
T_H	T helper
TNF-α	Tumor-necrosis factor-α

TSLP	Thymic stromal lymphopoietin
Vt	Tidal volumes
ΔC_{dyn}	Changes in dynamic compliance
ΔR_L	Changes in lung resistance

CHAPTER 1: INTRODUCTION

Allergic Asthma

Traditionally, asthma is divided into two general categories: the non-allergic (intrinsic) asthma and the allergic (extrinsic) asthma, depending upon the types of stimuli that trigger attacks. The non-allergic asthma is not allergy-related and is caused by inhalation of chemicals while the allergic asthma is triggered by allergen. The immunopathology of non-allergic asthma appears to be very similar to that of allergic asthma. However, there have been some differences reported in the relative proportion of the various inflammatory cells presents. The non-allergic asthma has been subject to careful comparative investigation but so far no clear pathogenic pathways have been identified (Humbert *et al.*, 1999; Corrigan, 2004; Holgate, 2008).

Allergic asthma is one of the most common diseases in developed countries, with an incidence of approximately 8% in the USA (2007). Allergic asthma is a chronic inflammatory disorder of the airways in which many cell types and cellular elements play a role (2002). Chronic inflammation in asthma patients causes an airway hyperresponsiveness (AHR) that leads to an episodic coughing, shortness of breath, and/or wheezing particularly at night and in the early morning (2002).

The development of allergic asthma is classified into three phases: sensitization, the early-phase asthmatic responses (EARs) and the late-phase asthmatic responses (LARs).

Sensitization

Sensitization is initiated by an initial inhalation of an allergen. The majority of allergen inhaled in the airways is cleared by the mucocililary escalator (Verstraelen *et al.*, 2008). However, the escaped allergen can be sampled by dendritic cells (DCs) in the airway lumen or can enter tissues through disrupted epithelium. Also, the airway epithelium tight junctions can be cleaved by allergens with protease activity, such as house dust mite (HDM) (Galli *et al.*, 2008). The allergen that passed through the epithelial lining in the airway is taken up by antigen-presenting cells (APCs), including DCs, macrophages, and B cells (Holt, 2000). APCs process the allergen in the local respiratory tract mucosa or in regional lymph nodes, and then present small peptides, bound to a class II major histocompatibility complex (MHC) on their membranes. After a T helper (T_H) cell recognizes and interacts with an antigen-MHC class II molecule complex, the T_H cell is activated and becomes a Type 2 effector cell, T_H2, which secretes the canonical cytokines interleukin (IL)-4, IL-5, and IL-13. The secreted cytokines drive IgE production by promoting immunoglobulin class-switch recombination in B cells (Gould *et al.*, 2008). After release into the blood, IgE binds to the high-affinity receptor, FcεRI, on the surface of mast cells or basophils, to prime them to respond to subsequent re-exposure to allergen.

EARs

EARs occur within minutes following re-exposure to allergen. Antigen-induced crosslinking of adjacent IgE and aggregated FcεRI on the surface of pulmonary mast cells results in immediate release of preformed inflammatory mediators including histamine, tryptase, tumor-necrosis factor-α (TNF-α), and newly synthesized mediators such as prostaglandin and leukotriene (Galli *et al.*, 2005; Verstraelen *et al.*, 2008; Brown *et al.*, 2008a). The immediate release of mediators contributes to bronchoconstriction by inducing airway smooth muscle contraction and increased goblet cell and tubulo-acinar gland mucus production and secretion into the airway (Bradding, 2003), leading to increased airway resistance and decreased lung compliance. Such symptoms are associated with early-phase asthmatic reactions. In addition to the mediators produced by mast cells, the recently described cytokine, thymic stromal lymphopoietin (TSLP), is released by pulmonary epithelial cells and also promotes the T_H2 response (Allakhverdi *et al.*, 2007; Corrigan *et al.*, 2009).

LARs

The late-phase asthmatic responses (LARs) occur 4-6 hours after antigen provocation and can be prolonged for over 7 days. During LARs, early IgE/Antigen (Ag) activated mast cells release newly synthesized cytokines, such as IL-4, IL-5, IL-13, granulocyte macrophage-colony stimulating factor (GM-CSF), and macrophage inflammatory protein-1α (MIP-1α), which recruit, prime, and activate inflammatory cells (Galli *et al.*, 2008). These inflammatory cells, including eosinophils, neutrophils, and CD4+ T cells, can release more cytokines, chemokines, and lipid mediators, which

induce airway bronchoconstriction, enhance mucus production, and increase airway responses to nonspecific bronchoconstrictors, such as methacholine.

T_H2 cells also play a very important role in LARs. Researchers have shown that allergic asthma predominantly results from an imbalance in favor of a T_H2 response. Allergen specific T_H2 cells are present in the lungs of almost all patients with asthma, particularly patients with allergic asthma (Kim *et al.*, 2010). T_H2 cells produce cytokines that regulate IgE synthesis (IL-4), recruit eosinophils (IL-5), recruit and develop mast cells (IL-9), and increase AHR (IL-13). T_H2 responses induce chronic eosinophil predominant inflammation in the airway, which is characteristic of LARs (Verstraelen *et al.*, 2008). One of the most common features in LARs is recurrent nocturnal bronchoconstriction, airway inflammation, AHR, dyspnea, cough and apnea (Ballard *et al.*, 1989; Kraft *et al.*, 1999b; Irvin *et al.*, 2000; Kelly *et al.*, 2004; Smolensky *et al.*, 2007).

Circadian Rhythm

The word “circadian” is from the Latin “circa” (approximately) and “diem” (a day). The physiology and behavior of most organisms from cyanobacteria to humans display a circadian rhythm, i.e., physiological processes and behavior oscillate with a periodicity of approximately 24 hours. Circadian rhythm is governed by an internal circadian timing system and is entrained by environmental cues (such as light, temperature, and magnetic field) (Dibner *et al.*, 2010; Paschos *et al.*, 2010). In mammals, the circadian

timing system consists of three components: sensors, pacemakers, and effectors. Sensors include both photic and non-photic receptors that entrain the central clock to environmental cues. Photic receptors located at the retina of the eye receive photic information from the environment and transmit information as neurotransmitters via axons of the retinohypothalamic tract (RHT) to the central clock, whereas non-photic information transmits to the suprachiasmatic nucleus (SCN) through the geniculohypothalamic (GHT), dorsal raphe nucleus (DRN) and median raphe nucleus (MRN). The master central pacemaker located in the SCN of the ventral hypothalamus (Gekakis *et al.*, 1998) generates an oscillation with a periodicity of about 24 hours and sends its rhythmic information to synchronize effectors via a variety of outputs. Peripheral organs, known as effectors, have been discovered to maintain circadian clocks (Schibler *et al.*, 2003; Cermakian *et al.*, 2009). A peripheral clock presents in lung (Gibbs *et al.*, 2009), liver (Akhtar, 2001), kidney (Wang *et al.*, 2006), adipose tissue(Wu *et al.*, 2007) and blood cells(Boivin *et al.*, 2003) and also exists in various non-SCN brain regions, such as hypothalamic nuclei, paraventricular nucleus (PVN), olfactory bulb and pineal gland(Abe *et al.*, 2002).

Circadian Central Clock

The circadian central clock resides in the SCN which thus plays a prominent role in circadian rhythmicity. The SCN is a paired neuronal nucleus located on both sides of the third ventricle, just above the optic chiasm (Abrahamson *et al.*, 2001). Complete lesions of SCN abolish circadian rhythmicity in physiological and behavioral parameters

(Welsh *et al.*, 2010; Chiesa *et al.*, 2010; Angeles-Castellanos *et al.*, 2010). Transplantation of SCN tissue into SCN lesions in animals can partially restore their circadian rhythm activity (LeSauter *et al.*, 1997). SCN neurons cultured *in vitro* maintain their circadian firing oscillation up to three weeks, which is partially mediated by circadian regulation of membrane potassium channels (Kuhlman *et al.*, 2006; Welsh *et al.*, 2010). The neuronal cells in the SCN communicate through paracrine and synaptic secretions to synchronize themselves when mammals are deprived of external timing cues (Dibner *et al.*, 2010). Therefore, the SCN contains the master clock in mammals and SCN neuronal firing coordinates the circadian oscillation in peripheral organs.

Circadian Peripheral Clock

Circadian clocks are present not only in the SCN but also in most peripheral organs (Akhtar, 2001; Boivin *et al.*, 2003; Wang *et al.*, 2006; Wu *et al.*, 2007; Gibbs *et al.*, 2009). Numerous physiological functions in peripheral organs are subject to circadian oscillation. In humans, pulmonary function, including tidal volume, minute ventilation, mean inspiratory flow, *et al*., exhibit circadian variations (Adamczyk *et al.*, 2008). Cardiovascular function, including heat rate and blood pressure, vary over 24 hours (Takeda *et al.*, 2010). Circadian rhythms have a profound influence on immune function (Esquifino *et al.*, 2007), including lymphocyte proliferation and cytokine production. Immune cells, such as peripheral blood mononuclear cells (PBMCs) (Born *et al.*, 1997; Murphy *et al.*, 2007), natural killer (NK) cells (Arjona *et al.*, 2006), and

peritoneal macrophages, have been found to exhibit circadian rhythmicity (Keller *et al.*, 2009).

Circadian oscillators are also self-sustained in established cell lines (Nagoshi *et al.*, 2004) and some primary cells (Keller *et al.*, 2009). However, circadian oscillation in individual cells that are not entrained to an external signal is uncoordinated, and therefore self-sustained oscillation in populations of cells is unsynchronized. Detuned circadian oscillators in cells can be synchronized by a serum shock (Balsalobre *et al.*, 1998; Nagoshi *et al.*, 2004; Durgan *et al.*, 2005) or a variety of pharmacological substances including cAMP analogs, glucocorticoids, and dexamethasone (Balsalobre *et al.*, 2000a; Wu *et al.*, 2007; Wu *et al.*, 2008; Huang *et al.*, 2009; Yagita *et al.*, 2010).

Circadian Molecular Clock

The molecular aspects of the circadian clock are similar in SCN and peripheral organs. The core circadian molecular clock in mammals consists of a series of delayed transcriptional and translational feedback loops involving *Period1-3*, *Clock*, *Bmal1*, and *Cry1-2* genes (Takahashi *et al.*, 2008). CLOCK and BMAL1 heterodimers translocate into the nucleus, binding to E-box motifs (CACGTG), and activate transcription of *Per* and *Cry* genes (Gekakis *et al.*, 1998). PER and CRY proteins are synthesized in the cytoplasm, accumulate to a critical level, and bind to CKIε/δ kinase. The phosphorylated heterotrimers translocate into the nucleus, and inhibit the CLOCK-BMAL1 heterodimers, thereby inhibiting transcription of their own genes as well as other

clock controlled genes (CCGs) (Kume *et al.*, 1999). Clock control genes encode a variety of proteins including transcription factors, metabolic enzymes, and receptors (Ganguly *et al.*, 2007; Cai *et al.*, 2008; Marcheva *et al.*, 2010). Another feedback loop directs alternating activation and repression of BMAL1 expression by the nuclear receptors RORα and REV-ERBϱ(Etchegaray *et al.*, 2003) respectively(Sato *et al.*, 2004; Emery *et al.*, 2004). The period for finishing entire transcriptional and translational feedback loops is approximately 24 hours.

Mast Cell

Mast cells arise from CD34+ pluripotential progenitor cells, circulate in the blood, and mature upon entering tissues (Brown *et al.*, 2008a). Mast cells, involved in both innate immune response and adaptive immune responses, reside in the connective tissue and in the mucous membranes. Mast cells play an important role in tissue homeostasis, wound healing, host defense to pathogens, autoimmune disease, and allergic inflammation (Abraham *et al.*, 2010). A common morphological feature of mast cells is prominent granules in their cytoplasm which contain preformed mediators including histamine, proteases, and cytokines. Diseases associated with mast cells include extrinsic mast cell disorders, such as IgE-mediated allergic disorders acting through FcεRI receptors on mast cells or direct mast cell activators acting through other receptors, and intrinsic mast cell disorders, such as mastocytosis and monoclonal mast cell activation syndrome (Stone *et al.*, 2010).

The mast cell is a crucial effector cell in the pathophysiology of allergic asthma (Brown *et al.*, 2008a). In allergic asthmatics, the infiltration of mast cells into the airway causes a significant increase of cell numbers within the bronchial epithelium (Pesci *et al.*, 1993) and the airway smooth muscle bundles (Brightling *et al.*, 2002; Carroll *et al.*, 2002; Brightling *et al.*, 2005). The localization of mast cells within the bronchial epithelium in asthmatics will facilitate activation of mast cell by exposure noxious stimuli such as allergen (Bradding *et al.*, 2006). Repeated exposure to allergen leads to crosslinking of IgE which binds to the FcεRI on the surface of mast cells by allergen, which triggers a complex activation of signaling cascades and causes mast cell degranulation (Metcalfe *et al.*, 2009), releasing the granule contents, including histamine, tryptases, chymases, and proinflammatory cytokines (TNF-α), into the external cellular environment. The released mediators induce airway smooth muscle contraction and mucus production, which contribute to the early asthmatic response. Mast cells responding to IgE/Ag also release numerous newly synthesized cytokines, chemokines, and growth factors. These mediators are released more slowly than the preformed mediators, however. The synthesized mediators induce airway constriction, enhance AHR, increase mucus secretion, and recruit inflammatory cells, such as eosinophils, neutrophils, and lymphocytes, into the airways several hours following allergen challenge (Verstraelen *et al.*, 2008). Such signs and symptoms are associated with the late asthmatic response.

The localization of mast cells within the airway smooth muscle bundles in asthmatics will facilitate interaction between mast cells and smooth muscles cells

through localized mediator release and direct cell-cell communicate, and thus will cause hypertrophy and hyperplasia of airway smooth muscle (Bradding *et al.*, 2006). Airway smooth muscle dysfunction will result in AHR to non-specific stimulus and airway obstruction.

FcεRI

A hallmark of mast cells is expression of the high affinity IgE receptor, FcεRI, on their cell surface (Gregory *et al.*, 2006). FcεRI is a heterotetrameric receptor composed of an α-subunit, a β-subunit, and two identical disulfide-linked γ-subunits (Kalesnikoff *et al.*, 2008). The α-subunit consists of two 90-aa extracellular domains, which bind to the C_H3/C_H3 and C_H4/C_H4 domains of IgE, a transmembrane domain, and a short cytoplasmic tail. The β-subunit spans the plasma membrane four times with a single immunoreceptor tyrosine-based activation motif (ITAM) and links the α subunit to the γ homodimer. The two γ subunits extend a considerable distance into the cytoplasm, and each subunit has an ITAM in its cytosolic domain which is important for initiating the downstream signaling events of FcεRI. Allergen mediated crosslinking of the bound IgE results in aggregation of the FcεRI receptors on the surface of mast cells and rapid tyrosine phosphorylation by Lyn kinase in the ITAMs of the β and γ subunits, which initiates the process of degranulation and synthesis of new mediators in mast cells.

Nocturnal Asthma

As mentioned above, nocturnal asthma is a component of the LAR and exhibits a circadian rhythm. The nocturnal asthmatic response (NAR) is characterized by bronchoconstriction, airway inflammation, dyspnea, wheezing, and cough associated with a decreased quality of sleep and an increased mortality during the night (Turner-Warwick, 1988; Kondo, 1993).

Epidemiology of Nocturnal Asthma

In 1988, Turner-Warwick surveyed 7729 asthmatic patients and reported that 74% awoke at least once a week due to bronchoconstriction, dyspnea, apnea, or cough. Sixty-four percent had nocturnal symptoms at least three times per week (Turner-Warwick, 1988). In 1994, Storms *et al*. reported that 67% of 325 subjects had nocturnal symptoms with 11% awakening every night and 16% awakening three to six nights per week (Storms *et al.*, 1994). In 1971, Cochrane and Clark reported that 68% of deaths attributed to asthma in Greater London hospitals occurred between midnight and 8 AM (Cochrane *et al.*, 1975).

Lung Function

Although lung function has been shown to fluctuate over 24 hours in non-asthmatics, these fluctuations are much more obvious in asthmatic patients with nocturnal symptoms (Smolensky *et al.*, 2007). In patients with nocturnal asthma, differences in forced expiratory volume in one second (FEV_1) exceeding 15% and decreases in peak expiratory flow rate (PEFR) have been shown between bedtime and

awakening (Martin *et al.*, 1990; Martin, 1999; Sutherland, 2005). In addition to increases in airway obstruction in nocturnal asthmatics during sleep, pronounced reductions in functional residual capacity (FRC) have been observed (Ballard *et al.*, 1990). Alteration of physiologic interaction between the airways and lung parenchyma has been reported in nocturnal asthma. During sleep, an immediate uncoupling of the parenchyma to the airway has been found in nocturnal asthmatics, associated with significant increases in lower airway resistance, decreases in FRC, and decreases in respiratory system compliance (Irvin *et al.*, 2000). Even though FRC restored to presleep values in nocturnal asthmatics, lower airway resistance did not significantly fall due to the decreases in respiratory system compliance.

Airway Inflammation

Airway inflammation in nocturnal asthmatics has been shown to oscillate over 24h with intensifying symptoms during the sleep period. Studies have shown the concentration of histamine (Jarjour *et al.*, 1992) and the numbers of eosinophils, neutrophils (Martin *et al.*, 1991), and macrophages (Kelly *et al.*, 2004) in bronchoalveolar lavage (BAL) fluid were significantly greater at 4 am than those at 4 pm in nocturnal asthmatics. Other studies by Kraft *et al.* have shown that numbers of eosinophils in the bronchial airway tissue and $CD4^+$ T cells in the alveolar tissue were significantly higher at 4 AM in nocturnal asthmatics than in asthmatic without nocturnal symptoms (Kraft *et al.*, 1996; Kraft *et al.*, 1999a).

Goal of Research and Statement of Hypothesis

Both pulmonary function and airway inflammation have shown circadian rhythmicity in nocturnal asthmatics, implicating that a circadian timing system may play a significant role in nocturnal asthma. However, whether the circadian timing system itself actually induces and/or exacerbates nocturnal asthma, and by what mechanisms, remains to be more fully elucidated.

The goal of my current research was to test the hypothesis that nocturnal symptoms in asthma are a fundamental characteristic of the disease manifested in both humans and animals, and arise from circadian clock activity in mast cells. Since circadian rhythm exists in almost all organisms, nocturnal asthmatic symptoms in humans might also present in HDM-sensitive NHP. Furthermore, cells associated with asthma, such as mast cells, might exhibit circadian gene oscillation and the circadian clock in these cells might regulate their functions.

Specific Aims:

Aim 1: Test the hypothesis that aerosol allergen exposure elicits a delayed, nocturnal response in HDM-sensitive NHP.

Aim 1.1 Identify respiratory inductive plethysmography (RIP) parameters which are highly correlated with conventional measures of lung resistance (R_L) and dynamic compliance (C_{dyn}).

Aim 1.2 Determine the nocturnal response in HDM-sensitive NHP by RIP measurement.

Aim 2: Test the hypothesis that a circadian clock expressed in murine bone marrow derived mast cells (BMMCs) modulates IgE-dependent activation *in vitro* (Figure 1.1).

