Restraint stress fails to modulate cutaneous hypersensitivity responses in mice lacking the adenosine A₁ receptor

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Abstract Psychological stress has long been associated with effects on immune function and disease. In particular, differential effects of acute and chronic stress on skin immunity occur in the rodent restraint stress model, with acute stress enhancing and chronic stress suppressing cutaneous hypersensitivity. Extracellular levels of adenosine are known to modulate diverse biological activities in the CNS and peripheral tissues and serve an important protective function against physiological stressors such as inflammation and ischemia. In this study, we utilized the restraint stress model and the skin sensitizer dinitrofluorobenzene to test the hypothesis that perceived stress influences contact hypersensitivity through an adenosine A₁ receptor-mediated mechanism. We subjected hapten-sensitized A₁ receptor knockout (A1 KO) mice and their wild-type (WT) littermates to either acute (2.5 h) or chronic (5 h daily × 4 weeks) restraint stress, followed by hapten re-challenge of the pinna. Daily measurements of the resulting pinna swellings from each group were compared to reactions in non-stressed controls. In WT mice, pinna swelling was augmented in acutely stressed mice and suppressed in the chronically stressed group. In contrast, contact hypersensitivity responses in the A1 KO mice failed to be affected by either acute or chronic stress. Absence of the adenosine A₁ receptor did not affect levels of plasma corticosterone or urine catecholamines under these stressful conditions but did lead to reduced numbers of circulating neutrophil granulocytes compared to stressed WT animals. These results suggest that the adenosine A₁ receptor pathway plays a role in the process

by which perceived psychological stress influences the contact hypersensitivity response.

Keywords Contract hypersensitivity · Adenosine · Adenosine receptor

Introduction

Effects are both beneficial and deleterious on systemic immunity and health which have been attributed to stress perceived by the CNS. In particular, differential effects of acute and chronic stress on skin immunity have been demonstrated in the rodent model of restraint stress [1], with acute stress enhancing and chronic stress suppressing cutaneous hypersensitivity, an antigen-specific immune response primarily mediated by T lymphocytes [2]. Directed leukocyte trafficking to skin and lymphoid organs that occurs with stress of short duration may provide protective, immune-surveillance under conditions of potential tissue injury. In contrast, the immune-suppressive effects of prolonged stress exposure may lead to increased susceptibility to disease.

Stress hormones, including catecholamines and corticosterone, are released locally and systemically in response to CNS perception of stress through activation of the sympathetic nervous system (SNS) and the hypothalamus–pituitary–adrenal (HPA) axis [3, 4]. Studies have shown that the dose and duration of stress hormones are critical factors in determining their downstream immune effects. For example, although corticosterone at high doses or when chronically administered can suppress immunity, smaller physiological doses of acutely administered corticosterone or the catecholamine epinephrine have immune enhancing properties [5]. The upstream signaling pathways within the

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nervous system that regulate stress-altered peripheral immunity remain unknown.

The purine adenosine is generated by degradation of released adenine nucleotides (ATP, ADP, AMP) and is constitutively present at low levels in the extracellular environment [6]. However, these adenosine levels markedly increase under metabolically stressful conditions, and biological systems have evolved to utilize fluctuations in adenosine levels to activate protective functions against physiological stress. Adenosine affects most cell types and tissues, acting through four purine receptor subtypes (A_1 , A_{2A} , A_{2B} , and A_3) to modulate a diverse range of biological activities that include sleep, behavior, and cognitive function, immunity, cell metabolism, vascular tone, and pain perception [7]. Within the CNS, the adenosine A_1 receptor is found in especially high concentrations in the hippocampus and the dorsal horns of the spinal column [8]. Through inhibitory effects on synaptic transmission, the A_1 receptor modulates neurotransmitter function, with downstream effects on neurobehavior. Mice lacking the A_1 receptor display increased aggressive behavior and anxiety, along with a lowered threshold for pain sensitivity [9]. Adenosine specific receptors in the CNS may also play a role in the stress hormone response. For example, studies in the rat have demonstrated that restraint stress increases both adenosine content in the anterior pituitary and the number of A_1 receptors in the hypothalamus [10, 11]. In addition, adenosine administration has been shown to increase plasma corticosterone levels independent of endogenous corticotrophin-releasing factor, suggesting a regulatory role for adenosine on adrenocortical secretion at the level of the anterior pituitary gland [11]. Adenosine can also inhibit or facilitate norepinephrine release into blood by nerve terminals through activation of pre-junctional A_1 or A_2 receptors, respectively [12]. Finally, evidence has shown that adenosine A_1 receptors within the CNS can directly modulate peripheral immunity. Direct activation of dorsal horn A_1 receptors in the spinal cord with cyclohexyladenosine was shown to suppress neutrophil accumulation in the skin and attenuate inflammation and joint destruction in an adjuvant arthritis rat model [13, 14].

Since adenosine levels modulate immune function and play a protective role against physiologic stress but are also involved in neurological function and behavior, we hypothesized that the adenosine signaling pathway plays a key role in the protective immunity occurring in response to perceived psychological stress. We describe here results from these initial experiments using adenosine A_1 receptor knockout (A_1 KO) mice in a contact hypersensitivity model subjected to restraint stress. We report here that stress, either acute or chronic, fails to alter contact hypersensitivity responses in previously sensitized A_1 KO mice exposed to hapten re-challenge. Furthermore, absence of the adenosine

A_1 receptor did not affect the release of stress hormones under these stressful conditions, but did appear to change how stress influences circulating granulocyte numbers.

Materials and methods

Animals A_1 KO mice used in this study were on a B129 background, derived from the C57/B16 strain [9]. Female wild-type (WT) and A_1 KO littermates of heterozygous parents were primarily used in all studies at an age range of 6–12 weeks. At weaning, a 0.5-cm-tail sample was removed for DNA collection and genotyping using RT-PCR as described previously [9]. Animals were maintained in the NYU School of Medicine animal facility with 12 h light–dark cycle and kept on a standard mouse diet. To maintain cleanly a differential model of perceived stress, careful attention was paid to any exposure to non-specific stress in the animals throughout the protocol. Stress and non-stressed animal cages were housed completely separately once the stress component was initiated. Manipulations (blood or urine collection) were done one animal at a time, with remaining animals in a cage left undisturbed in an adjacent room. All experiments were approved by the NYU School of Medicine Institutional Animal Care and Use Committee.

Cutaneous hypersensitivity Hypersensitivity was induced in animals according to established procedure [15] as follows: on day 1, the animals are clean-shaven ventrally. On day 0, they are sensitized by topical application of 50 μ l of 1% dinitrofluorobenzene (DNFB; Sigma Aldrich, St. Louis, MO) in acetone/olive oil (4:1), with a repeat application of the hapten on day 1. Depending on the protocol (day 5 for acute stress, variable later times for chronic stress), baseline ear measurements are taken using a micro-caliper (Ames Manufacturing Co., Chicopee, MA). Immediately following release from the stress apparatus, the animals are re-challenged with the hapten (10 μ l 0.5% DNFB, 4:1 acetone/olive oil) to each side of the left pinna, with the right pinna receiving vehicle only as a control. Pinna thickness for each animal was again measured by microcalipers at 24-h intervals, using minimal pressure and the reading at first resistance. Measured results for pinna swelling were calculated as Δ pinna thickness = pinna thickness – baseline thickness. All pinna measurements were made by a single individual in a blinded fashion throughout the study.