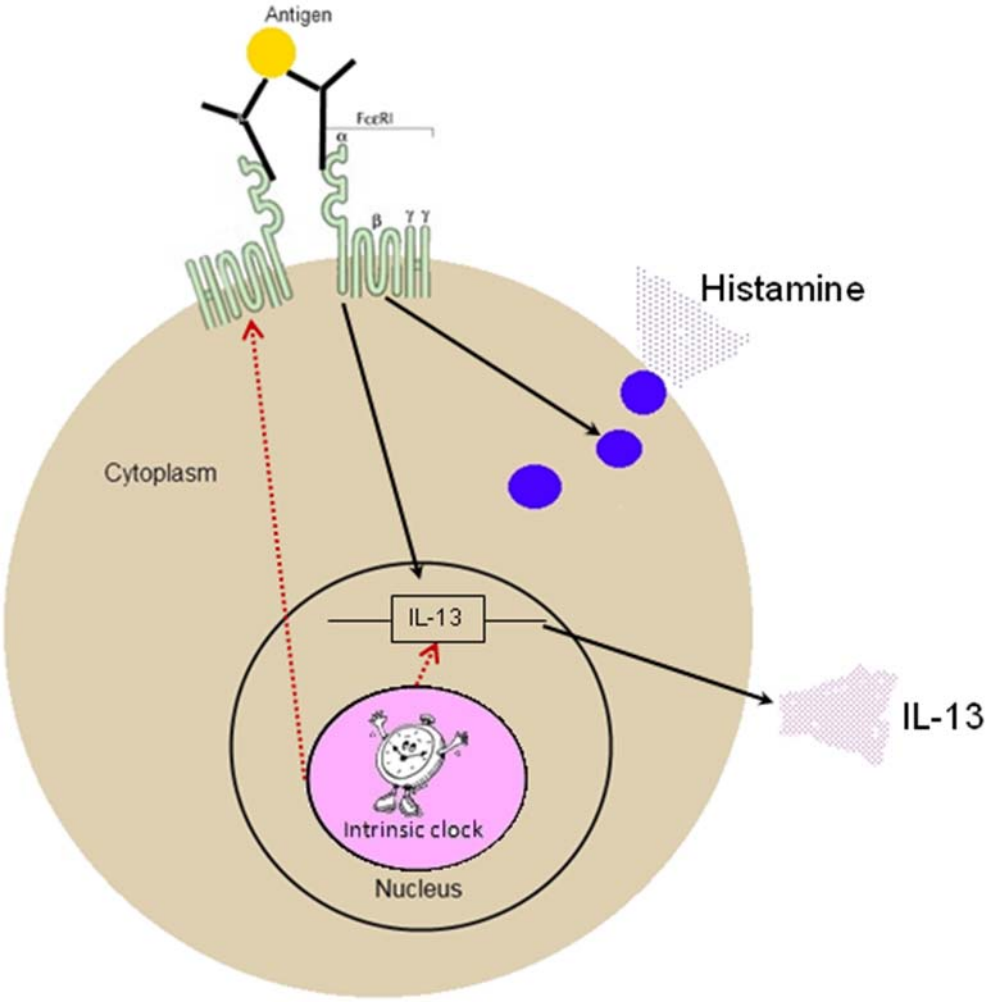
Aim 2.1 Demonstrate circadian clock gene expression in BMMCs following serum shock

Aim 2.2 Test whether activation of BMMCs display a circadian pattern (IgE-dependent and IgE-independent)

Aim 2.3 Determine the influence of BMMC activation on circadian clock gene expression

Aim 2.4 Examine whether IgE dependent circadian activation of BMMCs is due to circadian expression of IgE receptor (FcεRIα)

Figure 1.1 Proposed intrinsic circadian clock in mast cell


Antigen
FcεRI
α
β
γ γ
Histamine
Cytoplasm
IL-13
IL-13
Intrinsic clock
Nucleus

CHAPTER 2: NOCTURNAL THORACOABDOMINAL ASYNCHRONY IN THE HOUSE DUST MITE SENSITIVE NONHUMAN PRIMATES

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SUMMARY

Nocturnal bronchoconstriction is a common symptom of asthma in humans, but is poorly documented in animal models. Thoracoabdominal asynchrony (TAA) is a noninvasive clinical indication of airway obstruction. In this study, respiratory inductive plethysmography (RIP) was used to document nocturnal TAA in house dust mite (HDM) sensitive Cynomolgus macaques. Dynamic compliance (C_{dyn}) and lung resistance (R_L) measured in anesthetized animals at rest and following exposure to HDM allergen, methacholine, and albuterol, were highly correlated with 3 RIP parameters associated with TAA: Phase Angle of the rib cage and abdomen waveforms (PhAng), Baseline Effort Phase Relation (eBPRL) and Effort Phase Relation (ePhRL). 21 allergic subjects were challenged with HDM early in the morning, and eBPRL and ePhRL were monitored for 20 hours after provocation. 15 of the allergic subjects exhibited gradual increases in eBPRL and ePhRL between midnight and 6 AM, with peak activity at 4 AM. However, as in humans, this nocturnal response was highly variable both between subjects and within subjects over time. The results document that TAA in this nonhuman primate model of asthma is highly correlated with C_{dyn} and R_L; and demonstrate that animals exhibiting acute responses to allergen exposure during the day also exhibit nocturnal TAA.

INTRODUCTION

Allergic asthma is a common disease in developed countries, with an incidence of approximately 8% in the USA (2007). Nocturnal symptoms of asthma are common and include bronchoconstriction, airway inflammation and hyperreactivity, dyspnea, cough, and apnea (Ballard *et al.*, 1989; Kraft *et al.*, 1999b; Irvin *et al.*, 2000; Kelly *et al.*, 2004; Smolensky *et al.*, 2007). The incidence and magnitude of nocturnal asthma is variable. Approximately 70% of asthmatics experience nocturnal symptoms at least once per week, and 10% experience nocturnal symptoms at least 3 times per week (Turner-Warwick, 1988). Nocturnal symptoms can be severe, and a high incidence of respiratory arrest and deaths due to asthma is seen between midnight and 8 AM (Cochrane *et al.*, 1975; Hetzel *et al.*, 1977; Weitzman *et al.*, 1998). The mechanisms underlying nocturnal asthma have not been elucidated.

Early and late asthmatic responses are routinely investigated using animal models, but nocturnal symptoms are poorly documented in these models due in part to the nocturnal nature of rodents and to the techniques commonly used to assess bronchoconstriction in larger diurnal laboratory animals. Respiratory inductive plethysmography (RIP) is a noninvasive technique for monitoring breathing patterns(Pennock *et al.*, 1979; Abraham *et al.*, 1981; Tobin *et al.*, 1983; Whyte *et al.*, 1991; Cantineau *et al.*, 1992; Rusconi *et al.*, 1995; Springer *et al.*, 1996; Stromberg *et al.*, 1996a; Millard, 1999; Miller *et al.*, 2000; Kaplan *et al.*, 2000; Kohyama *et al.*, 2001; Black *et al.*, 2004; Willis *et al.*, 2004; Brown *et al.*, 2008b; Hammer *et al.*, 2009) and may be applicable to nocturnal studies in large animals. RIP uses deflection of the

ribcage and abdomen to estimate changes in lung volume, airflow, and phase relationship of abdominal and thoracic movements. Restricting airflow through the airways causes movements of the thorax and abdomen to be out of phase, giving rise to thoracoabdominal asynchrony (TAA). TAA is a common clinical indicator of airway obstruction (Ringel *et al.*, 1983; Sackner *et al.*, 1984; Hillman *et al.*, 1986; Rusconi *et al.*, 1995; Litman *et al.*, 2002; De *et al.*, 2002), and in this study is used to detect nocturnal respiratory disturbances.

Nonhuman primates (NHP) exhibit a high level of similarity to humans in genetic, anatomic, and physiological aspects, and are well suited to modeling human diseases (e.g. asthma) (Coffman *et al.*, 2005). The HDM model of allergic asthma in Cynomolgus macaques (referred to subsequently as "the NHP model") reproduces the key features of human asthma, including elevated serum IgE levels, β-agonist reversible bronchoconstriction, and late phase eosinophilic inflammation (Van Scott *et al.*, 2004). In addition, the airways become hyperresponsive to nonspecific bronchoconstrictors including histamine, methacholine, and adenosine (Van Scott *et al.*, 2004; Van Scott *et al.*, 2005). $CD4^+$ T-lymphocytes and NKT cells in BAL fluid are increased, and Th2 lymphocyte cytokines are up-regulated (Ayanoglu *et al.*, 2010). The severity of the asthma phenotype in the NHP model depends on the schedule of allergen challenges and the provocative dose of dust mite. At an exposure periodicity of 4 to 6 week intervals as used in this study, the animals have minimal symptoms between exposures, but on the day of provocation, exhibit a severe acute asthmatic response, including decrease in arterial O_2 saturation to less than 80% and in some animals less than 70%.

Upon resolution of the acute response the animals develop eosinophilia that is equivalent to what is observed following segmental allergen challenge of asthmatic humans (Erpenbeck *et al.*, 2006). As in humans, the symptoms are attenuated with corticosteroids (Van Scott *et al.*, 2004; Ayanoglu *et al.*, 2010). Extensive terminal protocols have not been conducted on these animals, but in a few animals that have been examined, airway wall remodeling and mucosal eosinophilia has been observed (Van Scott *et al.*, 2004). These characteristics are consistent with mild to moderate, intermittent asthma in humans that is well controlled by β-agonists and corticosteroids.

The goal of this study was to determine if aerosol allergen exposure elicits a delayed, nocturnal response in house dust mite (HDM) sensitive nonhuman primates. Non-Allergic and dust mite sensitive animals were identified from within an existing colony of Cynomolgus macaques. Correlation between RIP measurements and direct measures of lung resistance (R_L) and dynamic compliance (C_{dyn}) were explored by inducing acute bronchoconstriction with aerosolized methacholine and house dust mite allergen. RIP parameters indicative of TAA exhibited a high degree of correlation with the conventional measures of airway function. The animals were subsequently challenged with aerosolized saline or house dust mite allergen, and TAA was monitored by RIP for 20 hours to detect nocturnal disturbances in respiration.

METHODS

Overview

Cynomolgus macaques with documented sensitivity to aerosolized HDM for 2 or more years prior to the study were identified within an existing colony at East Carolina University. Animals with no history of allergic sensitivity were used as Non-Allergic controls. Allergic status at the time of the study was confirmed by serum levels of HDM-specific IgE, early phase bronchoconstriction to aerosolized HDM, and late phase elevation of eosinophils in bronchoalveolar lavage (BAL) fluid (Table 2.1).

Respiratory inductive plethysmography (RIP) parameters indicative of TAA were validated by simultaneous RIP and conventional determination of R_L and C_{dyn}. In an initial session, spontaneously breathing animals were challenged with increasing cumulative doses of aerosolized methacholine to induce graded changes in R_L and C_{dyn}. Four weeks later, bronchoconstriction was induced with aerosolized HDM allergen, and then reversed by aerosolized albuterol. Correlations between RIP parameters, R_L, and C_{dyn} were then determined.

Following the validation phase of the study, nocturnal responses to daytime allergen challenge were investigated. Animals were challenged with aerosolized saline or HDM allergen early in the morning. TAA was then monitored by RIP throughout the rest of the day and overnight.

Table 2.1 Characteristics for Non-Allergic and Allergic group

Different concentrations of HDM were diluted in sterile saline. Animals were exposed to increasing concentrations of HDM until the concentration presented in this table elicited the associated changes in lung resistance (ΔR_L) or dynamic compliance (ΔC_{dyn}) during 15 minutes following the HDM challenge.

	Serum α-HDM-IgE (% Allergic Control)	Eosinophils in BAL (%)	Total Cells in BAL Compartment × 10^6	HDM concentration (AU/ml)	ΔR_L (%)	ΔC_{dyn} (%)
Non-Allergic	15.8 ± 3.5	1 ± 0.4	9.58 ± 3.47	2500 ± 0	0.6 ± 5.3	-5.6 ± 4.9
Allergic	70.4 ± 5.1	35.1 ± 4.7	13.65 ± 1.76	360 ± 120.7	103.4 ± 16.7	-47.6 ±3.4
P-value	<0.0001	<0.0001	0.236	<0.0001	<0.0001	<0.0001

Values are Mean ± SE, Non-Allergic, n = 11, Allergic, n= 21.

Animals

Over a 10 year period prior to the study, thirty-eight cynomolgus macaques were obtained from Alpha Genesis, Inc. (Yemassee, SC) and sensitized to HDM as described previously (Van Scott *et al.*, 2004; Van Scott *et al.*, 2005). Briefly, animals were sensitized by intraperitoneal injections of *Dermatophagoides pteronyssinus* (Dp) and *Dermatophagoides farinae* (Df) extract adsorbed to Alum [156 units of each allergen (Greer Laboratories, Lenoir, NC); Imject Alum (Pierce, Rockford, IL)] at 2 to 4 week intervals for 6 months. Chronic airway disease was induced by subsequent periodic exposure to HDM aerosol at 4 to 6 week intervals. At the time of the study, the animals ranged from 3.6 to 9.4 years of age, weighed 2.3 to 7.5 kg, and exhibited allergic asthma symptoms for 1 to 8 years.

The animals were group housed in accordance with USDA guidelines within a BSL2 facility at East Carolina University, which is fully accredited by the Association for the Assessment and Accreditation for Laboratory Animal Care, International (AAALAC, Intl). All protocols were approved by the Institutional Animal Care and Use Committee of East Carolina University.

Exposure to allergen between intentional provocations was minimized by HEPA filtration of all air circulating through the animal rooms, rinsing the rooms and cages with pressurized water twice each day, and running the cages through an automated washer weekly. All personnel entering the rooms wore appropriate personal protective gear.

ELISA for Allergen-Specific IgE

Serum levels of HDM-specific IgE were measured as described previously.(Van Scott *et al.*, 2004) Ninety-six-well plates were coated with dust mite allergen (10 μg/ml per well, overnight at 4°C in 0.01 M sodium bicarbonate buffer, pH 9.6). The plates were blocked with 10% fetal bovine serum, 100 μl of serum (1:100 dilutions) was added to the wells. The plates were incubated overnight at 4°C. IgE was detected by incubating with 1 μg/ml biotinylated goat anti-human IgE (Vector Lab, Burlingame, CA) for 1 h at room temperature, and then developing with streptavidin-conjugated horseradish peroxidase (BD PharMingen, San Diego, CA) and 3,3',5,5'-tetramethylbenzidine (BD PharMingen). The reaction was stopped by addition of sulfuric acid, and the absorbance at 450 nm was determined. Relative IgE titers were determined by comparison to a serum sample pooled from a group of highly allergic animals.

Determination of R_L and C_{dyn}

Animals were anesthetized with Telazol (2.0 mg/kg, IM), stabilized on propofol (10 to 15 mg/kg/hr, IV), and intubated. R_L , C_{dyn} and respiratory rate (RR) were measured by a conventional pressure-flow technique.(Van Scott *et al.*, 2004; Van Scott *et al.*, 2005) Tracheal airflow was measured using a heated Fleisch Pneumotachograph (size 00, Fleisch, Lausanne, Switzerland) connected to a Validyne pressure transducer (Model DP 45-14, Validyne Engineering, Northridge, CA). Intrathoracic pressure was

measured using an esophageal balloon connected to a Model DP 45-24 Validyne pressure transducer. Flow and pressure signals were analyzed using a MuMed PR800 lung function recorder (MuMed, London, England). Baselines of R_L, C_{dyn}, and RR were determined during a one minute period of quiet breathing. Subsequent responses to aerosolized saline (vehicle), methacholine, HDM, and albuterol were expressed as raw data or as a percent change from baseline or saline as appropriate.

House Dust Mite Challenge

Baseline pulmonary function was recorded. The animals were challenged for 4 minutes with nebulized sterile saline delivered through the endotracheal tube (Devilbiss Ultrasonic Nebulizer, SUN-99HD, Red Lion, PA or Nouvag Ultrasonic 2000, Nouvag, Switzerland). Condensation on the inner surface of the endotracheal tube was cleared by aspiration and pulmonary function was monitored for 1 minute. The challenge was repeated with increasing concentrations of nebulized HDM allergen (1, 10, 100, 500, 2500 AU/ml) until a 100% increase in R_L or a 50% decrease in C_{dyn} was observed (Van Scott *et al.*, 2004). Once the target change in R_L or C_{dyn} was observed, pulmonary function was monitored at 5 minute intervals for 15 minutes. Following the last recording, the animals were treated with nebulized albuterol sulfate (4 minutes, 0.083% solution, Ventolin Nubules, Orlando, FL,).

Arterial oxygen saturation was monitored by pulse oximetry (SurgiVet Model V3304, Harvard Apparatus, Holliston, MA or Cardell® Model 9403 and 9405, Sharn

Veterinary, inc. Tampa, FL), and supplemental O_2 was delivered as needed to maintain O_2 saturation above 70%.

Methacholine Challenge

Animals were anesthetized and instrumented as described above. Baseline pulmonary function was recorded, and sterile saline was delivered for 2 minutes. Condensation on the tube was cleared by aspiration, and pulmonary function was monitored for one minute. Cumulative doses of methacholine (A2251, Sigma, St. Louis, MO) between 0.001 mg/ml to 10 mg/ml were delivered until a 100% increase in R_L and/or a 50% decrease in C_{dyn} was observed (Van Scott *et al.*, 2004). The range of aerosolized methacholine doses was based on previous experience with this model (Van Scott *et al.*, 2004), published results from tissue bath experiments (Kott *et al.*, 2002; Joad *et al.*, 2006), and empirical observations with this cohort of animals. A methacholine concentration of 0.001 mg/ml did not induce bronchoconstriction, whereas concentrations greater than 10 mg/ml induced high levels of secretions in the airways. Therefore, 10 mg/ml methacholine was chosen as the upper level. The provocative concentration of methacholine leading to a 50% maximal response (PC_{50}) for R_L, C_{dyn}, and RR was calculated for individual animals, and then mean PC_{50} was determined for all animals.

Endotracheal bronchoalveolar lavages

24 hours after HDM challenge, the animals were anesthetized with Telazol and propofol, and intubated. A sterile 2.5 mm OD Tygon tube was wiped with Surgilube®, advanced through the endotracheal tube, and gently wedged into an airway. The lung segment beyond the tip of the catheter was lavaged with 7 ml of sterile saline, which was collected by gentle aspiration. Total cell counts were determined using a Coulter Counter (Beckman Coulter Instruments, Miami, FL). Slides were generated using a Cytospin and stained with DiffQuik (VWR Scientific, So. Plainfield, NJ). Manual differential cell counts were acquired by counting 200 cells per sample.

Respiratory Inductive Plethysmography (RIP)

RIP parameters were measured using the LifeShirt System (Vivometrics, Inc., Ventura, CA). The LifeShirt incorporated two parallel sinusoidal wire arrays encircling the thorax and abdomen. A small alternating current was passed through the wires, inducing a magnetic field. Changes in the shape of the arrays during breathing altered the magnetic field, inducing a current that was detected as a change in the frequency of the applied current, which translated into lung volumes and flows on a breath by breath basis through coefficients obtained during calibration procedures. Changes in animal posture and activity were monitored by accelerometers in the jackets allowing movement artifacts to be filtered out of the dataset (Prisk *et al.*, 2002; Keenan *et al.*, 2005a; Keenan *et al.*, 2005b).

Animals were fitted with the LifeShirt jackets such that the rib cage band was positioned over the sternum and the abdominal band positioned immediately cephalad

to the pelvic girdle (Prisk *et al.*, 2002). The circumference of each band was adjusted to provide rib cage (RC) and abdomen (AB) deflections of 20 to 200 assumed milliliters (Aml) prior to calibration. The LifeShirt jackets were fitted on those animals before any airway challenges began and were left on for about 24 hours. Raw data records were analyzed with VivoLogic software (Vivometrics, Inc.). The default settings were overridden to permit analysis of the small tidal volumes (100% tidal volume (Vt) = 40 Aml; minimum acceptable Vt = 2.5%; minimum acceptable effort volume = 2.5%). A Fixed Volume Calibration was then performed using the tidal volumes recorded by the Mumed lung function recorder during the 1 minute baseline period (Sackner *et al.*, 1989; Sartene *et al.*, 1993). The calibrated records were used to determine the RIP parameters (expressed as calibrated raw data or as a percent change from baseline; see below) to be compared with simultaneously determined conventional measures of R_L and C_{dyn}.

Three RIP-derived parameters analyzed by the LifeShirt software were used as indicators of thoracoabdominal asynchrony: Phase Angle (PhAng) of the AB and RC waveforms, Baseline Effort Phase Relation (eBPRL), and Effort Phase Relation (ePhRL). PhAng is a common parameter used to compare the initial slopes of two sinusoidal waveforms (Allen *et al.*, 1990). As abdominal and thoracic movement become asynchronous, PhAng increases. Effort parameters provide an indication of the amount of time during a breath that deflection of the abdomen and chest wall are asynchronous (i.e., the two are moving in opposite directions instead of in unison). Baseline Effort Phase Relation (eBPRL) is the percentage of time during inspiration that the rib cage is moving in the opposite direction from the abdomen; and Effort Phase

Relation (ePhRL) is the percentage of time during the total breath that the rib cage and abdomen are moving in opposite directions. Thus, thoracoabdominal asynchrony is associated with increases in PhAng, eBPRL, and ePhRL.

Nocturnal Study

Following HDM provocation, the animals were recovered from anesthesia and returned to their cages. RIP readouts were monitored continuously until the next morning. The lower threshold for movement artifact was set as the maximum acceleration values associated with quiet breathing for each animal while under anesthesia. All records associated with accelerations greater than this maximum value were removed from the dataset.

Thirty minute averages of eBPRL and ePhRL (but not PhAng: see Results) were plotted from 13:00 the day of HDM provocation to 6:00 the next day. A 30 minute period between 13:00 and 22:00 was identified during which there was little animal movement and low incidence of TAA (i.e., minimum average eBPRL or ePhRL for the period, labeled as Baseline in Figure 2.4). This relatively normal, quiet breathing period served as a baseline for the nocturnal studies. eBPRL and ePhRL were plotted against time and the Area Under the Curve (AUC) between 22:00 the day of HDM challenge and 5:00 the next day was determined using SigmaPlot Version 10 (Systat Software, Inc., San Jose, CA; see Figure 2.4 for details). The AUC was used as an indicator of TAA incidence and to compare nocturnal responses among subjects.

Statistics

Recorded respiratory parameters were expressed as calibrated raw data or as a percent change from the baseline control period. The relationships between R_L, C_{dyn}, RR, and RIP parameters were investigated by simple and multiple linear regressions using SPSS software (SPSS, Inc., Chicago, IL). Methacholine PC_{50}'s of each parameter were determined by Hill fit analysis for each animal (Systat Software, Inc., San Jose, CA). Differences between two means were assessed by Student's t-test for independent observations ($P \leq 0.05$). Differences between three or more means were assessed by ANOVA with Tukey HSD post-hoc test ($P \leq 0.05$). Values are reported as Mean ± Standard Error.

RESULTS

Methacholine and HDM Challenges

To validate RIP as a measure of TAA associated with underlying airway responses, the relationships between R_L, C_{dyn}, and RIP parameters indicative of TAA were defined under controlled conditions where methacholine and HDM were used to induce acute bronchoconstriction, and albuterol to induce bronchodilation.

Non-allergic animals had minimal changes in all parameters at the highest concentration of methacholine (Data not show), whereas in allergic animals the highest concentration of methacholine increased RR and R_L and decreased C_{dyn}. RR exhibited a higher sensitivity to methacholine than C_{dyn} and R_L; C_{dyn} exhibited sensitivity to methacholine that was intermediate between RR and R_L (Table 2.2). These results were consistent with independent modulation of RR and R_L, and with changes in C_{dyn} that were due to reduced alveolar filling time as RR increased. Multiple linear regression of values recorded before and after methacholine challenge revealed significant associations of R_L and C_{dyn} with eBPRL, ePhRL and PhAng (eBPRL: $r = 0.49$, $P < 0.05$; ePhRL: $r = 0.41$, $P < 0.05$; PhAng: $r = 0.45$, $P < 0.05$).

At a later date, the animals were challenged with aerosolized HDM antigen to induce acute bronchoconstriction, and then were treated with albuterol to reverse the HDM-induced bronchoconstriction. When the effects of HDM and albuterol were separated, the RIP parameters tracked closely with changes in C_{dyn} and R_L (Figure 2.1). Although the directions of C_{dyn} and R_L were opposite, the relative magnitude of HDM-

induced changes were similar (ΔC_{dyn}: p = 0.0001 and ΔR_L: p = 0.001, HDM vs. saline) and both parameters exhibited at least partial reversal with albuterol (Figures 2.1B and 2.1C). HDM provoked increases in eBPRL, ePhRL, and PhAng ($p < 0.01$, HDM vs. saline) that were reversed by albuterol (Figures 2.1D – 2.1F). In contrast, RR increased with HDM ($p < 0.001$, HDM vs. saline), but did not reverse with albuterol (Figure 2.1A). These results demonstrated that TAA, as measured by changes in RIP, coincided with changes in R_L and C_{dyn} induced by diverse agents.

The aggregate HDM and albuterol data were analyzed by multiple linear regression, which revealed associations of R_L and C_{dyn} with eBPRL, ePhRL, and PhAng as described by the following equations (See Table 2.3):

$$eBPRL = 26.8 – 3.8\ C_{dyn} + 1.7\ R_L$$

$$ePhRL = 30.2 – 4.4\ C_{dyn} + 1.7\ R_L$$

$$PhAng = 98.6 – 11.4\ C_{dyn} + 2.1\ R_L$$

Thus, the RIP parameters increased as C_{dyn} decreased; and independently, after adjustment for C_{dyn}, the RIP parameters increased as R_L increased. The regression equations were consistent with an inverse relation between R_L and C_{dyn}. This relationship was verified by plotting coincident values of R_L against C_{dyn} for data collected during both the methacholine and HDM challenges. The resulting curve exhibited an inverse first order relationship ($r = 0.69$, $P < 0.05$ by simple linear regression of $1/R_L$ vs. C_{dyn}).

Nocturnal Asthmatic Response to House Dust Mite Challenge

In follow-up experiments to those discussed above, animals were challenged with aerosol HDM between 6:00 AM and 10:00 AM, and then acute bronchoconstriction was reversed with albuterol. The animals were recovered from anesthesia, and returned to their cages. Excursions of the rib cage and the abdomen were monitored continuously until the next morning. Figure 2.2 shows representative RIP recordings for one animal while anesthetized during the baseline and the early asthmatic response (Figure 2.2, left panels), and later while conscious during the afternoon or evening baseline and the nocturnal asthmatic response (Figure 2.2, right panels). PhAng (calculated from the Konno-Mead diagram) has been used in previous studies to assess TAA (Allen *et al.*, 1990; Rusconi *et al.*, 1995; Prisk *et al.*, 2002), however, PhAng is affected by the shape of the abdominal and ribcage waveforms, with a triangular waveform being associated with increased error (Rusconi *et al.*, 1995; Prisk *et al.*, 2002). Triangular waveforms were commonly observed in the current study (Figure 2.2), and therefore, PhAng was not used as an indicator of TAA at night. The effort parameters, eBPRL and ePhRL, correlated well with labored breathing exhibited during the acute response to HDM and methacholine provocation, and were therefore used to monitor for nocturnal respiratory disturbances.

To assess the long-term stability of eBPRL and ePhRL, both parameters were recorded in Non-Allergic animals overnight. No significant change was observed in eBPRL and ePhRL over the nocturnal time period between 22:00 and 5:00 (Figure 2.3), whereas in most Allergic animals eBPRL and ePhRL gradually increased from the

baseline, reaching a peak value around 3:30 (Figure 2.4). To quantify nocturnal TAA, we determined the Area Under the Curve (AUC) between 22:00 and 5:00 and determined the 99% confidence intervals for Non-Allergic animals (dashed line in Figure 2.5A and B). Plots of eBPRL and ePhRL against time for Allergic animals revealed 15 of 21 animals with AUCs greater than the upper boundary of the 99% confidence interval; these 15 animals were therefore classified as nocturnal asthmatic responders (NAR). The Allergic group mean AUCs for changes in eBPRL and ePhRL were greater than Non-Allergic control group mean AUCs (Figure 5A and B, ΔeBPRL: $p < 0.001$, ΔePhRL: $p = 0.001$). In control experiments, the NAR group did not exhibit nocturnal responses following challenges between 6:00 AM and 10:00 AM with aerosolized saline alone (data not shown).

To evaluate the reproducibility of the nocturnal response, eight animals exhibiting acute responses to HDM were rechallenged at a later date (Figure 2.6). Six of the animals had been classified as NARs in the previous trial (shown as six symbols above the dashed lines for challenge number 1, Figure 2.6) and two animals had not been classified as NARs. Four of these six NARs from the first trial exhibited nocturnal responses following the second challenge (Figure 2.6). Both the animals that had not been classified as NARs in the previous trial exhibited nocturnal responses for challenge number 2. Conversely, two animals that exhibited a nocturnal response following the first challenge exhibited minimal increase in eBPRL and ePhRL following the second challenge. Manifestation of a nocturnal response and its magnitude could not be attributed to the provocative concentration of HDM used to elicit the early phase

response or the magnitude of the change in R_L and C_{dyn} during early phase response (data not shown).

Table 2.2 Methacholine PC50's of conventional respiratory indexes

	Methacholine PC_{50} (mg/ml)		ANOVA (*P* value)		
	Mean	SE	RR	C_{dyn}	R_L
RR	0.07	0.01	--	0.031	0.001
C_{dyn}	0.23	0.05	0.031	--	0.001
R_L	0.63	0.09	0.001	0.001	--

Figure 2.1 Responses to sequential exposure of aerosolized saline, the maximum concentration of house dust mite extract (HDM) and albuterol

Shown are changes in (A) respiratory rate (RR), (B) dynamic compliance ($C_{dyn,}$), (C) lung resistance (R_L), (D) Baseline Effort Phase Relation (eBPRL), (E) Effort Phase Relation (ePhRL), and (F) Phase Angle (PhAng). Responses to saline and HDM were recorded in 23 animals. Albuterol was administered to 8 animals exhibiting severe and prolonged increases in airway resistance. The changes were expressed as a percentage of the baseline measurement recorded prior to saline delivery. Values are means ± SE.

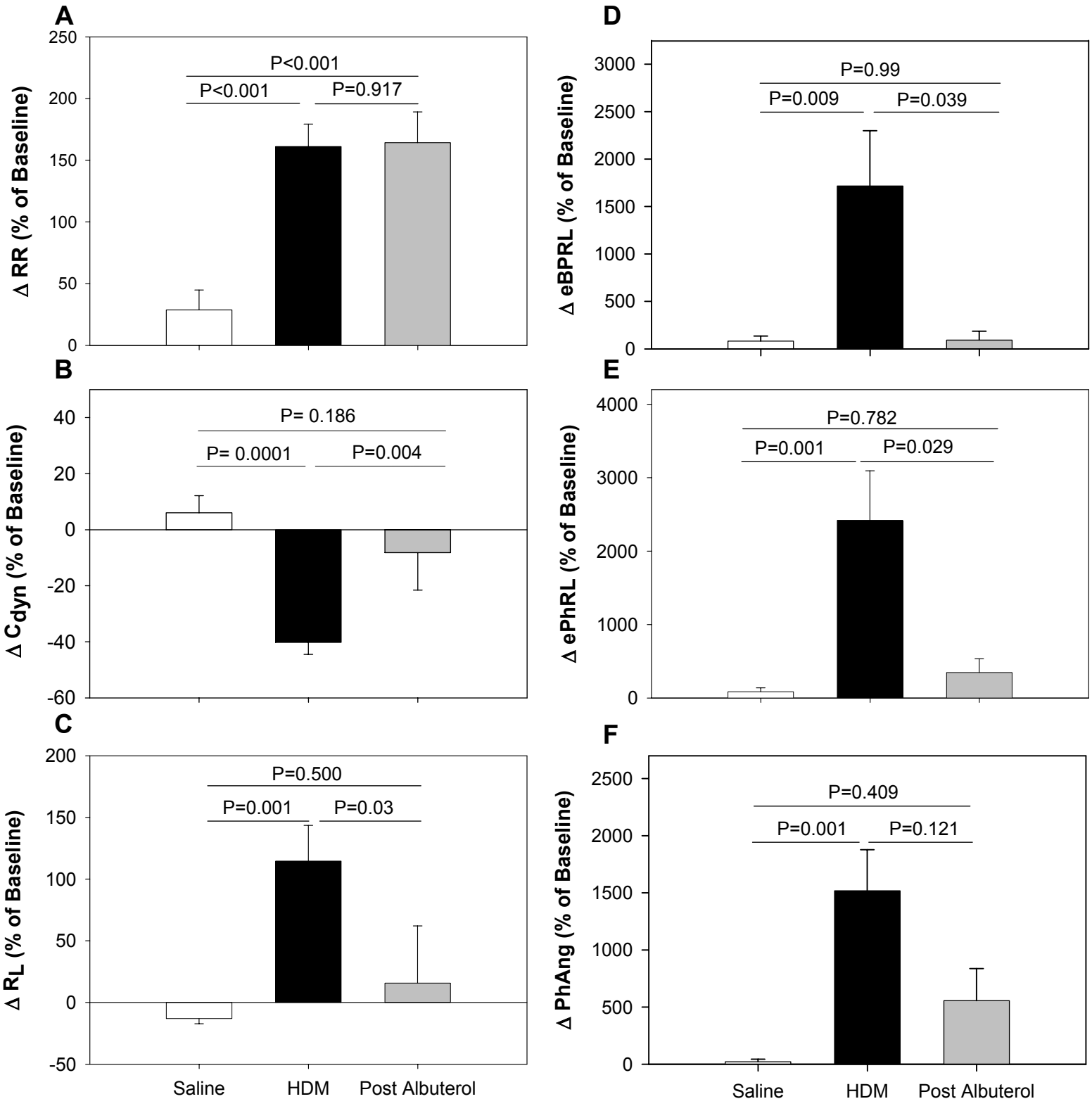

A
Δ RR (% of Baseline)
P<0.001
P<0.001
P=0.917
B
Δ Cdyn (% of Baseline)
P= 0.186
P= 0.0001
P=0.004
C
Δ RL (% of Baseline)
P=0.500
P=0.001
P=0.03
D
Δ eBPRL (% of Baseline)
P=0.99
P=0.009
P=0.039
E
Δ ePhRL (% of Baseline)
P=0.782
P=0.001
P=0.029
F
Δ PhAng (% of Baseline)
P=0.409
P=0.001
P=0.121
Saline
HDM
Post Albuterol

Table 2.3 Multiple linear regression results of eBPRL, ePhRL, and PhAng recorded during the first 30 minutes following a HDM challenge

		Regression Coefficients			
Dependent variable	Independent variable	b_j	Std. Error s_{bj}	t_{bj}	*P*
	Intercept	26.80	13.72	1.95	0.056
	C_{dyn}	-3.85	1.25	-3.08	0.003
eBPRL	R_L	1.65	.41	4.08	<0.001
	Intercept	30.15	13.99	2.16	0.035
	C_{dyn}	-4.43	1.27	-3.48	0.001
ePhRL	R_L	1.68	.41	4.06	<0.001
	Intercept	98.62	25.20	3.91	<0.001
	C_{dyn}	-11.42	2.29	-4.98	<0.001
PhAng	R_L	2.07	.74	2.782	0.007

The *p*-value is a test of H_0: $\beta_j = 0$ against H_a: $\beta_j \neq 0$

Figure 2.2 Representative RIP recordings of one animal (ID: 8065) during both the early asthmatic response (EAR) and the nocturnal asthmatic response (NAR)

Excursions of the rib cage (RC) and the abdomen (AB), as well as corresponding eBPRL, ePhRL, and PhAng are shown for multiple breaths. Note the triangular RC and AB wave forms, and that eBPRL and ePhRL are better reflections of TAA than PhAng during the EAR peak and also are good reflections of TAA during NAR peak. The Konno-Mead diagram (bottom of each vertical panel) shows the direction of RC movement (dRC) and the direction of AB movement (dAB) during inhalation and exhalation in three breaths.

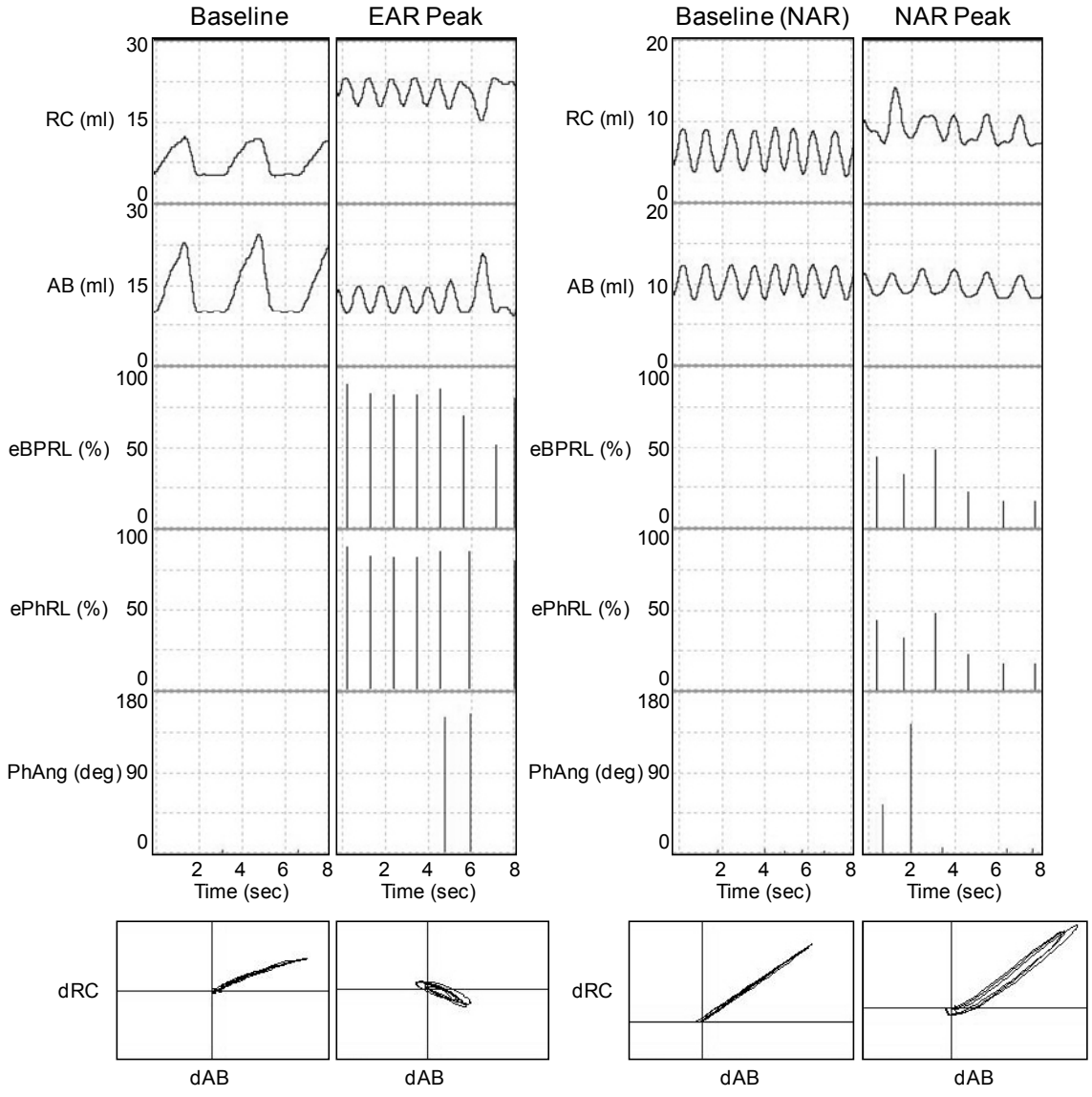

Baseline
EAR Peak
Baseline (NAR)
NAR Peak
RC (ml)
AB (ml)
eBPRL (%)
ePhRL (%)
PhAng (deg)
Time (sec)
dRC
dAB

Figure 2.3 Long-term stability of nocturnal eBPRL and ePhRL

Plots of eBPRL and ePhRL (30 minute averages) starting at 22:00 and ending at 5:00 AM next morning in 11 Non-Allergic animals. The changes were expressed as a percentage of the baseline measurement recorded during afternoon. Values are means ± SE at each time point.