Restraint stress Mice react stressfully to immobilization in tight quarters, perhaps due to the ever-present dangers of entrapment in their natural burrowing environment [16]. Following the example set by Dhabhar and McEwen [1],

reusable restraint devices were constructed of flexible stainless steel wire mesh with latex borders and used to individually and painlessly enclose the mice, enabling them to breath freely with minimal movement possible. The overall effect is a sense of confinement that is psychologically stressful, activating both the SNS and the HPA axis [17]. The mouse is introduced into the space without undo force and maintained in this immobilized position for varying periods according to protocol. Because of the potential for inducing bystander stress, mice undergoing restraint stress are kept separate from non-stressed controls. To avoid unwanted, exogenous stress exposure, mice must be delicately handled with care by the same investigator in a consistent manner, with stress measurements made on days when cage cleaning and other routine animal maintenance activities do not take place. Due to the potential variability in extraneous non-specific stress factors between individual experiments, non-stressed control mice are required for each experiment. For acute stress protocols, mice were introduced into the restraint devices on day 5 after hapten sensitization for a period of 2.5 h. Immediately upon leaving the restraint device, mice were re-challenged with hapten and returned to their cages. For mice in the chronic stress protocol, restraint stress was also started on day 5 post-hapten sensitization, but the period of restraint stress was extended to 5 h and repeated daily for up to 4 weeks before hapten re-challenge and measurement of contact hypersensitivity responses.

Phlebotomy Blood specimens were obtained by nicking the mouse tail vein with a #11 scalpel blade and removing approximately 200 µl of blood using a micropipet for transfer into a heparin-coated polypropylene eppendorf tube on ice for subsequent analyses as follows:

1. *Circulating blood leukocyte phenotyping* was performed by two-color staining of 60 µl aliquots of blood with either GR-1 FITC (granulocytes) and CD3e PE (T lymphocytes), or CD11b FITC (monocytes) and CD45R/B220 PE (B lymphocytes). Cell acquisition of RBC-lysed specimens was performed on a flow cytometer (FACSCaliber, BD Biosciences, San Jose, CA) and analyzed by Flowjo (TreeStar, Inc., Ashland, OR). Gating was performed in the forward scatter/side scatter plot for the appropriate region containing each leukocyte subset, with subsequent gating on the positively staining cells in the FL1/FL2 dot plot. Final leukocyte subset quantification was calculated using the percent total cells of a given subset multiplied by the total cell count of the drawn blood specimen as concurrently determined by hemocytometer counting of nucleated cells within the whole blood specimen (Turk's Solution, Sigma Aldrich).

2. *Plasma corticosterone levels* were determined from plasma separated from the remaining aliquot of whole blood and frozen for later analysis by a competitive radioimmunoassay (RIA) according to the manufacturer's instructions (MP Biomedicals, Santa Ana, CA).

Urine catecholamine levels Urine was collected from mice by placing the animal in the clear plastic packaging material that typically accompanies multi-well culture plates (Costar, Corning Life Sciences, Corning, NY). In brief, mice are placed on the inverted clean plastic packaging material and gently stroked along the back while raising the tail to induce urination that collects on the underlying plastic surface. The urine is then transferred by pipet to polypropylene eppendorf tubes containing 0.1N HCl at a 1:2 dilution to maintain an acid pH. Frozen (−80 C) specimens were later analyzed for epinephrine and norepinephrine levels in duplicate using a two-stage kit (Rocky Mountain Diagnostics, Colorado Springs, CO) according to manufacturer's instructions that involved an extraction step and subsequent ELISA. Absorption levels at the manufacturer's specified wavelengths were determined by spectrophotometer (Spectramax 384Plus, Molecular Devices Corp., Sunnyvale, CA), with results generated against a concurrently run standard curve.

Statistics

Data are presented graphically as mean and SEM, unless otherwise indicated. Nonparametric comparisons between group data were performed using SPSS software (version 11.0, SPSS, Inc., Chicago, IL) employing the Wilcoxon signed-ranks test and Mann–Whitney test for paired and independent data, respectively. Significance indicates a $p < 0.05$.

Results

Acute and chronic restraint stress on cutaneous hypersensitivity immune responses To determine the optimal duration of perceived stress in our contact hypersensitivity experiments, we exposed hapten-sensitized mice to restraint stress of varying duration and measured their hypersensitivity responses after re-challenge (data not shown). Acute stress of 2.5-h duration was sufficient to consistently augment hapten-induced pinna swelling in these WT mice. However, with increasing duration of stress exposure, this augmented hypersensitivity response was progressively diminished. Daily exposure to 5 h of restraint stress for 4 weeks resulted in markedly suppressed hypersensitivity responses in WT mice, and this time duration was used for subsequent chronic stress experiments.

A1 KO mice fail to augment contact hypersensitivity responses after acute stress exposure To study the role played by the adenosine A₁ receptor pathway in acute stress-altered hypersensitivity responses, we exposed hapten-sensitized mice genetically lacking the A₁ receptor and WT littermates to acute stress, followed immediately by hapten challenge. Pinna measurements made at 24-h intervals thereafter (Fig. 1) showed that hypersensitivity responses in acutely stressed WT mice were significantly increased compared to WT mice that were not exposed to stress. In the non-stressed A1 KO group, pinna swelling was non-significantly elevated compared to the non-stressed WT control mice. However, in striking contrast to the augmented cutaneous hypersensitivity responses in the acutely stressed WT mice, no additional increases in pinna swelling were observed in the A1 KO mice exposed to acute stress compared to their non-stressed controls. Thus, cutaneous immunity failed to augment in A1 KO mice exposed to acute restraint stress.

Blood neutrophil granulocyte levels fail to increase in acutely stressed A1 KO mice Studies have shown that perceived stress and stress hormones cause large and transient shifts in the absolute numbers and relative proportions of circulating leukocyte subsets within the blood of rodents [18–20]. Blood lymphocyte numbers decrease in response to stress-elevated corticosterone levels and are redistributed into the surrounding lymphoid tissues

and dermal tissues [18, 21]. In contrast, circulating granulocytes rapidly increase in number in response to stress-induced release of catecholamines, largely through recruitment from the vascular-marginated cell pool [22, 23].

We compared the changes in circulating leukocyte subsets occurring in response to acutely stressed WT and A1 KO mice. As expected, WT mice exposed to acute stress typically had marked decreases in circulating levels of lymphocytes and monocytes and increases in granulocyte levels (Fig. 2a–d). In the A1 KO mice, reduced circulating blood lymphocytes and monocytes were similar to those observed in the WT mice (Fig. 2a, b, d). In contrast, circulating numbers of granulocyte in the blood of A1 KO mice did not increase after exposure to acute stress (Fig. 2c).

A1 KO mice have a normal corticosterone response to acute stress Acute stress-induced shifts in blood levels of circulating lymphocytes and monocytes occur in response to corticosterone release from an activated HPA axis [18, 21]. We measured plasma corticosterone levels in WT and A1 KO mice at rest and immediately after acute stress (Fig. 3). There was a non-significant trend toward lower non-stressed baseline levels of corticosterone in A1 KO mice compared to levels in resting WT mice. However, significant increases in plasma corticosterone occurred in both WT and A1 KO mice in response to acute stress exposure, suggesting that this stress-induced outcome of the HPA axis remained intact in the absence of the A₁ receptor.

Both WT and A1 KO mice have increased urine epinephrine levels in response to acute stress Catecholamines are rapidly released into the blood stream in response to stress, making it difficult to obtain accurate, non-stressed catecholamine blood levels in mice by using a stress-inducing venipuncture procedure. Catecholamines levels in the urine are less subject to rapid shifts, and so we chose to measure epinephrine and norepinephrine levels in urine specimens collected from WT and A1 KO mice prior to and immediately following exposure to acute stress. Mice in both WT and A1 KO groups all showed large increases in urine epinephrine levels after experiencing 2.5 h of restraint stress (Fig. 4a), with a trend towards greater increases in the A1 KO mice compared to WT mice. In contrast, there were no significant changes in urine norepinephrine levels in either WT or A1 KO mice in response to acute stress (Fig. 4b). Thus, A1 KO and WT mice were similar in their respective epinephrine and norepinephrine responses to acute perceived stress.