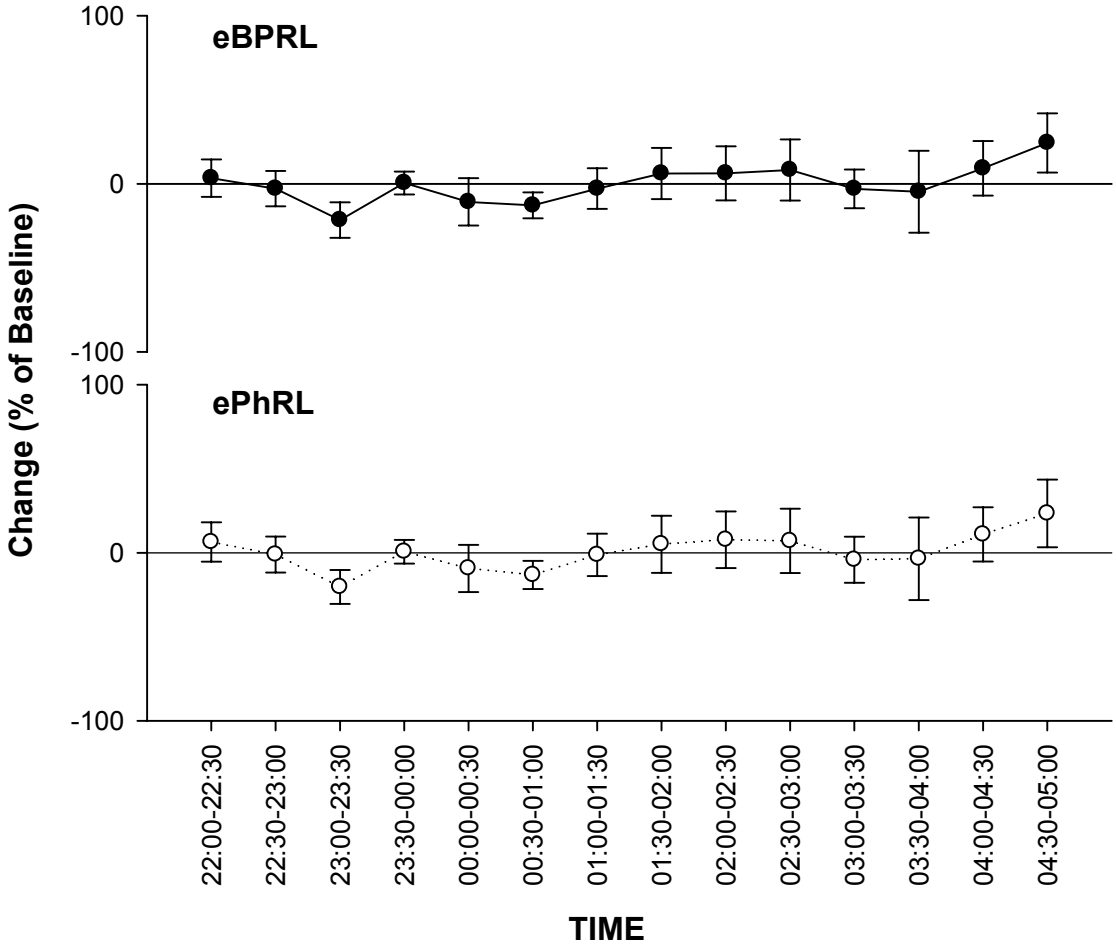
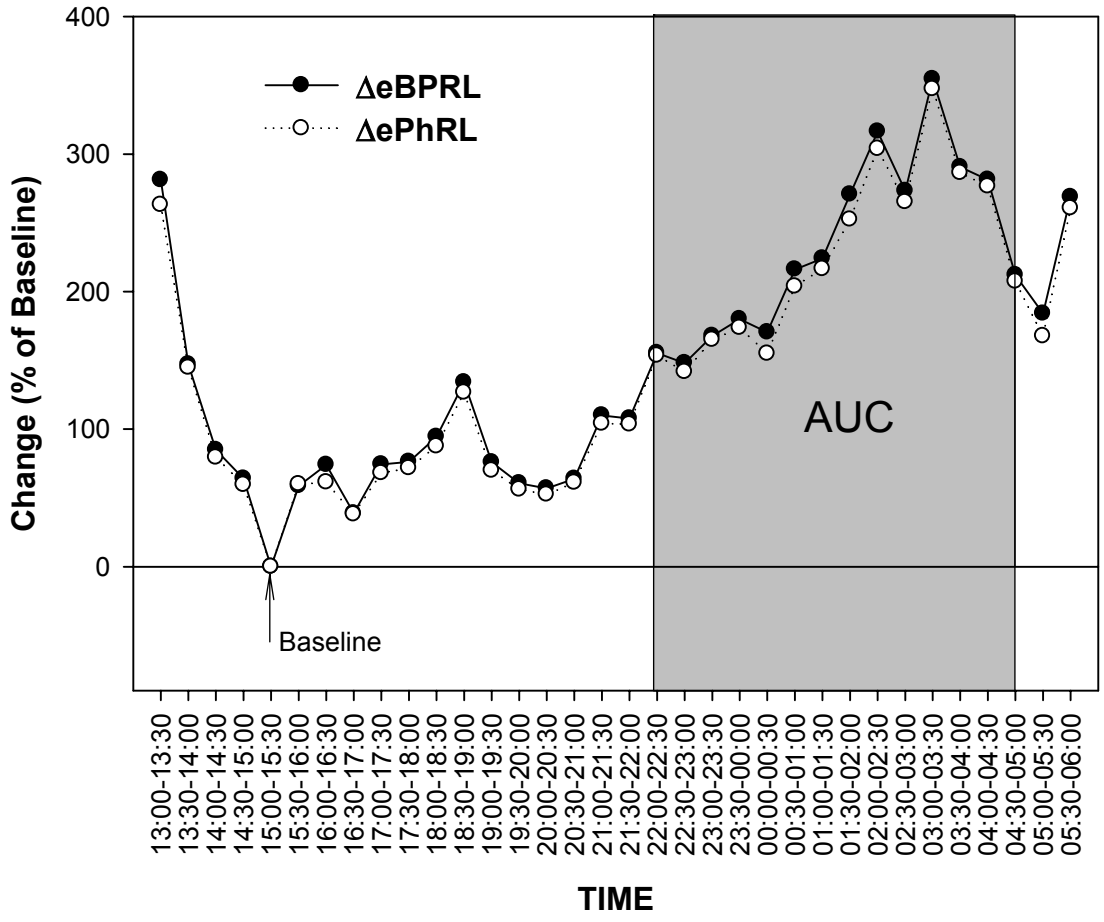

eBPRL
ePhRL
100
0
-100
Change (% of Baseline)
22:00-22:30
22:30-23:00
23:00-23:30
23:30-00:00
00:00-00:30
00:30-01:00
01:00-01:30
01:30-02:00
02:00-02:30
02:30-03:00
03:00-03:30
03:30-04:00
04:00-04:30
04:30-05:00
TIME

Figure 2.4 Representative nocturnal eBPRL and ePhRL response of the same animal represented in figure 2 (ID: 8065)

Plot of eBPRL (filled circle) and ePhRL (open circle) (30 minute averages) starting at 13:00 and ending at 6:00 AM next morning. Area under the curve (AUC) was started at 22:00 and ended at 5:00 AM.


400
300
200
100
0
Change (% of Baseline)
ΔeBPRL
ΔePhRL
AUC
Baseline
13:00-13:30
13:30-14:00
14:00-14:30
14:30-15:00
15:00-15:30
15:30-16:00
16:00-16:30
16:30-17:00
17:00-17:30
17:30-18:00
18:00-18:30
18:30-19:00
19:00-19:30
19:30-20:00
20:00-20:30
20:30-21:00
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21:30-22:00
22:00-22:30
22:30-23:00
23:00-23:30
23:30-00:00
00:00-00:30
00:30-01:00
01:00-01:30
01:30-02:00
02:00-02:30
02:30-03:00
03:00-03:30
03:30-04:00
04:00-04:30
04:30-05:00
05:00-05:30
05:30-06:00
TIME

Figure 2.5 Changes in eBPRL (A) and ePhRL (B) between 22:00 and 5:00 for each individual animal in both the Allergic group (n=21) and the Non-Allergic group (n=11)

(A and B) The Allergic group mean AUCs for eBPRL and ePhRL were significantly greater than Non-Allergic control group mean AUCs. The arrow indicates values for animal ID: 8065 that is also represented in Figure 2 and 4. Dashed lines represent the upper bound of the 99% confidence interval for the Non-Allergic group.

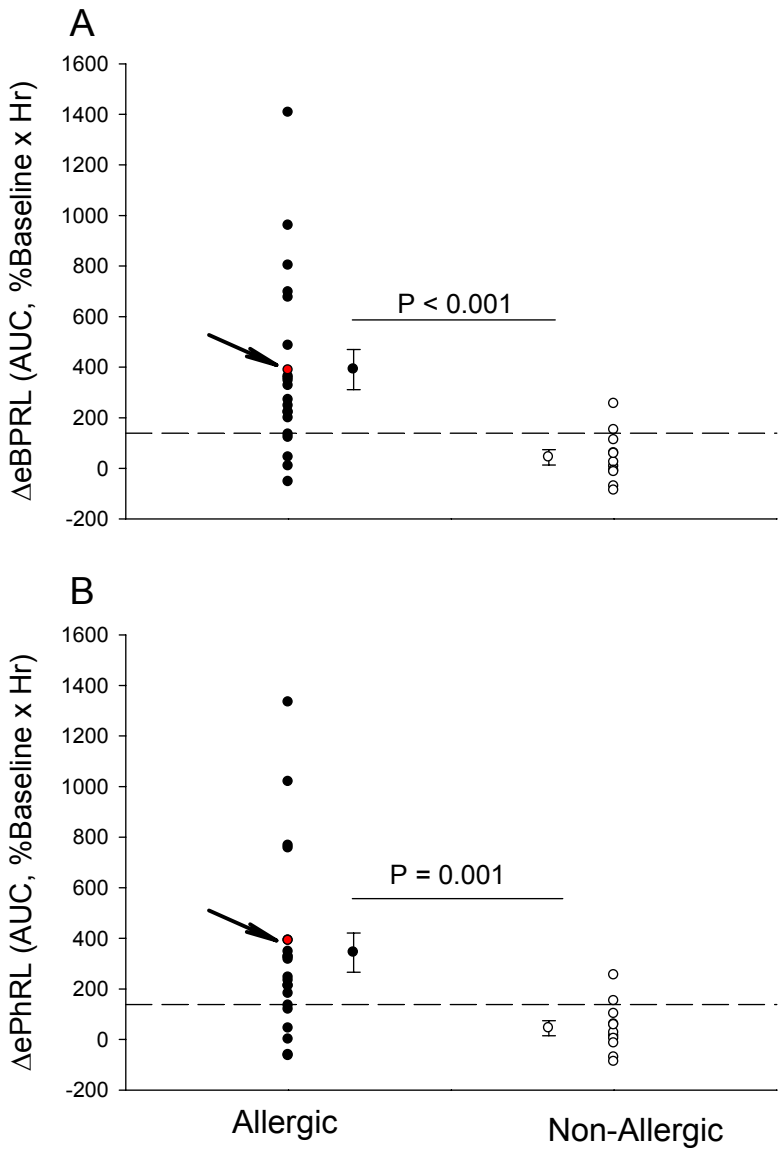
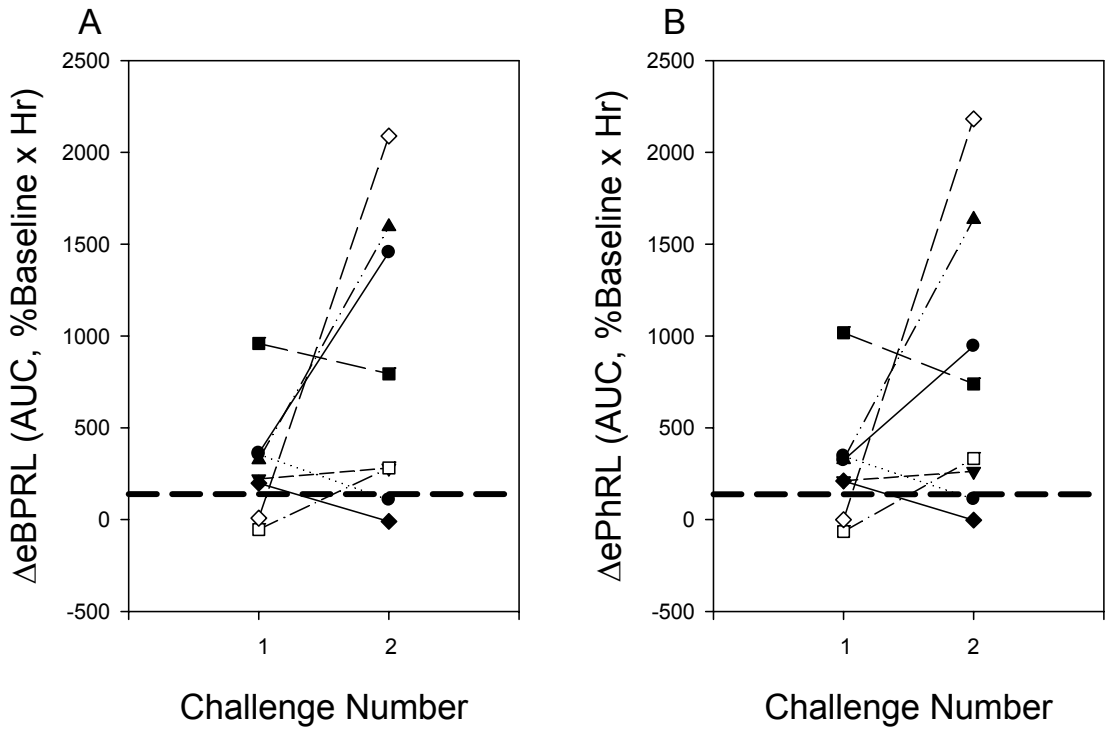

A
ΔeBPRL (AUC, %Baseline x Hr)
1600
1400
1200
1000
800
600
400
200
0
-200
P < 0.001
B
ΔePhRL (AUC, %Baseline x Hr)
1600
1400
1200
1000
800
600
400
200
0
-200
P = 0.001
Allergic
Non-Allergic

Figure 2.6 Reproducibility of the nocturnal response to house dust mite challenge

Eight animals were challenged with HDM on two different dates. AUC for eBPRL (A) and ePhRL (B) are shown. Symbols represent individual animals. Dash lines represent the upper bound of the 99% confidence intervals for the Non-Allergic group. The symbols above the dashed lines were classified as nocturnal asthmatic responders (NARs).


A
B
ΔeBPRL (AUC, %Baseline x Hr)
ΔePhRL (AUC, %Baseline x Hr)
2500
2000
1500
1000
500
0
-500
1
2
Challenge Number
Challenge Number

DISCUSSION

In spontaneously breathing animals, large and small airway constriction manifest as changes in resistance and compliance. These changes may contribute to thoracoabdominal asynchrony (TAA) which results from inability of thoracic volume to change in phase with abdominal volume due to restricted airflow through the lungs. To our knowledge, this is the first report on the use of RIP to measure TAA in a nonhuman primate model of allergic asthma and to apply the technique to document nocturnal responses in an animal model of asthma. TAA as indicated by RIP Effort Phase Relation parameters correlated with changes in C_{dyn} and R_L in Cynomolgus macaques during bronchoconstriction induced by methacholine or HDM, and bronchodilation induced by albuterol. Following HDM challenge in the early morning, TAA was shown to increase with a peak between 3:00 and 4:00 the next morning. The nocturnal response exhibited intra-animal variability in both incidence and magnitude, which is consistent with manifestation of nocturnal asthma symptoms in humans.

RIP has been used extensively to assess apnea and breathing patterns during sleep in humans (Whyte *et al.*, 1991; Cantineau *et al.*, 1992; Kaplan *et al.*, 2000; Kohyama *et al.*, 2001; Brown *et al.*, 2008b) and animals (Warren *et al.*, 1989). Multiple human studies have demonstrated correlations among TAA, bronchoconstriction, and bronchodilation (Allen *et al.*, 1990; Rusconi *et al.*, 1995; Springer *et al.*, 1996). In contrast to TAA, other RIP parameters, particularly ventilation, do not appear to be useful in assessing pulmonary function in asthmatic subjects (Stromberg *et al.*, 1996b; Black *et al.*, 2004), possibly due to artifact induced in volume measurements by

asynchronous movement of the chest wall and abdomen during bronchoconstriction.(Fujimori *et al.*, 1996) In this study of allergic nonhuman primates, TAA as measured by RIP correlated with changes in R_L and C_{dyn} during HDM- and methacholine-induced bronchoconstriction. In contrast, a previous study in foals did not observe these correlations during histamine-induced bronchoconstriction (Miller *et al.*, 2000). The reason for this discrepancy is not clear.

In asthmatics there is a high prevalence of nocturnal symptoms (Ballard *et al.*, 1989; Irvin *et al.*, 2000) with peak pulmonary resistance (Irvin *et al.*, 2000) and airway inflammation (Kraft *et al.*, 1999b; Kelly *et al.*, 2004) observed around 4:00 AM. In this study of allergic nonhuman primates, both eBPRL and ePhRL gradually increase during the night following an early morning HDM challenge, which is consistent with the time course of nocturnal symptoms in humans. These nocturnal increases in TAA may be linked to underlying bronchoconstriction, but this cannot be proven without overt intervention at night. TAA is also impacted by changes in respiratory muscle load, control of auxiliary muscle of breathing, and respiratory muscle dysfunction (Hammer *et al.*, 2009). These factors cannot be excluded as underlying causes of nocturnal TAA. Regardless, the data provide compelling evidence that daytime exposure to allergen results in nocturnal disturbances in breathing.

The incidence and severity of nocturnal asthma symptoms in humans is variable, both between and within subjects (Turner-Warwick, 1988; Storms *et al.*, 1994). Likewise, nocturnal TAA following HDM challenge in allergic nonhuman primates was variable between animals and within individual animals over time. Shigemitsu and

colleagues (Shigemitsu *et al.*, 2007) reported that 60% of asthmatic subjects manifest nocturnal symptoms. This number compares favorably with the 71% incidence observed in the current study. Fix *et al*. reported that occurrence of nocturnal symptoms is linked to the severity of asthma (Fix *et al.*, 1997), but other investigators failed to substantiate a definitive link between any particular phenotypic feature of early acute allergic responses and late asthmatic responses in humans and animals (Robertson *et al.*, 1974; O'Byrne *et al.*, 1987; Nabe *et al.*, 2005), including the occurrence of nocturnal symptoms (Turner-Warwick, 1988). In the NHP model, nocturnal TAA could not be attributed to any of the functional parameters measured during the early asthmatic response, including the provocative concentration of HDM or the magnitude of the acute bronchoconstriction.

In summary, RIP parameters indicative of TAA correlated with decrease in pulmonary function during methacholine challenge and the early asthmatic response in HDM-sensitive Cynomolgus macaques. Using RIP as a measure of TAA revealed nocturnal changes in pulmonary function following daytime exposure to aeroallergen. While increased TAA is consistent with bronchoconstriction, the changes may not be due solely to changes in R_L and C_{dyn}. Regardless, the results provide compelling evidence that daytime exposure to aeroallergen in allergic nonhuman primates is associated with increased incidence of nocturnal disturbances in breathing. Taken together, the results indicate that symptoms of nocturnal asthma can be induced in nonhuman primates, providing a model for elucidating the mechanisms underlying circadian manifestation of asthma symptoms.

CHAPTER 3: A CIRCADIAN CLOCK IN MURINE BONE MARROW-DERIVED MAST CELLS (BMMCs) MODULATES IgE-DEPENDENT ACTIVATION *in vitro*

JOURNAL SUBMISSION PENDING

SUMMARY

Circadian rhythm is expressed in most organisms, and many functions and parameters in the immune system are associated with time-of-day. For instance, blood histamine levels in patients with mastocytosis display a diurnal variation. In addition, pulmonary function and inflammation in patients with nocturnal asthma is more severe during the night than the daytime. However, it is largely unknown if local circadian clocks in immune cells directly control physiological outcomes. We hypothesized that a circadian clock in murine bone marrow derived mast cells (BMMCs) modulates IgE-dependent activation *in vitro*.

Mature BMMCs, grown from bone marrow of C57BL/6 mice, were synchronized with serum rich media (50% horse serum). Total RNA was harvested from BMMCs at 4 hour intervals for up to 72 hours following synchronization and expression of circadian genes (*mPer1*, *mPer2*, *Bmal1*, *Rev-erbα*, and *Dbp*) and IgE receptor gene (*fcer1a*) were amplified by quantitative PCR. The surface expression of FcεRIα was analyzed by flow cytometry every 4 hours for up to 72 hours following synchronization. Synchronized BMMCs were activated by IgE/Antigen or ionomycin at given time points. Total RNA of

cytokines was harvested 30 minutes after BMMCs activation and was amplified by quantitative PCR. Cytokines were collected from supernatant 4 hours after BMMCs activation and were analyzed by ELISA.

Serum shock synchronized rhythmic expressions of circadian genes (mPer2, Bmal1, Rev-erbα, and Dbp) in BMMCs. Synchronized BMMCs stimulated with IgE/Ag at different circadian times displayed circadian rhythms in IL-13 mRNA. The expression of *fcer1a* gene and FcεRIα protein displayed a circadian pattern following serum shock, with mean periods of 18.9 and 28.6 hours respectively. These results demonstrate that synchronized BMMCs provide an *in vitro* model to study circadian mechanism associated with allergic disease and that circadian oscillation of FcεRIα may contribute to the circadian oscillation of cytokine production following IgE-dependent activation.

INTRODUCTION

Circadian rhythm is a conserved feature of organisms ranging from cyanobacteria to humans (Dunlap, 1999) that is manifest as oscillations of biological processes with a periodicity of approximately 24 hours. The circadian system consists of three components: sensors, pacemakers, and effectors. Sensors include both photic and non-photic receptors that entrain the central clock to environmental cues. Afferent pathways convey information from the sensors to pacemakers that establish the rhythm, and efferent pathways alter effector organ function according to the established rhythm. In mammals, a central circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the ventral hypothalamus (Gekakis *et al.*, 1998) coordinates oscillators in peripheral organs (Reppert *et al.*, 2002).

The core circadian molecular clock in mammals consists of a series of transcriptional and translational loops involving *Period1-3*, *Clock*, *Bmal1*, and *Cry1-2* genes (Takahashi *et al.*, 2008). CLOCK and BMAL1 heterodimers translocate into the nucleus, binding to E-box motifs (CACGTG), and activating transcription of *Per* and *Cry* genes (Gekakis *et al.*, 1998). PER and CRY proteins synthesized in the cytoplasm accumulate to a critical level, and bind to CKIε/δ kinase. The phosphorylated heterotrimers translocate into the nucleus, and inhibits CLOCK-BMAL1 heterodimers, thereby inhibiting transcription of their own genes and other clock controlled genes (CCGs) (Kume *et al.*, 1999). Another feedback loop directs alternating activation and repression of BMAL1 expression by the nuclear receptors RORα and REV-ERBα (Etchegaray *et al.*, 2003), respectively(Sato *et al.*, 2004; Emery *et al.*, 2004).

Circadian oscillations in individual cells that are not entrained to an external signal is uncoordinated, and therefore self-sustained oscillation in populations of cells is unsynchronized. Detuned circadian oscillators observed in cell lines (Nagoshi *et al.*, 2004) and some primary cells (Keller *et al.*, 2009) can be synchronized by a serum shock (Balsalobre *et al.*, 1998; Nagoshi *et al.*, 2004; Durgan *et al.*, 2005) or pharmacological substances (Balsalobre *et al.*, 2000a; Wu *et al.*, 2007; Wu *et al.*, 2008; Huang *et al.*, 2009; Yagita *et al.*, 2010).

Many diseases, including allergic asthma, exhibit circadian variation of symptoms. Nocturnal symptoms of asthma are common and include bronchoconstriction, airway inflammation and hyperreactivity, dyspnea, cough, and apnea during the night (Ballard *et al.*, 1989; Kraft *et al.*, 1999b; Irvin *et al.*, 2000; Kelly *et al.*, 2004; Smolensky *et al.*, 2007). Mast cells play a central role in allergic diseases (Brown *et al.*, 2008a); and circadian rhythmicity is an established feature of many immune cells, including peripheral blood mononuclear cells (PBMCs) Born *et al*1997.,) (Murphy *et al.*, 2007), natural killer (NK) cells (Arjona *et al.*, 2006), and peritoneal macrophages(Keller *et al.*, 2009). While it remains unknown how circadian variation may directly influence mast cell function, it has been reported that plasma histamine levels in patients with mastocytosis exhibit a circadian variation (Friedman *et al.*, 1989), and circadian variation in mast cell number has been observed in thyroid gland(Catini *et al.*, 1994), ovaries, (Gaytan *et al.*, 1991), tongue, pinna and dorsal skin(Chen *et al.*, 1989). Despite this evidence, it remains unknown whether circadian clock genes are expressed in the mast cells and whether these genes influence mast cell function.

Crosslinking of FcεRI (high-affinity IgE receptor) on the surface of mast cells by Ag/IgE results in immediate release of preformed inflammatory mediators including histamine and proteases, that initiate an immediate hypersensitivity reaction (Metcalfe *et al.*, 2009). In addition, activation of mast cells stimulates production of cytokines and chemokines, including IL-6, IL-13, CXCL8 and CCL3, which promote the late-phase inflammatory reactions.

The present study was conducted to test the hypothesis that a circadian clock expressed in murine bone marrow derived mast cells (BMMCs) modulates IgE-dependent activation in vitro. BMMCs were serum shocked with a high concentration of horse serum, and expression of circadian clock genes (*mPer1, mPer2, Bmal1, Rev-erbα, and Dbp*) were monitored for up to 72 hours. Inflammatory cytokines were measured following FcεRI stimulation to examine the influence of circadian rhythm on mast cell activation. Lastly, rhythmic expression of FcεRI, the high affinity IgE receptor, was evaluated by both quantitative real-time PCR and flow cytometry.

METHODS

Animals

Four week-old C57BL/6 mice were obtained from The Jackson Laboratories (Bar Harbor, ME). Mice were euthanized by CO_2 asphyxiation followed by collection of femoral bone marrow for generation of BMMCs. All animal protocols were approved by the Institutional Animal Care and Use Committee of East Carolina University.

Cell Culture

Mouse BMMCs were cultured from femoral bone marrow of C57BL/6 mice. Bone marrow was collected from 3-4 mice per BMMC culture and all experiments were repeated 6 times, each with different culture of BMMCs. Cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES, 1 mM sodium pyruvate, nonessential amino acids (Sigma-Aldrich, St. Louis, MO), 0.0035% 2-mercaptoethanol, and 300 ng/ml recombinant murine IL-3 (PeproTech, Rocky Hill, NJ). BMMCs were used after 4-6 weeks of culture, a time at which >95% of the cells are mast cells as determined by granule content and high expression of FcεRI.

Synchronization of BMMC by Serum Shock

Bone marrow-derived mast cells were plated in 25 cm^2 cell culture flask. At time t = 0, the medium was exchanged with serum rich medium (RPMI 1640 supplemented

with 50% horse serum (GIBCO), 100 U/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES, 1 mM sodium pyruvate, nonessential amino acids, 0.0035% 2-mercaptoethanol, and 300 ng/ml recombinant murine IL-3), and after 2 hours this medium was replaced with 1% FBS RPMI with IL-3 (Balsalobre *et al.*, 1998). At the time points indicated in Figure 3.1A, BMMCs were collected for subsequent analysis as described below.

Quantitative Real-Time PCR

Total RNA from BMMCs was isolated at 4 hour intervals after serum shock using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was reverse transcribed to obtain cDNA using a QuantiTect reverse transcription kit (Qiagen). Quantitative real-time PCR was performed using QuantiTect primer assays and SYBR green master mix (Qiagen). A Bio-Rad iCycler was used to obtain cycle threshold (Ct) values for target and internal reference cDNA levels. Target cDNA levels were normalized to GAPDH, an internal reference using the equation $2^{-[\Delta Ct]}$, where ΔCt is defined as $Ct_{target} - Ct_{internal\ reference}$. Values shown are the average of 6 independent experiments conducted with 6 different batches of BMMCs.

FcεRI-mediated BMMC activation

Mouse BMMCs were seeded at 1×10^6 cells/well in a 6-well plate and sensitized 1 hour with 1 µg/ml mouse IgE anti-DNP (Sigma-Aldrich) at each given time point

following serum shock. Following 1 hour sensitization, cells were washed with PBS and 100 ng/ml DNP-HSA was added to the cells (Sigma-Aldrich) (see Figure 3.1B for time course). Total RNA from BMMCs was isolated 30 min following addition of DNP using a Qiagen RNeasy Mini Kit. Supernatants of BMMCs that had been incubated with IgE and stimulated with DNP for 4 hours were analyzed for IL-6, IL-13, MIP-1α and TNF-α using a mouse Duo-Set ELISA system (R&D Systems, Minneapolis, MN).

In addition to FcεRI-mediated activation, BMMCs were seeded at 1×10^6 cells/well and stimulated with 0.5 μm ionomycin (Sigma-Aldrich) at each given time point following serum shock (Figure 3.1B). Total RNA and supernatants from BMMC cultures were collected respectively at 30 min and 4 hours after incubation with ionomycin as described above.

Flow Cytometry

For measurement of FcεR1α, BMMCs were harvested at indicated time points after serum shock and incubated with 2 μg/ml phycoerythrin (PE)-conjugated anti-mouse FcεR1α (MAR-1, eBioscience) at room temperature for 30 min. PE-labeled Armenian Hamster IgG (eBioscience) was used as isotype control. Flow cytometric analysis of the stained cells was performed with a FACScan flow cytometer (Becton, Dickinson and Company) equipped with CELLQUEST software. 10,000 BMMCs per sample were analyzed and mean fluorescence intensity (MFI) values at each time point are reported.

Periodicity Analysis

The time series data of circadian gene expression, fcer1a gene expression, and FcεR1α mean fluorescence intensity (MFI) were analyzed for circadian rhythmicity by the cosinor method (Nelson *et al.*, 1979; Bingham *et al.*, 1982). The time series of the parameters of the cosinor, i.e. amplitude (half the difference between the minimum and maximum of the fitted cosine function), MESOR (middle value of the fitted cosine curve representing the rhythm adjusted mean) and acrophase (time of peak value of the fitted cosine function), were tested by the cosinor parameters test designed by Bingham et al (Bingham *et al.*, 1982).

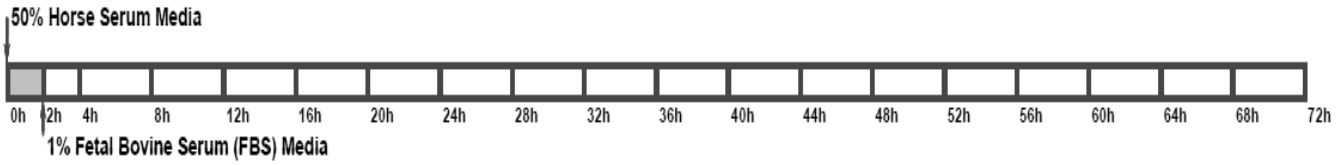
Statistical Analysis

Variation in cytokine expression and circadian gene expression in activated BMMC data were analyzed by one-way ANOVA or two-way ANOVA using SPSS software (SPSS, Inc., Chicago, IL) followed by Bonferroni post hoc tests where appropriate. Values are reported as Mean ± Standard Error. A value of $p < 0.05$ was considered significant.

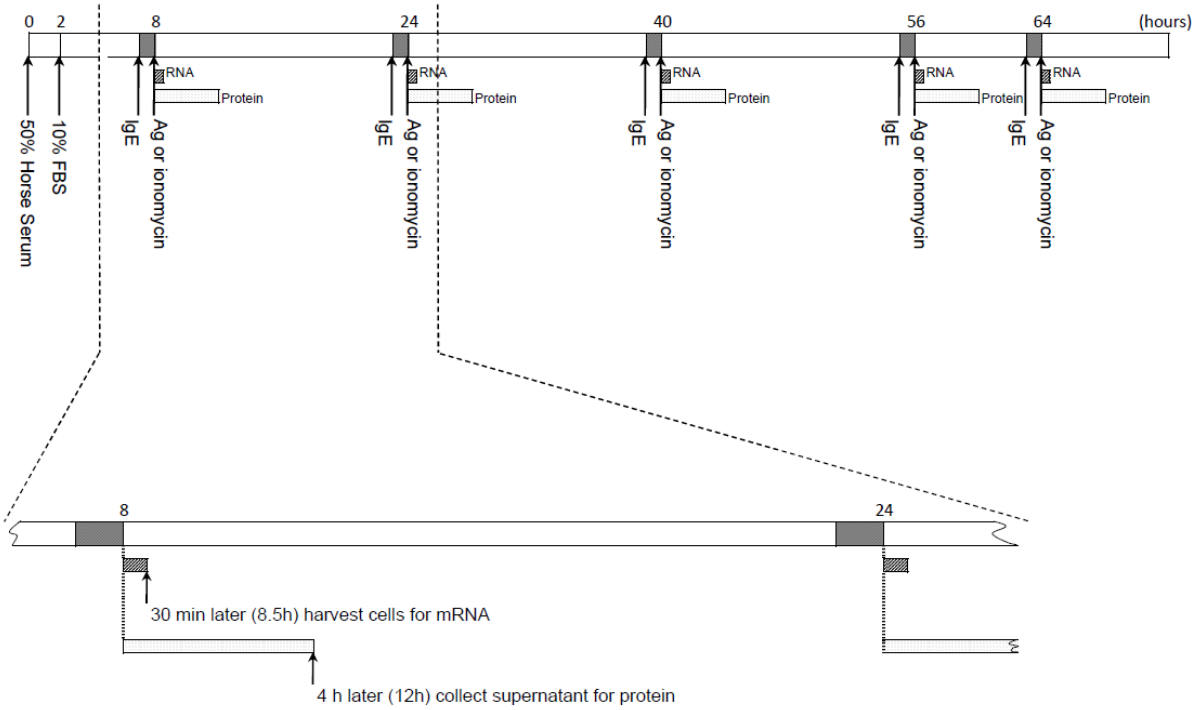
Figure 3.1 Protocols

Serum shock synchronization protocol (A) and IgE/Ag or ionomycin stimulation protocol (B). Samples were collected at each time point indicated.

A


50% Horse Serum Media
0h
2h
4h
8h
12h
16h
20h
24h
28h
32h
36h
40h
44h
48h
52h
56h
60h
64h
68h
72h
1% Fetal Bovine Serum (FBS) Media

B


0
2
8
24
40
56
64
(hours)
RNA
Protein
50% Horse Serum
10% FBS
IgE
Ag or ionomycin
8
24
30 min later (8.5h) harvest cells for mRNA
4 h later (12h) collect supernatant for protein

RESULTS

Expression of Circadian Clock Genes in Serum Synchronized BMMCs

Following a 2 hour synchronization with serum rich media (50% horse serum), expression of *mPer2*, *Bmal1*, *Rev-erbα*, and *Dbp* exhibited a robust oscillation over a 72 hour period (Figure 3.2). *mPer2* and *Rev-erbα*, which are negative regulatory arms of the circadian transcriptional complex, displayed peak expression levels at 19.8 and 18.5 hours following synchronization. In contrast, *Bmal1*, a positive regulatory arm of the circadian transcriptional complex, exhibited peak phase at 5.9 hours after synchronization (Table 3.1). The peak expression of *Bmal1* was 14 hours in advance of the peak phase of *mPer2*. Overall, serum shock synchronized an oscillating expression profile of circadian gene mRNAs as a function of time in BMMC.

Cytokine Production in Serum Synchronized BMMCs following IgE-Dependent and IgE–Independent Activation

Since we demonstrated circadian clock genes are rhythmically expressed in BMMC, we next determined if they influence activation of BMMCs. To address this question, cytokine mRNA expression profiles were investigated following either IgE sensitization and Ag challenge or ionomycin stimulation in synchronized BMMCs at the indicated time points (0 hour, 8 hour, 24 hour, 40 hour, 56 hour, and 64 hour). IL-13 mRNA expression levels peaked at 0 and 56 hours following FcεRI stimulation, while expression was lowest at 8 hours ($p < 0.05$, Figure 3.3A). Interestingly, IL-6 mRNA

expression levels displayed the same pattern following IgE/Ag stimulation as IL-13 mRNA expression following serum shock (Figure 3.3B). However, the peak and nadir of IL-6 mRNA expression levels in FcεRI stimulated BMMCs did not reach statistical significance. In synchronized BMMCs, ionomycin stimulated IL-13 mRNA production did not display any circadian oscillation (Figure 3.3C). Likewise, IL-6 mRNA expression levels in ionomycin treated synchronized BMMCs displayed no oscillation over 72h (Figure 3.3D). In synchronized BMMCs, TNFα and MIP1α mRNA expression following activation by IgE/Ag or ionomycin did not result in circadian oscillation (data not shown).

In contrast to mRNA expression levels, IL-13 (Figure 3.4 A and C), IL-6 (Figure 3.4 B and D), TNFα (data not shown), and MIP1α (data not shown) protein secretion from FcεRI or ionomycin stimulated synchronized BMMCs did not display any circadian oscillation.

Effects of FcεRI-mediated BMMC Activation on *mPer2* Expression

To test whether FcεRI stimulation influences the oscillating expression of the circadian gene *mPer2,* mRNA expression profiles were investigated in 3 treatment groups: serum shocked BMMCs, FcεRI activated BMMCs synchronized by serum shock, and FcεRI activated BMMCs in the absence of serum shock. FcεRI stimulation had little effect on *mPer2* expression over 72 hours in unsynchronized cells (Figure 3.5). Serum shock alone, induced/unmasked a circadian oscillation in mPer2 levels ($F(5,90)$=3.760, p=0.004). However, FcεRI activation in combination with serum shock

did not affect the amplitude or phase of *mPer2* mRNA oscillation. A significant effect of three groups on *mPer2* levels were observed ($F(2,90)$=4.197, p=0.018).

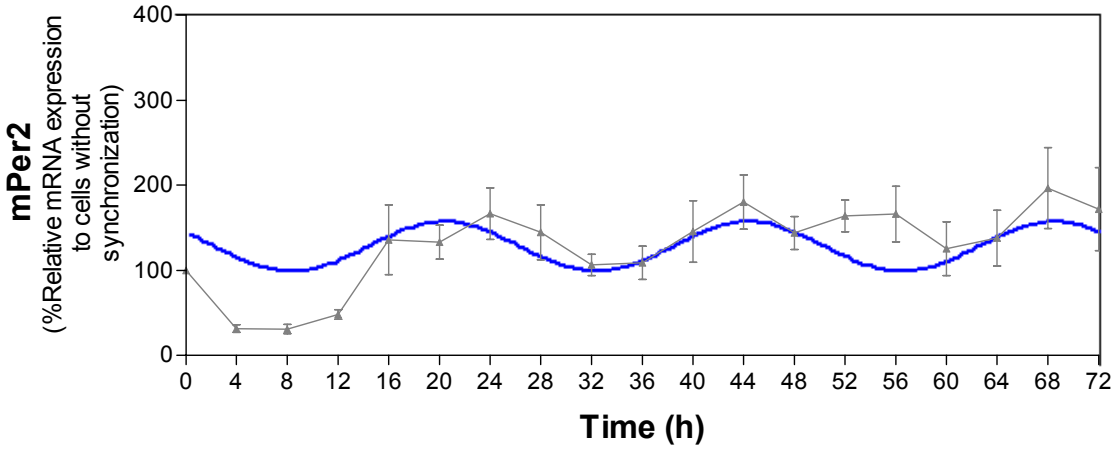
Circadian Expression of FcεRIα in Serum Synchronized BMMCs

To test if FcεRIα expression is under circadian control in mast cells, FcεR1α mRNA levels were determined by quantitative real-time PCR in BMMC every 4 hours up to 72 hours following synchronization. FcεR1α mRNA expression displayed a circadian oscillation, with a robust peak at 12 hours and a depression around 22 hours following synchronization (Figure 3.6). Further, this circadian oscillation of FcεR1α mRNA levels was maintained up to 72 hours following synchronization.

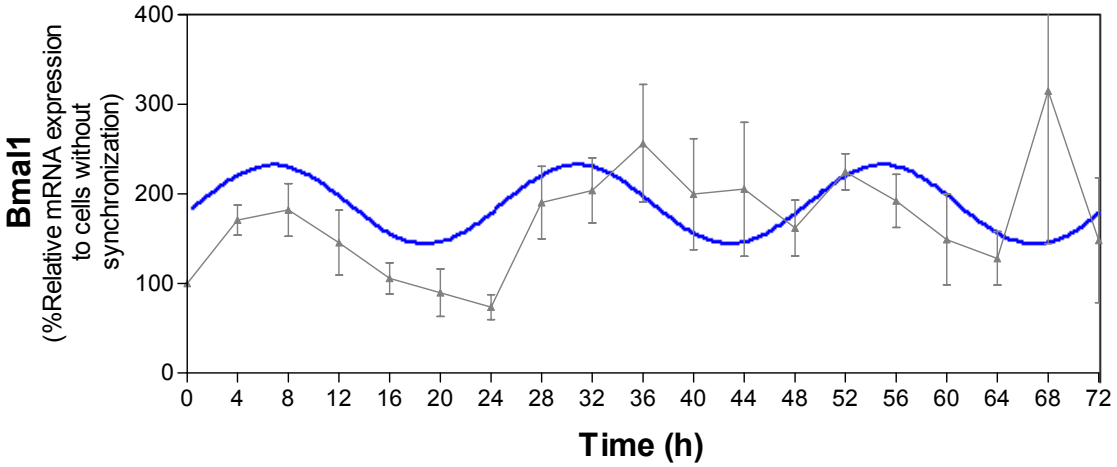
To investigate whether FcεRIα protein is expressed in a circadian pattern, we measured surface expression of FcεRI by flow cytometry every 4 hours following synchronization. Figure 3.7A shows the surface expression profiles of FcεRI at representative time points following synchronization of BMMCs. FcεRIα expression at 32 hours displayed a left shift as compared to 8 hours or 64 hours indicating that receptor expression at 32 hours is higher than the other 2 time points. Mean fluorescence intensity (MFI) of FcεRIα expression showed a circadian oscillation, with a robust peak at 28 hours following synchronization (Figure 3.7). The rhythm in FcεRIα surface expression levels was out of phase with mRNA levels (Figure 3.6 and Figure 3.7B). Without synchronization, MFI values of FcεRIα expression on BMMCs did not show any oscillation over 72 hours (data not shown).

Figure 3.2 Circadian clock gene expression in BMMCs following serum shock. mRNA expression of circadian clock genes (A) mPer2, (B) Bmal1, (C) Reverba, and (D) Dbp. mRNA expression was normalized to non-oscillating gapdh expression levels and expressed as a percentage of mRNA expression in non-serum shocked cells. Gray lines represent mean ± S.E.M. for expression data. The blue line displays best-fitting 24 hour cosine analysis predicted by Time Series Analysis-Single Cosinor v. 6.3 software. n = 6 independent experiments.