Chronic stress does not suppress contact hypersensitivity responses in A1 KO mice We next investigated whether the adenosine A₁ receptor plays a role in the immune-suppressive changes associated with exposure to chronic stress. Mice were subjected to 5-h restraint stress on a daily

Fig. 1 Acute stress fails to augment cutaneous hypersensitivity responses in A1 KO mice. Hapten-sensitized WT and A1 KO mice ($n=5/\text{group}$) were subjected to 2.5-h restraint stress, followed immediately by hapten re-challenge of the left pinna. Outcome was measured daily for the subsequent 3 days and shown as increment (Δ ; change in) pinna thickness from base line. Results are compared to concurrently studied non-stressed control mice. Group means and SEM shown in this representative example of four experiments. (* $p=0.016$, ** $p=0.008$)

Fig. 2 Shifts in circulating levels of blood leukocyte subsets in response to acute stress. Whole blood obtained by tail vein phlebotomy from WT and A1 KO mice ($n=5/\text{group}$) immediately following acute stress (shaded white box) and compared to non-stressed control mice of same genetic background (black box) by flow cytometry for leukocyte markers. Results are shown for B lymphocytes (a), T lymphocytes (b), granulocyte neutrophils (c), and monocytes (d). Note difference in y-axis scales used for panels a, b and c, d. Group means and SEM shown. $*p\leq 0.002$ versus non-stressed controls

basis for 4 weeks, followed by hapten re-challenge of the pinna. Compared to concurrently studied, non-stressed controls, WT mice exposed to chronic stress had significantly reduced cutaneous hypersensitivity responses (Fig. 5). However, chronically stressed A1 KO mice maintained levels of pinna swelling comparable to their non-stressed

control group. These findings suggested that A1 KO mice were resistant to the cutaneous immune-suppressive effects normally associated with chronic stress exposure.

Neutrophil granulocyte numbers decrease in the blood of A1 KO mice exposed to chronic stress To help elucidate the role of the adenosine A_1 receptor in chronic stress-altered immunity, we measured the peripheral blood leukocyte differential in mice subjected to 4 weeks of daily restraint stress. Comparison was made to control mice that did not undergo restraint stress but were otherwise similarly exposed to our animal facility conditions over the same time period. Circulating levels of lymphocytes (Fig. 6a, b) and monocytes (Fig. 6d) were significantly decreased in A1 KO mice and their WT littermates after exposure to chronic stress when compared to their respective non-stressed control mice. Granulocyte numbers in the chronically stressed WT mice remained relatively stable to levels found in non-stressed control mice (Fig. 6c). In contrast, circulating granulocyte numbers in the A1 KO mice were significantly decreased after chronic stress exposure. Thus, as was observed under conditions of acute stress, we found that chronic stress also had differential effects on circulating blood granulocytes in A1 KO mice compared to their WT littermates.

A1 KO mice have normal elevations in plasma corticosterone in response to chronic stress Plasma corticosterone levels were measured in mice subjected to chronic restraint stress and compared to concurrently obtained levels in non-

Fig. 3 Plasma corticosterone response to acute stress is not affected by lack of adenosine A_1 receptor. Plasma is obtained by tail vein phlebotomy from WT and A1 KO mice ($n=5/\text{group}$) immediately following acute stress and compared to plasma from non-stressed control mice. Specimens were frozen until later analysis by RIA. Group means and SEM shown in representative data from one of three experiments. $*p\leq 0.008$ versus respective non-stressed controls

Fig. 4 Urine catecholamine response to acute stress is not affected by lack of adenosine A₁ receptor. Urine specimens obtained from mice before (white circle) and immediately after (white up-pointing triangle) 2.5-h acute stress were frozen until later determination by ELISA of epinephrine (a) and norepinephrine (b) levels. Individual mouse data points and median values are shown for both wild-type (WT) and A1 KO mice (KO). Representative data shown from one of three experiments ($n=5$ /group; $*p=0.043$)

stressed controls. As with our findings in acute stress (Fig. 3), both A1 KO and WT littermates experienced similar elevations of plasma corticosterone levels in response to 4-week daily stress (Fig. 7). Thus, A1 KO mice and their WT littermates respond similarly to both acute and chronic stress with elevations in their plasma corticosterone levels.