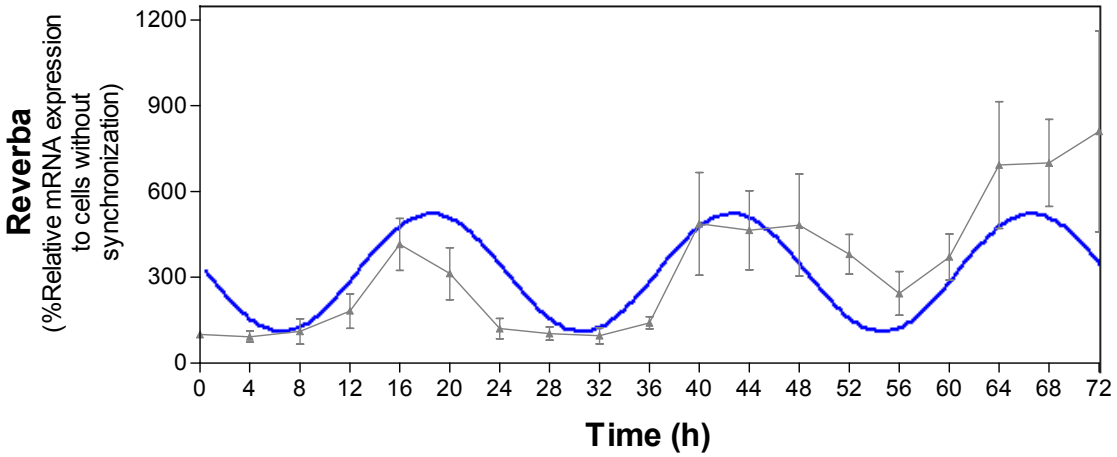
A


mPer2
(%Relative mRNA expression to cells without synchronization)
400
300
200
100
0
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72
Time (h)

B


Bmal1
(%Relative mRNA expression to cells without synchronization)
400
300
200
100
0
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72
Time (h)

C


Reverba
(%Relative mRNA expression to cells without synchronization)
1200
900
600
300
0
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72
Time (h)

D

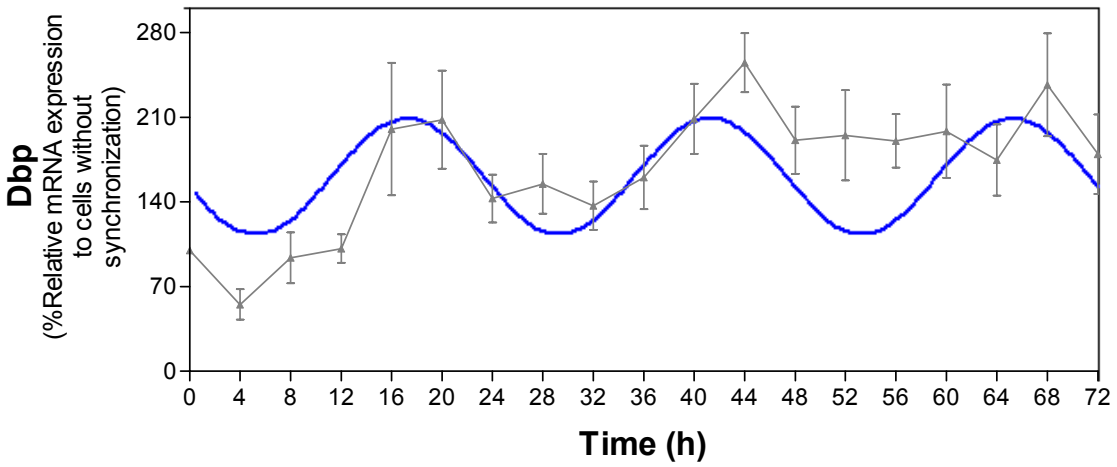

Dbp
(%Relative mRNA expression to cells without synchronization)
280
210
140
70
0
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72
Time (h)

Table 3.1 Peak Phases and Amplitudes of Circadian Clock Gene Expression in BMMCs

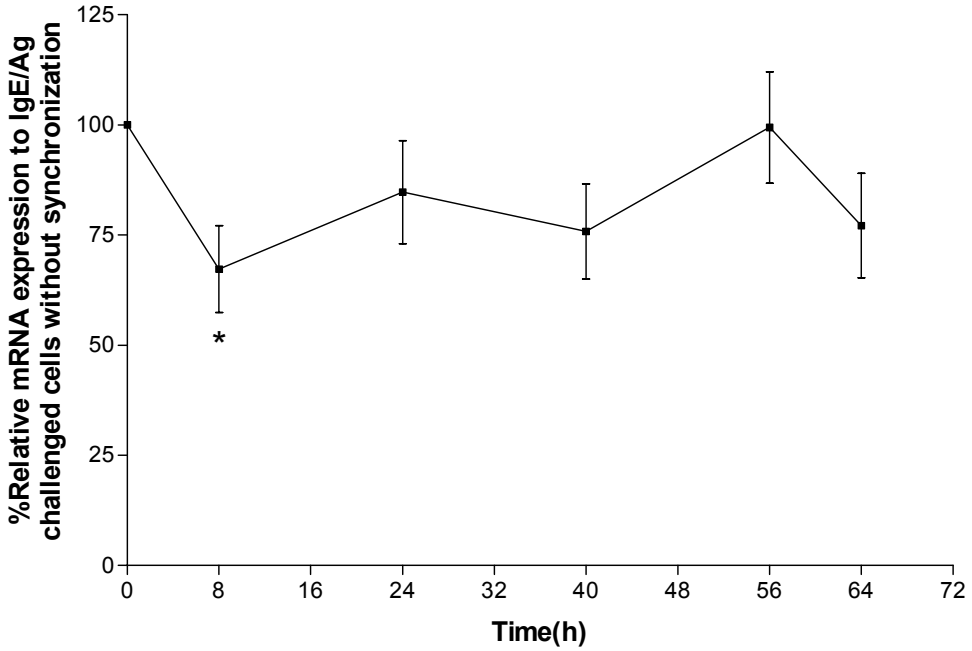
Circadian Gene	Peak-phase (h)	Amplitude*	*P* value ¶
Bmal1	5.93	85.30	0.0098
Dbp	17.27	48.00	0.0002
Rev-erbα	18.53	206.00	0.0001
mPer2	19.8	33.1	0.0056

*Amplitudes and ¶P values (H0: Amplitude=0) have been calculated by Time Series Analysis-Single Cosinor v. 6.3 software (Expert Soft Technologie). n = 6 independent experiments

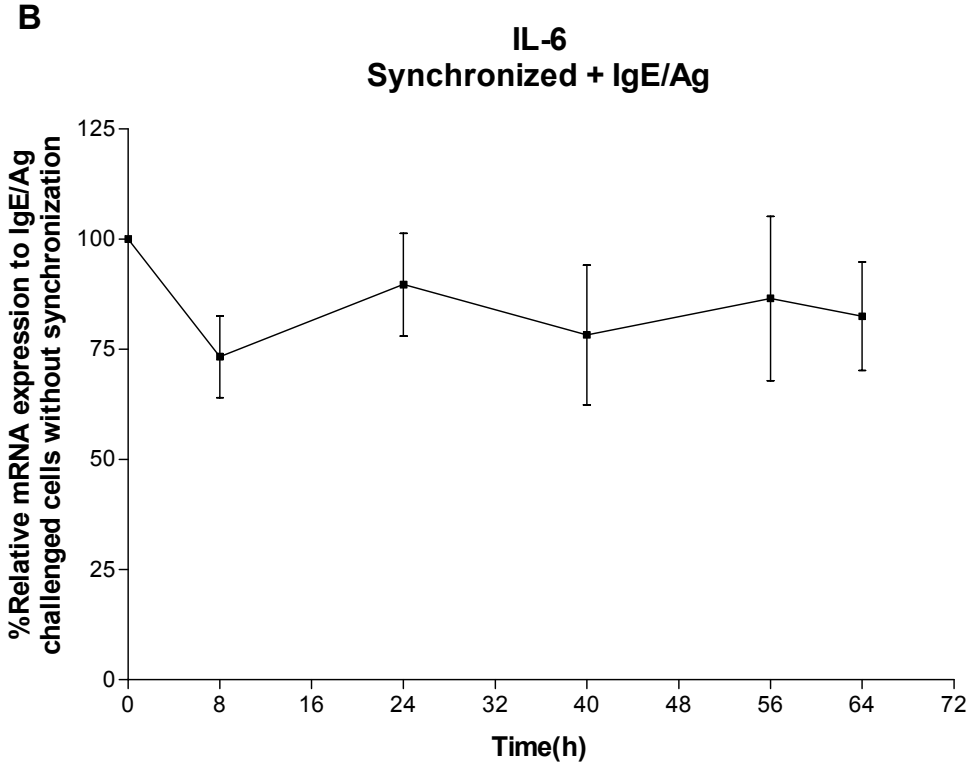
Figure 3.3 Cytokine mRNA expression in synchronized BMMCs following IgE-dependent and –independent activation.

BMMCs, following serum shock, were sensitized with IgE anti-DNP and stimulated for 30 min with (A & C) DNP or (B & D) ionomycin, and cells were collected. (A & B) IL-13 and (C & D) IL-6 mRNA expression following activation were normalized to non-oscillating Gapdh expression levels and expressed as a percentage of the non-serum shocked controls. Data is presented as mean ± S.E.M. n = 6 independent experiments.

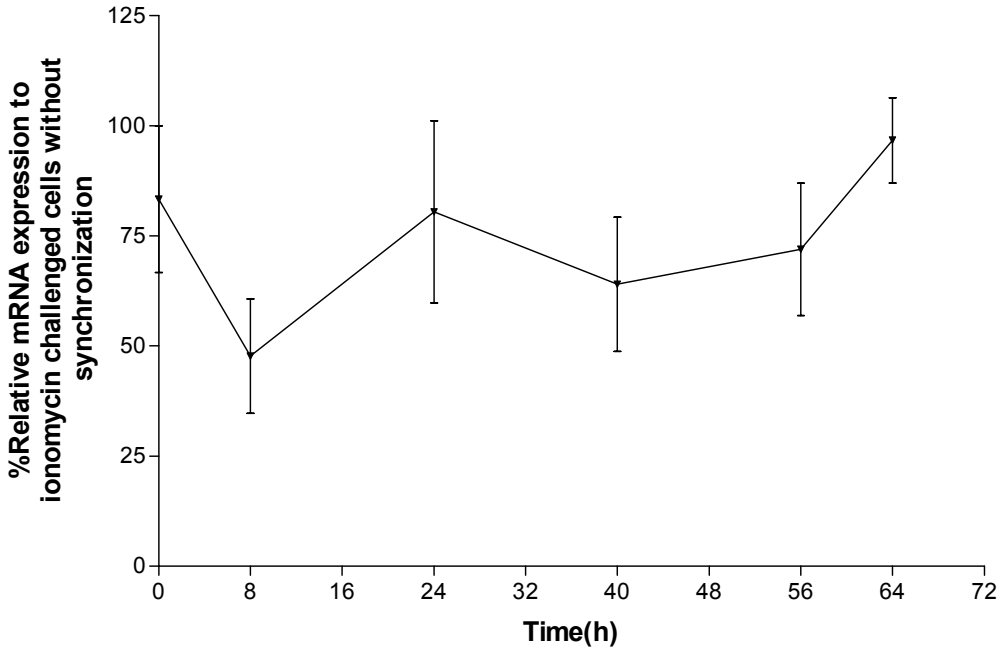
A


IL-13
Synchronized + IgE/Ag
%Relative mRNA expression to IgE/Ag challenged cells without synchronization
125
100
75
50
25
0
*
0
8
16
24
32
40
48
56
64
72
Time(h)

B


IL-6
Synchronized + IgE/Ag
%Relative mRNA expression to IgE/Ag challenged cells without synchronization
125
100
75
50
25
0
0
8
16
24
32
40
48
56
64
72
Time(h)

C


IL-13
Synchronized + ionomycin
%Relative mRNA expression to ionomycin challenged cells without synchronization
125
100
75
50
25
0
0
8
16
24
32
40
48
56
64
72
Time(h)

D

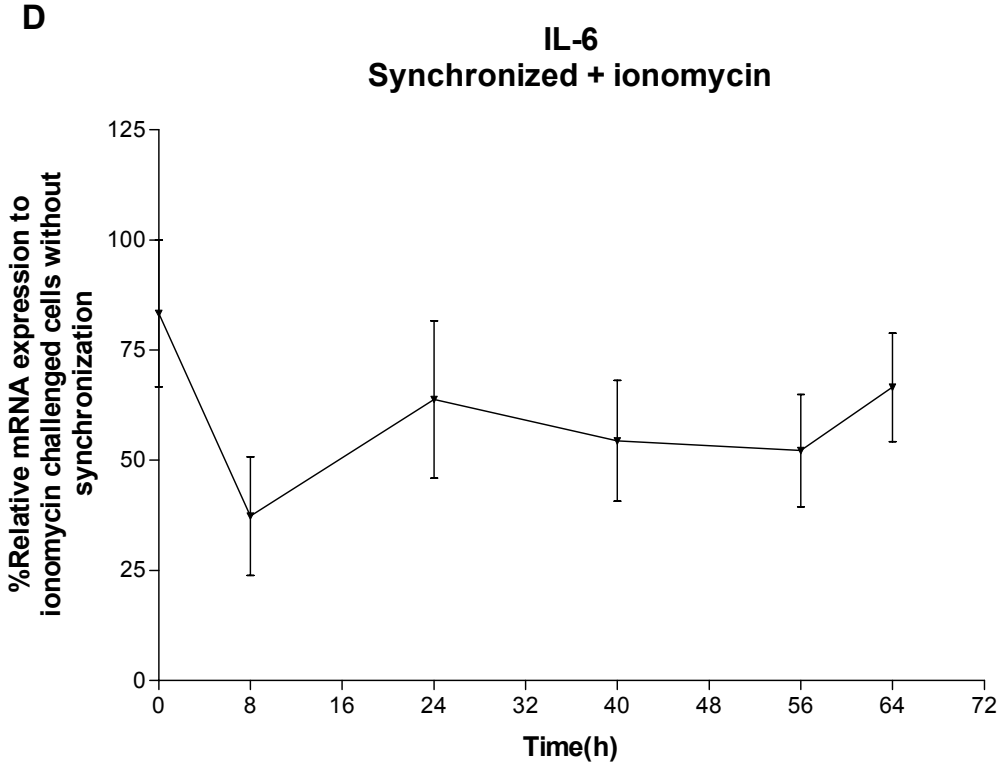
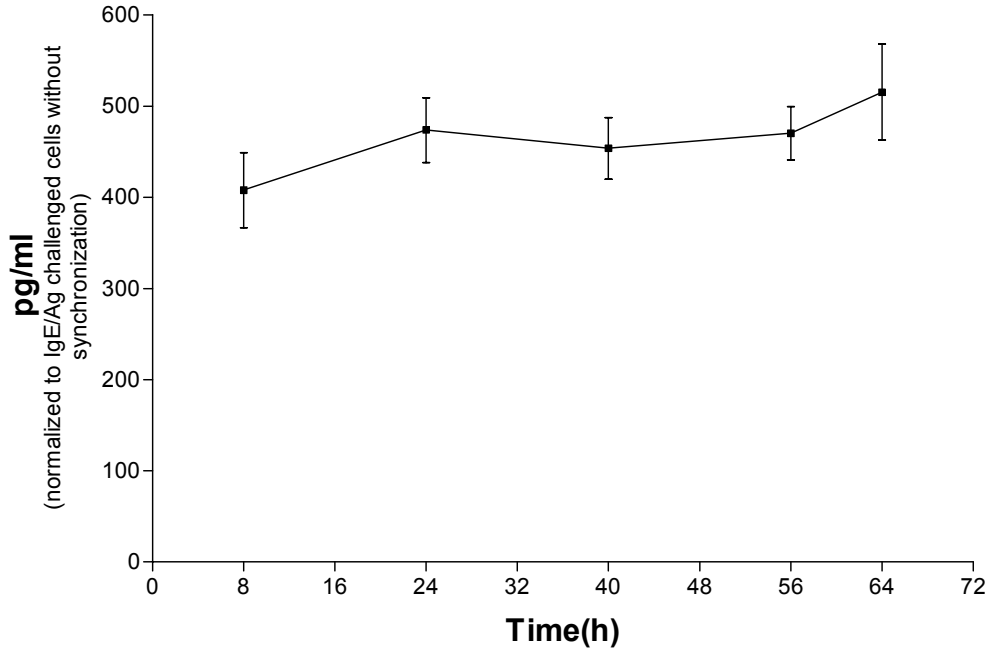

IL-6
Synchronized + ionomycin
%Relative mRNA expression to ionomycin challenged cells without synchronization
125
100
75
50
25
0
0
8
16
24
32
40
48
56
64
72
Time(h)

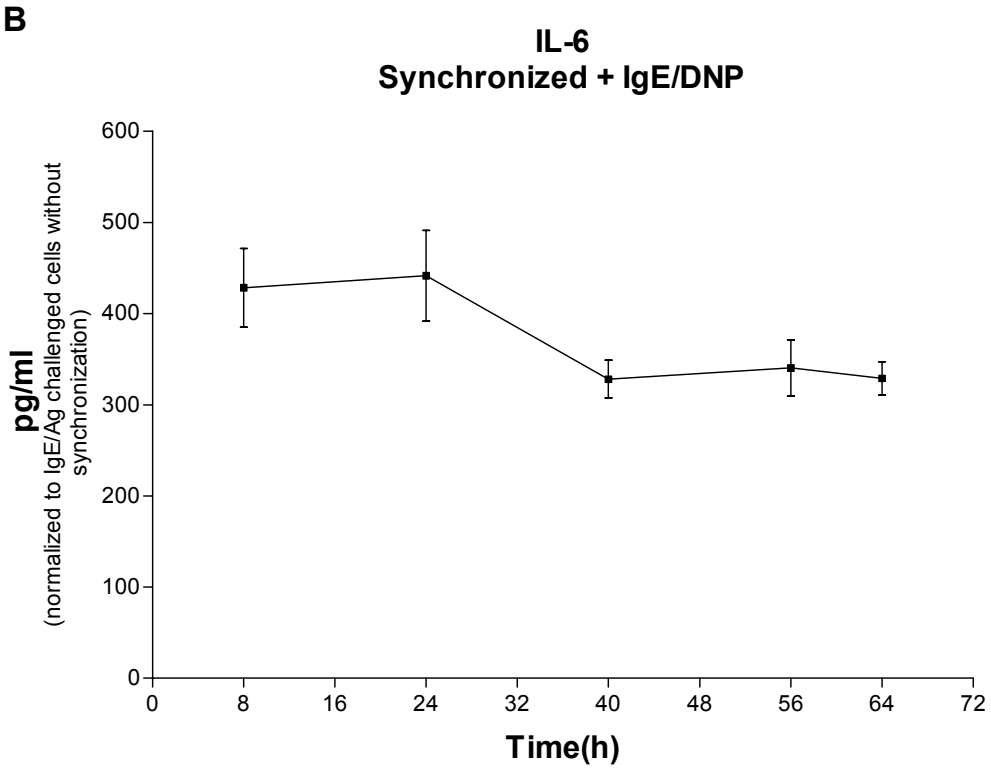
Figure 3.4 Cytokine expression in synchronized BMMCs following IgE-dependent and –independent activation.

BMMCs, following serum shock, were sensitized with IgE anti-DNP and stimulated for 4 hours with (A & C) DNP or (B & D) ionomycin, and supernatant were collected. (A & B) IL-13 and (C & D) IL-6 expression following activation were normalized to non-oscillating Gapdh expression levels and expressed as a percentage of the non-serum shocked controls. Data is presented as mean ± S.E.M. n = 3 independent experiments.