A1 KO mice urine catecholamine levels To evaluate the effect of chronic stress on catecholamine release by WT and A1 KO mice, we measured urine levels of epinephrine and norepinephrine in mice after having subjected them

to 5-h restraint stress daily over a 4-week period. Urine was collected from the mice just before and immediately after their exposure to 5 h of restraint stress. Despite exposure to 4 weeks of chronic stress, animals in both A1 KO and WT groups had low urine catecholamine levels just prior to undergoing a 5-h restraint stress session (Fig. 8) that were similar to urine levels detected in non-stressed mice (Fig. 4). Also similar to changes seen in acutely stressed mice, epinephrine levels in urine specimens obtained immediately after 5 h in the restraint devices significantly increased in all mice from both groups (Fig. 8a). These levels were comparable to epinephrine levels achieved with acute stress, suggesting that minimal accommodation in this response had occurred with repeated exposure to the restraint devices.

No consistent changes were observed in urine norepinephrine levels from either the WT or A1 KO groups (Fig. 8b). Interestingly, the attenuated norepinephrine response to perceived stress in our mice reflect findings in human studies in which responses of these two catecholamines differ according to the type of stress stimulus [24, 25]. In these studies, physical stress occurring with exercise was found to be associated mainly with the release of norepinephrine, whereas mental stress leads primarily to an increase in epinephrine concentration. Our results would suggest that mice are similar to humans in their selective release of epinephrine over norepinephrine in response to psychological stress.

Fig. 5 Chronically applied stress fails to suppress contact hypersensitivity responses in A1 KO mice. Hapten-sensitized WT and A1 KO ($n=5$ /group) were subjected to 5-h restraint stress daily for 4 weeks, after which the right pinna was re-challenged with the hapten. The resulting pinna swelling was measured daily and shown as increment (Δ ; change in) pinna thickness from base line. Results were compared to concurrently studied control mice. Group means and SEM shown. $*p<0.04$ versus non-stressed control

Discussion

Stress is defined as any state challenging an organism's homeostasis [26]. Stress response involves a coordinated series of physiological and behavioral responses from an organism to protect itself and restore homeostasis. A variety of stressors, including emotional, physical and immunolog-

Fig. 6 Shifts in circulating levels of blood leukocyte subsets in response to chronic stress. Whole blood obtained by tail vein phlebotomy from WT and A1 KO mice ($n=5/\text{group}$) following chronic stress daily of 4-week duration (white box) were compared to concurrently studied control mice of same genetic background (black box) by flow cytometry for leukocyte markers. Results are shown for B lymphocytes (a), T lymphocytes (b), neutrophil granulocytes (c), and monocytes (d). Note difference in y-axis scales used for panels a, b and c, d. Group means and SEM shown. $*p\leq 0.047$ versus non-stressed controls

ical, can activate the CNS and lead to neuroendocrine and autonomic responses. In peripheral tissues, the body's response to a broad range of physiological stressors can be modulated by extracellular adenosine levels [27]. Within the CNS, receptors of extracellular adenosine are key modulators of neurotransmitter functioning and have neuro-

protective and neurobehavioral effects [8, 28]. In this study, we present data suggesting that the adenosine A_1 receptor pathway is a modulatory element in the CNS regulation of peripheral immunity under conditions of acute and chronic, psychologically perceived stress. Our results show that perceived stress, both acute and chronic, fails to alter contact hypersensitivity-associated inflammation and swelling in mice genetically lacking the A_1 receptor. While acute stress augments and chronic stress suppresses contact hypersensitivity responses in WT mice, similarly stressed A1 KO mice do not vary from non-stressed A1 KO controls to hapten re-challenge.

Biological responses to perceived stress are thought to be primarily driven by the HPA axis and the SNS [3, 4]. Activation of these effector arms results in systemic release of endogenous glucocorticoids and catecholamines, which have diverse effects on immune function and other biological processes. Although adenosine and its receptor pathways have reported associations with these neuroendocrine mediators [10–12], lack of the A_1 receptor in the mice of our study did not affect their measured levels of corticosterone or catecholamines following stress exposure. This suggests that the adenosine A_1 receptor pathway is involved in stress-altered cutaneous immunity through either downstream signaling of corticosterone or catecholamines, or through a separate mechanism independent of these stress hormones.

Contact hypersensitivity is considered to be a model of antigen-specific, T-cell-mediated immunity consisting of

Fig. 7 Plasma corticosterone response to chronic stress is not affected by lack of adenosine A_1 receptor. Plasma was obtained by tail vein phlebotomy from WT and A1 KO mice ($n=5/\text{group}$) immediately following a 4-week period of restraint stress of 5-h daily duration and compared to plasma from concurrently studied control mice. Specimens were frozen at -80°C for later analysis by RIA. $*p<0.0001$ versus respective non-stressed controls

Fig. 8 Urine catecholamine levels in response to chronic stress are not affected by lack of adenosine A₁ receptor. Following 4 weeks of daily exposure to chronic stress, mouse urine specimens were obtained before (white circle) and immediately after (white triangle) exposure to 5-h chronic stress and frozen until later determination by ELISA of

epinephrine (a) and norepinephrine (b) levels. Individual data points and median values are shown for both wild-type (WT) and A₁ KO mice (KO). Representative data shown from one of three experiments ($n=5$ /group; $*p=0.043$)

two stages that include sensitization and an effector phase [15]. During sensitization, topically applied, low-molecular-weight chemical haptens bind to proteins. These hapten-carrier adducts are taken up by epidermal Langerhan's cells and dermal dendritic cells which then migrate to the draining lymph nodes to prime hapten-specific T cells [29, 30]. Following cutaneous re-exposure to the hapten, the effector inflammatory phase is mediated by IFN- γ producing CD4⁺ and CD8⁺ T cells and down-regulated by CD4⁺ T cells producing IL-4 and IL-10 [31]. Mice depleted of CD4⁺ T cells have been shown to have an increased baseline contact hypersensitivity reaction similar to that observed in our non-stressed A₁ KO mice [32]. However, acute stress exposure further augmented contact hypersensitivity responses in these CD4⁺ T cell depleted mice, while the acutely stressed A₁ KO mice in our study failed to alter their contact hypersensitivity responses. In addition, chronic stress exposure did not suppress contact hypersensitivity inflammation in the A₁ KO mice as was observed in their similarly stressed WT littermates. Thus, our data provides evidence that there is an uncoupling in the A₁ KO mice of the pathway linking perception of acute or chronic perceived stress with its downstream effects on the contact hypersensitivity reaction.

How does one explain the dual absences in A₁ KO mice of both the augmented contact hypersensitivity response under conditions of acute stress and the suppressive immune effects of chronic stress? An intriguing possibility lies in the inflammation occurring during the initial, innate immune response of contact hypersensitivity. Recent studies in mice lacking either innate immunity (Fc γ R^{-/-}) or adaptive immunity (athymic) have suggested that activation of the innate defense mechanisms by the irritant

effects of chemical haptens are critical for the development of cutaneous hypersensitivity [33]. More specific to our findings, other investigators have demonstrated that restraint stress modulation of the ear swelling response in contact hypersensitivity is strongly dependent on the irritancy/inflammatory potential of the chemical sensitizer [34]. If acute or chronic perceived stress modulates hypersensitivity responses primarily via direct effects on the chemical irritant-induced inflammation rather than through a later action upon T cells responding to the hapten re-challenge, it is possible that the adenosine A₁ receptor acts at this early key stage to modulate stress-altered cutaneous immune responses. This model would also explain the confusing and sometimes contradictory findings of how perceived stress affects immunity when other types of chemical sensitizers are used or immune outcomes are measured.