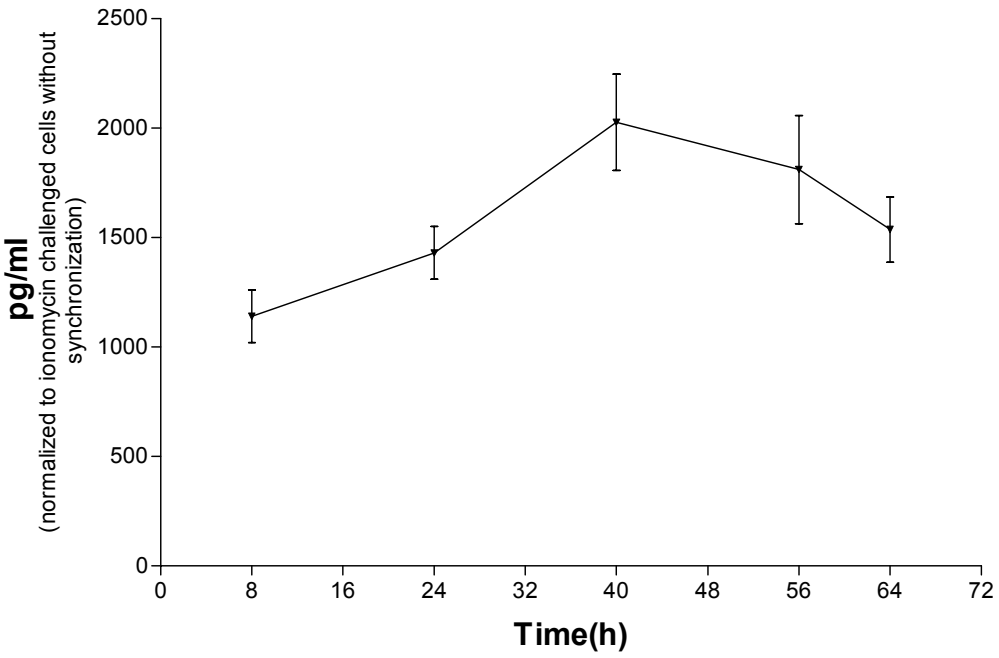
A


IL-13
Synchronized + IgE/DNP
pg/ml
(normalized to IgE/Ag challenged cells without synchronization)
600
500
400
300
200
100
0
0
8
16
24
32
40
48
56
64
72
Time(h)

B


IL-6
Synchronized + IgE/DNP
pg/ml
(normalized to IgE/Ag challenged cells without synchronization)
600
500
400
300
200
100
0
0
8
16
24
32
40
48
56
64
72
Time(h)

C


IL-13
Synchronized + ionomycin
pg/ml
(normalized to ionomycin challenged cells without synchronization)
2500
2000
1500
1000
500
0
0
8
16
24
32
40
48
56
64
72
Time(h)

D

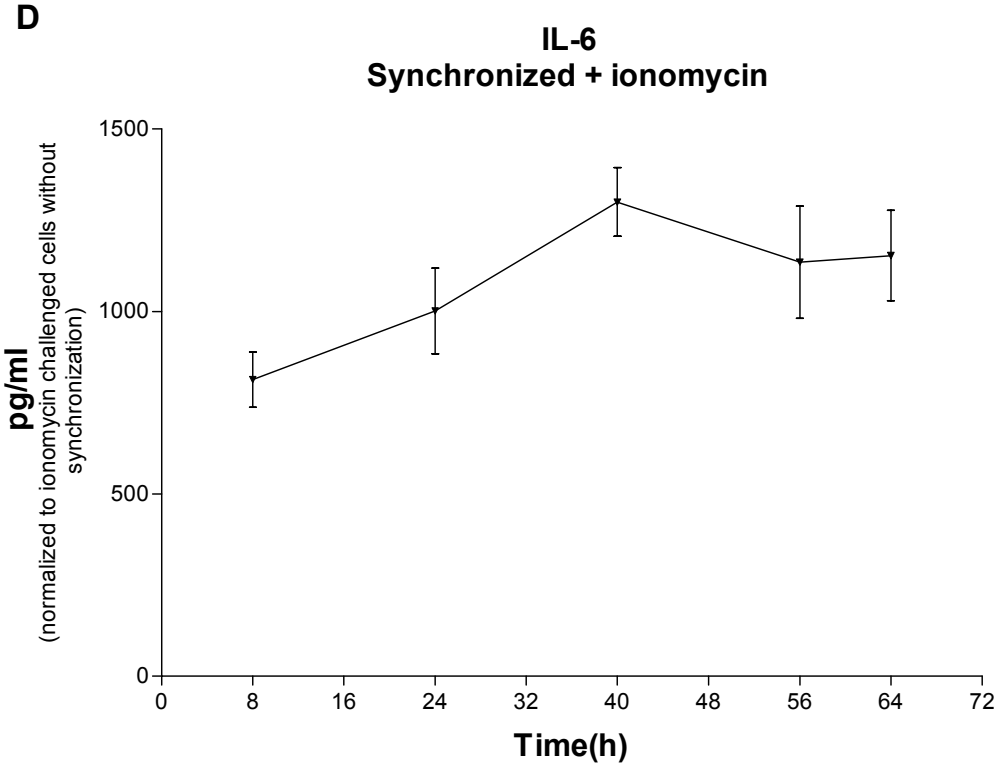

IL-6
Synchronized + ionomycin
pg/ml
(normalized to ionomycin challenged cells without synchronization)
1500
1000
500
0
0
8
16
24
32
40
48
56
64
72
Time(h)

Figure 3.5 Effect of FcεRIα-dependent activation on circadian gene expression. BMMCs were treated with serum, IgE+DNP, or both. Serum shock alone (solid line), IgE +DNP alone (dash line), serum shock in combination with IgE+DNP (dot line). *mPer2* expression were determined by quantitative real-time PCR, normalized to non-oscillating Gapdh, and expressed as percentage of 0 hour without serum shock. Data is presented as means ± S.E.M. n = 6 independent experiments. *: p <0.05 IgE+DNP vs50% HS or 50% HS + IgE/DNP

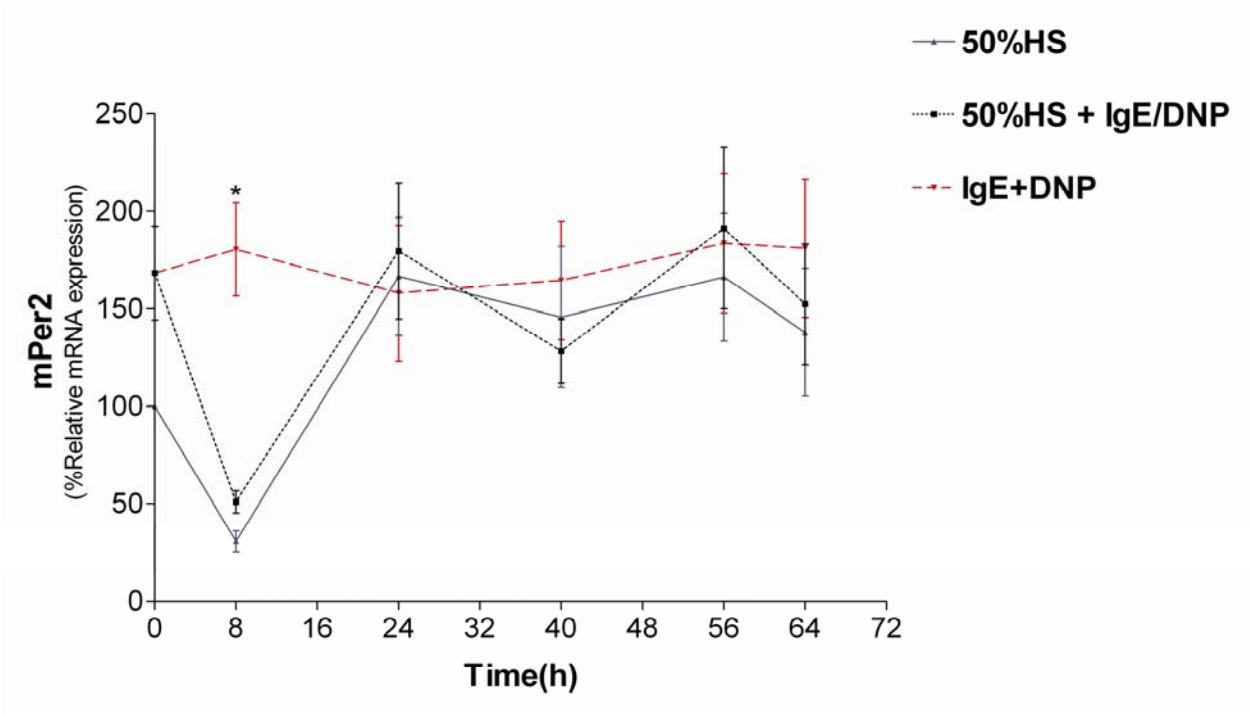

50%HS
50%HS + IgE/DNP
IgE+DNP
mPer2
(%Relative mRNA expression)
250
200
150
100
50
0
*
0
8
16
24
32
40
48
56
64
72
Time(h)

Figure 3.6 FcεRIα mRNA expression in BMMCs following synchronization.

mRNA levels of fcer1a in BMMCs following serum shock (mean ± S.E.M., n = 6, best-fitting cosine blue line).

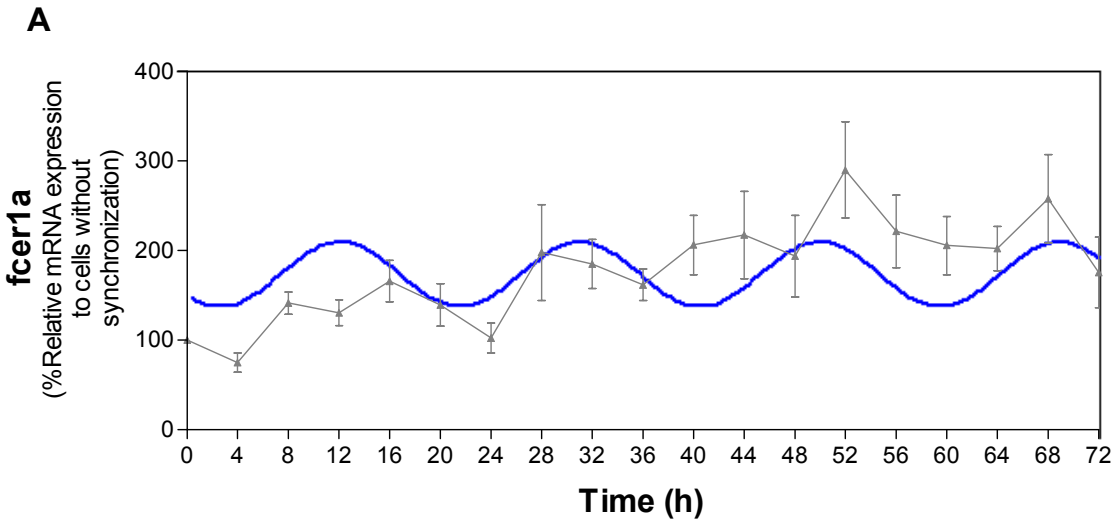
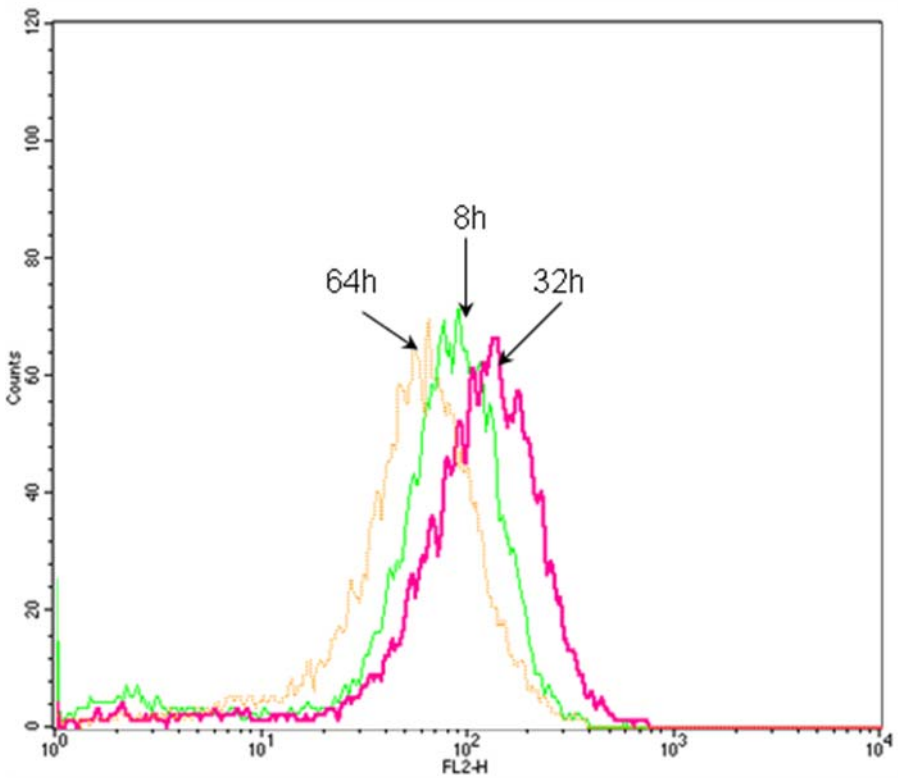

A
fcer1a
(%Relative mRNA expression to cells without synchronization)
400
300
200
100
0
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
Time (h)

Figure 3.7 FcεRIα expression in BMMCs following synchronization.

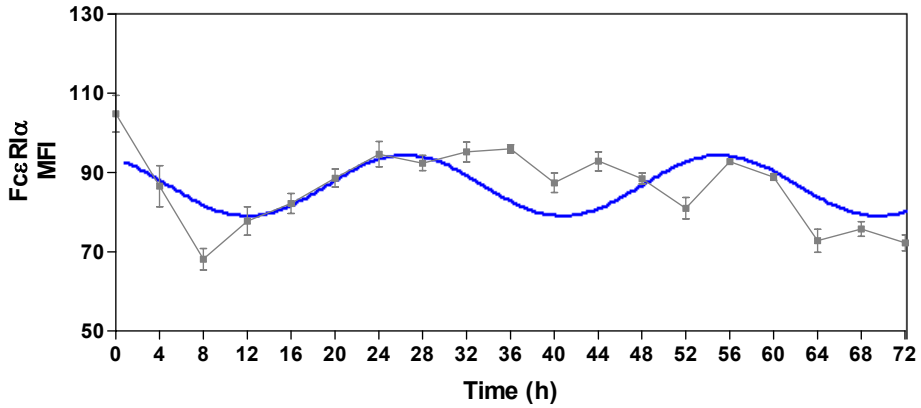
(A) Surface protein expression of FcεRIα in BMMCs following serum shock. Histogram on the left shows 3 representative time points (8h, 32h, and 64h). (B) Mean fluorescence intensity (MFI) values are shown as mean ± S.E.M. (gray line) at each time point with best-fitting cosine blue line (n=3).

A

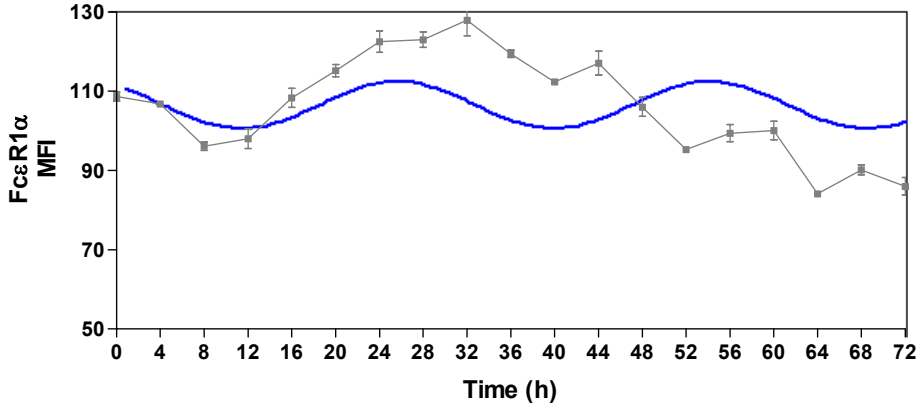


B

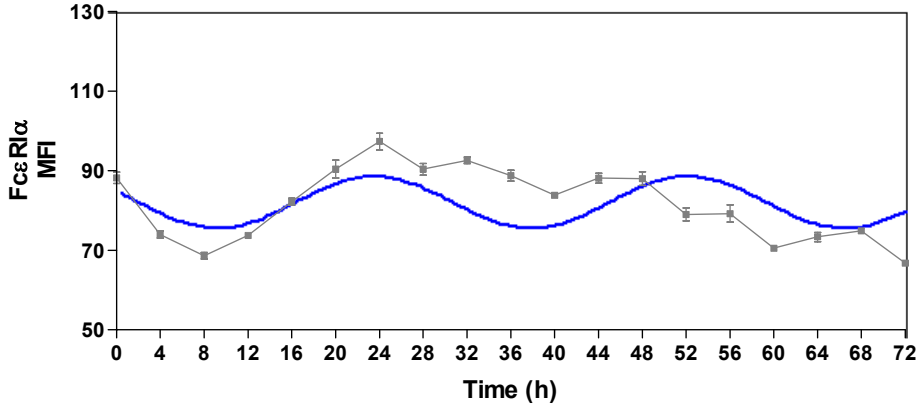
1st experiment



2nd experiment



3rd experiment



DISCUSSION

The most important finding in this study is that circadian clock genes are rhythmically expressed in serum synchronized mouse bone marrow-derived mast cells. Cytokine mRNA expression following IgE-dependent activation of BMMCs and serum synchronization exhibited a trend towards a circadian pattern. However, IgE/Ag stimulation of BMMCs did not shift existing circadian expression of a key circadian gene, *mPer2*. Following serum synchronization, the expression of FcεRIα displayed a circadian pattern that was associated with changes in cytokine mRNA expression in activated BMMCs.

Circadian clock genes are expressed in a number of immune cells including natural killer (NK) cells (Arjona *et al.*, 2005; Arjona *et al.*, 2006; Arjona *et al.*, 2008), macrophages (Keller *et al.*, 2009), and T-cell subsets (Dimitrov *et al.*, 2009). In this study, we provide the first evidence that circadian clock genes are expressed in BMMCs in a circadian pattern for up to 72 hours following serum shock. Serum shock is a common method utilized to synchronize the circadian clock in cell cultures and is equivalent to the ability of light cycles to entrain the circadian clock *in vivo*(Hastings *et al.*, 2003). It does not jumpstart damped circadian oscillators but instead synchronizes dephased circadian oscillators. This has been demonstrated in fibroblasts, where individual cells have been found capable of generating self-sustained circadian rhythms of gene expression (Balsalobre *et al.*, 1998; Nagoshi *et al.*, 2004). Here we report that serum shock synchronizes individual rhythms in BMMCs to a common phase as does it in fibroblasts (Balsalobre *et al.*, 1998; Nagoshi *et al.*, 2004), adipose-derived stem cells (Wu *et al.*, 2007), natural killer cells (Murphy *et al.*, 2007), and cardiomyocytes (Durgan

et al., 2005). The mechanism(s) underlying serum shock synchronization of circadian gene expression *in vitro* is still unknown. Neither the activation of cAMP-dependent kinases nor the glucocorticoid receptor appear to be required for the synchronization of circadian gene expression by serum shock (Balsalobre *et al.*, 2000b).

The phase relationship between rhythms of circadian genes in BMMCs is consistent with that in other cells. The heterodimer Bmal1 and Clock binding to E-box in the promoter region of other circadian genes to initiate their expression parallels with other cell types. In BMMCs, the expression of *Bmal1* occurs earlier than *Rev-erbα* and *mPer2*. Further, the increased expression of *Rev-erbα* correlates with decreased expression of *Bmal1*. Lastly, the expression of *mPer2* appears to inhibit the formation of the heterodimer BMAL1 and CLOCK. Taken together, these results indicate that mast cells contain a local intrinsic clock.

The function of a local intrinsic clock in BMMCs was investigated by measuring the activation of cells at specific time points following serum shock synchronization. Several studies have shown a circadian variation of cytokine levels in NK cells and macrophages (Arjona *et al.*, 2005; Hayashi *et al.*, 2007; Keller *et al.*, 2009). TNF-α and IL-6 secretion patterns exhibit a circadian oscillation in *ex vivo* LPS-stimulated macrophages (Keller *et al.*, 2009). Here, we report rhythms in mRNA levels of IL-13 and IL-6 in IgE-mediated activation of synchronized BMMCs. Mast cells, activated through antigen- and IgE-dependent aggregation of the high affinity IgE receptor, FcεRI, produce a broad range of mediators, including preformed mediators and newly synthesized cytokines/chemokines (Galli *et al.*, 2008; Kalesnikoff *et al.*, 2008; Brown *et*

al., 2008a). Newly synthesized cytokines produced by mast cells include, but are not limited to, IL-6, IL-13, and TNF-α. We tested IL-13 production over time due to the important role of IL-13 in allergic asthma by initiation of a late phase response(Toru *et al.*, 1998), induction of IgE synthesis by B cells(Punnonen *et al.*, 1994), proliferation of mast cells, and increasing FcεRI expression(Kaur *et al.*, 2006). The phases of IL-13 mRNA were oscillated with nadirs at 8 hours and 40 hours and peaks at 24 hours and 56 hours. These observations suggest mast cells stimulated by IgE/Ag along the circadian cycle could possibly have different outcomes. The oscillation of cytokine mRNA produced by IgE-dependent activation of mast cells may be associated with circadian pathological responses that have been observed in patients with mastocytosis(Catini *et al.*, 1994) and nocturnal responses in allergic asthma (Smolensky *et al.*, 2007).