Support for a potential innate immune target by the A₁ receptor pathway in stress-altered, cutaneous hypersensitivity responses includes our finding in the A₁ KO mice of attenuated neutrophil granulocytosis in response to stress, both acute and chronic. In our study, neutrophil granulocyte numbers in the A₁ KO mice failed to increase with acute stress and did not maintain their numbers after chronically applied stress. Neutrophils are typically the first cells to infiltrate an inflammatory site and are known to accumulate at the site of epicutaneous chemical application, persisting as the predominant cellular infiltrate even 72 h after chemical challenge [33, 35]. Studies with mice genetically lacking the neutrophil chemokine CXCL1 have shown that manipulation of neutrophil recruitment can modulate subsequent contact hypersensitivity responses, possibly through production of cytokines and chemoattractants

targeting elements of the later, adaptive immune response [36]. Neutrophils also express A₁ and A₂ adenosine receptors on their surfaces [37]. Previous studies have shown that A₁ receptors promote neutrophil chemotaxis and adhesion of stimulated neutrophils to cultured endothelial cells and A₂ receptors inhibit neutrophil adhesion [38, 39], suggesting that adenosine regulates accumulation of neutrophils at sites of inflammation.

Neutrophil trafficking in peripheral tissues can also be affected by adenosine acting within the central nervous system. In a series of studies in the rat, investigators have shown that intrathecal spinal administration of an adenosine A₁ selective agonist suppressed neutrophil accumulation in the skin and markedly decreased joint swelling and destruction in adjuvant induced arthritis [13, 14, 40]. In a complex neural network dependent on intact sensory fibers and deactivation of NMDA receptors, dorsal horn A₁ receptors within the spinal cord are thought to modulate peripheral generation of adenosine within the inflamed tissue site, which in turn acts through the adenosine A_{2A} receptor to suppress neutrophil trafficking.

An additional means by which adenosine A₁ receptor signaling may influence the effects of stress on cutaneous immunity is via dendritic cells. These antigen-presenting cells have been reported by others to play a key role in modulating contact hypersensitivity responses under perceived stress conditions [32]. Although their studies were limited to the sensitization phase of the hypersensitivity response, investigators found that acute restraint stress was associated with increased migration of hapten-bearing skin dendritic cells to draining lymph nodes in a manner dependent on norepinephrine release by sympathetic nerve endings. In light of these findings, it is interesting to note that accumulation of dendritic cells at target sites is modulated through the adenosine receptor pathway. In studies not involving perceived stress [41], immature dendritic cells migrated to inflammatory sites along an adenosine gradient via an A₁ receptor-mediated mechanism, with subsequent down-regulation of these surface A₁ receptors upon dendritic cell maturation. Thus, the adenosine A₁ receptor pathway could potentially affect how the contact hypersensitivity reaction responds to perceived stress via modulation of either neutrophil or dendritic cell trafficking; cellular activities that are known to be affected under conditions of stress.

The stress response is comprised of a complex, multi-tiered system that has evolved over time to protect biological systems against injury and alterations in homeostasis. Adenosine and its receptors provide a continuous measure of metabolic activity within the body's tissues that can be used to modulate diverse biological processes to maintain a relative homeostasis. The goal of our study was to determine if absence of the adenosine A₁ receptor would

alter outcomes typically measured in the restraint stress contact hypersensitivity model. Because of the exploratory design of our study, these findings must be taken as preliminary and for use in generating hypotheses for future studies. In summary, our results show that absence of the adenosine A₁ receptor eliminates the