Other than activation through FcεRI, mast cells can also participate in physiological and pathological processes as a result of their activation by certain chemicals. To verify whether circadian oscillation of mast cell activation is dependent upon FcεRI expression or represents an intrinisic change in mast cell state, we used ionomycin to activate synchronized BMMCs independent of FcεRI and measured cytokine production over 72 hours. Interestingly, IL-13 mRNA did not display an oscillation pattern following ionomycin challenge as observed following IgE/Ag stimulation of BMMCs. This result provides evidence that circadian activation of mast cells may be FcεRI-dependent.

To further investigate IgE-dependent circadian activation of BMMCs, FcεRI expression was measured up to 72 hours. The diurnal variations in FcεRIα expression first observed in rat pineal glands suggests a biological significance of the timing of receptor function (Ganguly *et al.*, 2007). Although we cannot formally exclude other influences, we speculate that the circadian oscillation of cytokine production in IgE/Ag stimulated BMMCs is due to circadian variation in FcεRIα expression. In fact, the IL-13 mRNA levels observed in this study from IgE/Ag stimulated BMMCs peak at 24 hours and 56 hours, when the surface expression of FcεRIα peaks. Further experiments are required to unravel the underlying mechanism and determine which circadian factors regulate the oscillation of FcεRIα in BMMCs.

Even though it appears that the circadian system can influence IgE/Ag activated BMMCs, the influence of IgE/Ag stimulation on the phases, phase relations, and amplitudes of circadian genes is still unknown in mast cells. Evidence suggests that the relationship of the immune system and the circadian system is bidirectional (Coogan *et al.*, 2008). In this study, we show circadian expression of *mPer2* can be triggered by serum shock but not IgE/Ag stimulation alone. Meanwhile, the initiated circadian phase of *mPer2* by serum shock cannot be shifted via IgE/Ag stimulation. We cannot, however, exclude the prolonged effects of IgE/Ag on BMMCs, including the production of proinflammatory cytokines, such as TNF-α, which could phase shift the clock in BMMCs.

In conclusion, BMMCs rhythmically express circadian clock genes, and BMMC activation through IgE/Ag exhibits a trend towards a circadian pattern which is linked to

circadian expression of FcεRIα. The local conserved circadian clock in mast cells begins to provide further understanding of the dynamics in allergic disease and in particular diseases such as allergic asthma and mastocytosis where diurnal patterns have been observed. By increasing our understanding of the circadian regulation of mast cells, we may begin to explore novel therapeutic strategies in the treatment of allergic disease.

CHAPTER 4: GENERAL DISCUSSION

The present study was undertaken to determine the effects of circadian output on nocturnal asthma, and to identify potential mechanisms of circadian rhythm in nocturnal asthma by studying the circadian characteristics of mast cells.

In Aim 1, we used nonhuman primates (NHP), which exhibit a high level of similarity to humans in genetic, anatomic, and physiological aspects, and are well suited to modeling human diseases such as asthma (Coffman *et al.*, 2005). The HDM model of allergic asthma in Cynomolgus macaques (referred to subsequently as "the NHP model") reproduces the key features of human asthma, including elevated serum IgE levels (Table2.1) , β-agonist reversible bronchoconstriction (Figure 2.1), and late phase eosinophilic inflammation (Table 2.1) (Van Scott *et al.*, 2004). In addition, the airways become hyperresponsive to nonspecific bronchoconstrictors including histamine, methacholine (Table 2.2), and adenosine(Van Scott *et al.*, 2004; Van Scott *et al.*, 2005). $CD4^+$ T-lymphocytes and NKT cells in BAL fluid are increased, and Th2 lymphocyte cytokines are up-regulated (Ayanoglu *et al.*, 2010). The severity of the asthma phenotype in the NHP model depends on the schedule of allergen challenges and the provocative dose of dust mite. At an exposure periodicity of 4 to 6 week intervals as used in this study, the animals have minimal symptoms between exposures, but on the day of provocation, exhibit a severe acute asthmatic response, including decrease in arterial O_2 saturation to less than 80% and in some animals less than 70%. Upon resolution of the acute response the animals develop eosinophilia that is equivalent to

what is observed following segmental allergen challenge of asthmatic humans(Erpenbeck *et al.*, 2006). As in humans, the symptoms are attenuated with corticosteroids (Van Scott *et al.*, 2004; Ayanoglu *et al.*, 2010). Extensive terminal protocols have not been conducted on these animals, but in a few animals that have been examined, airway wall remodeling and mucosal eosinophilia has been observed (Van Scott *et al.*, 2004). These characteristics are consistent with mild to moderate, intermittent asthma in humans that is well controlled by β-agonists and corticosteroids.

In the present study, we used RIP parameters (eBPRL and ePhRL), which exhibited a high degree of correlation with the conventional measures of airway function, to document the nocturnal asthmatic response in the NHP model of asthma. Both eBPRL and ePhRL gradually increase during the night following an early morning HDM challenge with a peak around 4 AM (Figure 2.4), which is consistent with the time course of nocturnal symptoms in asthmatic patients (Irvin *et al.*, 2000). Nocturnal increase in RIP parameters may therefore be linked to underlying bronchoconstriction. To fully verify the nocturnal bronchoconstriction in allergic NHP, however, further studies must be conducted.

To further study nocturnal responses in allergic NHP, nocturnal bronchoconstriction ideally should be measured by forced oscillatory technique (Scireq Flexivent system) and nocturnal inflammation should be determined by the mediators in BAL samples and blood samples when eBPRL and ePhRL peak during the night. The forced oscillatory technique determines pulmonary mechanics by superimposing small external pressure signals on the spontaneous breathing of the subjects (Oostveen *et al.*,

2003). In our study, the measurement of nocturnal bronchoconstriction was conducted by forced oscillatory technique as well as RIP. Unfortunately, animals awakening during the night increases stress levels in the subjects. Stress has been considered a powerful manipulator in human airway function (Chrousos, 1995) due to the increased release of cortisol and epinephrine. Cortisol exerts anti-inflammatory effects and upregulates β-adrenergic receptor function; epinephrine induces bronchodilation and stabilizes the mast cell membrane attenuating the release of proinflammatory mediators (Smolensky *et al.*, 2007). In these cohort animals, strong stresses have been found to limit inflammation resulting in the attenuation of symptoms for up to 6 months. In addition to the stress produced in animal subjects by awakening, studies in asthmatics with nocturnal symptoms have shown that the disturbance of sleep has profound effects on nocturnal bronchoconstriction by decreasing airway resistance during the night (Ballard *et al.*, 1989). Therefore, nocturnal bronchoconstriction in allergic NHP could not be measured directly by conventional pressure flow analysis or the forced oscillatory technique.

Isolation of mast cells from NHP's on regular basis was not feasible, and therefore we transitioned from a monkey model to a mouse model. However, as mice are nocturnal animals, they are not suitable to study nocturnal asthma. To further study the cellular mechanisms underlying circadian variation in asthma symptoms, bone marrow-derived mast cells (BMMCs) were collected from femoral bone marrow in C57/BL6 mice and cultured for 4 weeks *in vitro.* These cells have been dissociated from the body, and thus are uninfluenced by nocturnal features of the animals. Cultured BMMCs remain growth factor-dependent (IL-3 dependent), express high levels of c-kit

and high-affinity IgE receptor, FcεRI, contain preformed mediators that are present in the characteristic granules, and express some inducible mediators. Thus, BMMCs provide a powerful tool for investigating the underlying mechanisms of asthma *in vitro*.

In Aim 2, serum shock induced periodic expression of circadian genes in BMMCs up to 72 hours, which indicated an endogenous circadian clock in BMMCs. *In vitro*, circadian oscillations have been documented in individual cells of NIH3T3 fibroblast lines (Nagoshi *et al.*, 2004). However, self-sustained oscillation in populations of cells is unsynchronized and can be synchronized by serum shock(Balsalobre *et al.*, 1998; Nagoshi *et al.*, 2004). In the present study, serum shock synchronized individual rhythms in BMMCs to a common phase as does it in fibroblasts (Balsalobre *et al.*, 1998; Nagoshi *et al.*, 2004), adipose-derived stem cells (Wu *et al.*, 2007), natural killer cells (Murphy *et al.*, 2007), and cardiomyocytes (Durgan *et al.*, 2005). Even though the mechanism(s) underlying serum shock synchronization of circadian gene expression in vitro is still unclear, the ability of serum shock is thought to be equivalent to the ability of light cycles to entrain the circadian clock *in vivo* (Hastings *et al.*, 2003). Other than serum shock, certain pharmacological substances, including β_2 –adrenoceptor agonists and dexamethasone, can synchronize the uncoordinated circadian oscillation in cells (Takata *et al.*, 2005; Wu *et al.*, 2007; Wu *et al.*, 2008). In our study, we used serum shock to synchronize the BMMCs because such pharmacological substances synchronize the BMMCs at the expense of proper mast cell functioning.

The current study has demonstrated that circadian clock genes are not only expressed in BMMCs but also oscillate in a circadian pattern following serum shock.

The expression of *Bmal1* is earlier than the expressions of *Rev-erbα* and *mPer2* because BMAL1 and CLOCK heterodimers bind to the E-box on the promoter of the genes and initiate *Rev-erbα* and *mPer2* expressions. On the other hand, both the expressions of *Rev-erba* and *mPer2* inhibit *Bmal1* gene expression or BMAL1 protein binding with CLOCK, respectively (Figure 4.1). This phase relationship among circadian genes in BMMCs is consistent with that in other cell types. In summary, these findings indicate that mast cells contain a local intrinsic clock.

In Aim 2, we also investigated the effects of the endogenous clock on mast cell functions including cytokine production and surface receptor expression. Cytokine levels were measured at different time points along the circadian clock gene oscillation. IL-13 mRNA produced by IgE-dependent activation in synchronized BMMCs showed circadian changes in their levels over 72 hours (Figure 3.3 A and B). However, IL-13 mRNA produced by ionomycin activated synchronized BMMCs did not exhibit circadian changes (Figure 3.3 C and D). These results indicated that cytokine mRNAs, which are associated with allergic asthma and are produced by mast cells through an IgE-dependent pathway, exhibit a trend towards a circadian pattern.

In human asthma, IL-13 mRNA in BAL cells and IL-13 in BAL fluid are significantly increased (Huang *et al.*, 1995). The increased IL-13 is associated predominantly with the late asthmatic response and is correlated with eosinophil numbers. In present study, we showed that IL-13 mRNA oscillated in mast cells, which are predominant cellular sources of IL-13. Studies have shown that the number of BAL cells expressing IL-13 mRNA in nocturnal asthma was increased at 4:00 AM as

compared with 4:00 PM (Kraft *et al.*, 2001). The circadian oscillation of IL-13 mRNA in mast cells might directly influence nocturnal expression of asthma symptoms in human.

To further study the mechanism behind the IgE-dependent cytokine mRNA oscillation in BMMCs, the surface expression of FcεRIα was measured for up to 72 hours. Both mRNA levels and protein levels of FcεRIα exhibit periodic oscillation following serum shock. Interestingly, the IL-13 mRNA levels observed in this study from IgE/Ag stimulated BMMCs peak at 24 hours and 56 hours, paralleling peak surface expression of FcεRIα (Figure 3.3, Figure 3.6 and Figure 3.7). These results indicate that circadian oscillation of IL-13 mRNA following IgE-dependent activation is at least in part due to circadian oscillation of FcεRIα.

However, the cytokine proteins produced by IgE-dependent activation of synchronized BMMCs did not show any circadian oscillation due to a 4 hour delay in supernatant collection. In mast cells, IgE receptors can be recruited to cell surface and stabilized by exposing the cells to IgE *in vitro* (Kitaura *et al.*, 2004). This time consuming process might diminish the oscillation of receptor surface expression *in vitro*. In addition to IgE receptor recruitment, 4 hours of cytokine release will lead to an accumulation of cytokine levels in the supernatant, perhaps dampening circadian effects on cytokine release. Nevertheless, cytokine mRNA levels were measured in a much shorter period than that of protein levels. Therefore, without time disturbance, the circadian oscillation of cytokine mRNA was observed in synchronized BMMC through an IgE-dependent pathway.

In the current study, we investigated the influence of IgE/Ag simulation on the phases, phase relations, and amplitudes of circadian genes. Previous studies have shown that the relationship of the immune system and the circadian system is bidirectional (Coogan *et al.*, 2008). Circadian expression of *mPer2* can be triggered by serum shock but not IgE/Ag stimulation alone (Figure 3.5). Meanwhile, the initiated circadian phase of *mPer2* by serum shock cannot be shifted via IgE/Ag stimulation. We cannot, however, exclude the prolonged effects of IgE/Ag on BMMCs, including the production of proinflammatory cytokines, such as TNF-α, which could phase shift the clock in BMMCs.

Overall, this dissertation has increased our understanding of how the circadian clock system underlies the nocturnal expression of asthma symptoms (Figure 4.2). The results have provided first direct evidence that the circadian time system in mast cells modulates their function. However, the exact mechanism of circadian variation in mast cells directly affects nocturnal asthma need further studies.

Future Studies

Future studies must be conducted to further characterize the mechanism for augmenting nocturnal inflammation in asthmatics. One possibility is the evening reduction in cortisol, which may allow the inflammatory cascade to proceed. If the technique for remotely drawing the blood is available, we could collect the blood from

NHP at different time points to study the serum cortisol levels without disturbing the animals.

In our study, we demonstrate an effect of time of day on cytokine production and receptor expression in mast cells, but it is still unknown whether these time-dependent effects are induced directly by a local endogenous circadian clock. To answer this question, we would need knockout/knockdown circadian clock genes to determine whether there is a phase shift or attenuated expression of cytokines or receptor.

To further prove the effects of the mast cell circadian clock on nocturnal asthma *in vivo*, lung mast cells in NHP need to be studied by sacrificing animals and analyzing the circadian clock in lung tissues. However, it is too expensive to conduct such experiments. Alternatively, if the technique for remotely drawing the blood is available, basophils, which share many features with mast cells such as expression of FcεRI (Stone *et al.*, 2010), could be collected through blood and studied for circadian effects on nocturnal asthma.

Figure 4.1 Schematic representation of molecular circadian clock in mast cell

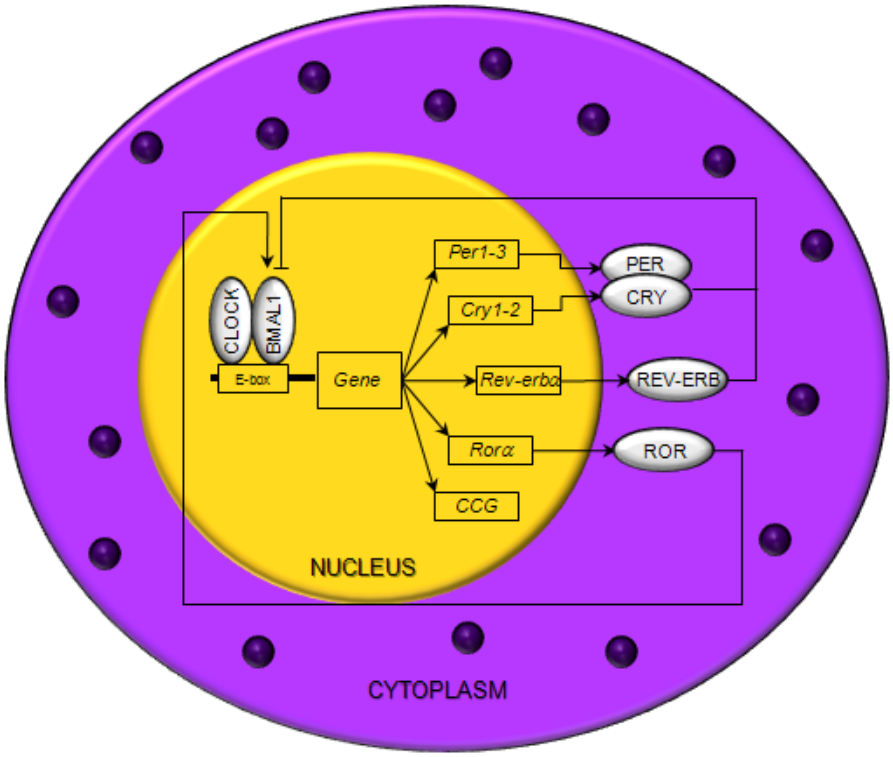
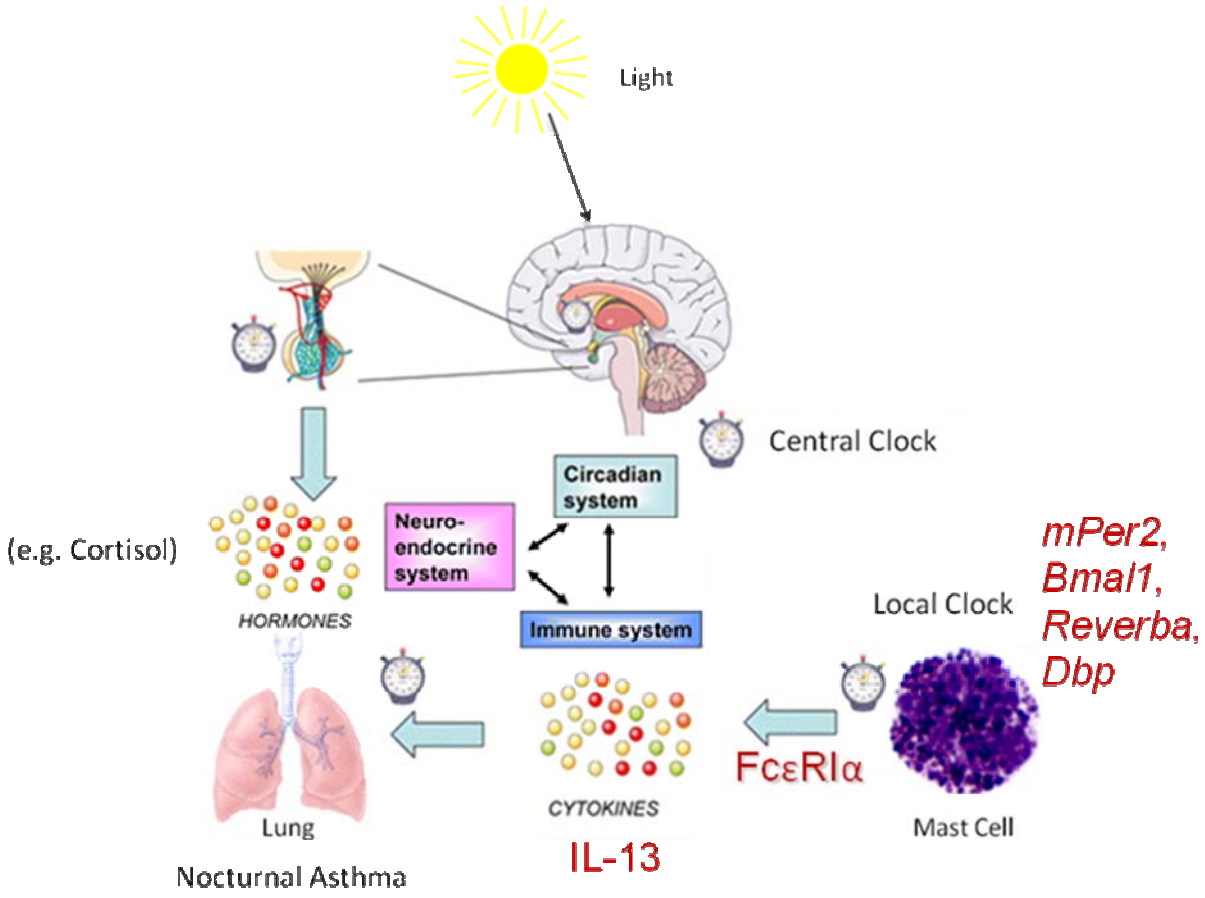

CLOCK
BMAL1
E-box
Gene
Per1-3
Cry1-2
Rev-erbα
Rorα
CCG
PER
CRY
REV-ERB
ROR
NUCLEUS
CYTOPLASM

Figure 4.2 Circadian outputs from mast cells mediate nocturnal symptoms in asthma



Modified from Esquifino et al., 2007

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Ref Type: Generic

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APPENDIX A: ANIMAL CARE AND USE COMMITTEE PROTOCOL APPROVAL

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

December 19, 2007

Michael Van Scott, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Van Scott:

Your Animal Use Protocol entitled, "Nocturnal Asthma in Dust Mite-Sensitive Macaques," (AUP #Q249) was reviewed by this institution's Animal Care and Use Committee on 12/19/07. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G Carroll, Ph.D

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

EAST CAROLINA UNIVERSITY

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

September 25, 2008

Jared Brown, Ph.D.
Department of Pharmacology
Brody 6S-10
ECU Brody School of Medicine

Dear Dr. Brown:

Your Animal Use Protocol entitled, "Generation of Murine Bone Marrow Derived Mast Cells," (AUP #W215) was reviewed by this institution's Animal Care and Use Committee on 9/25/08. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to biohazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure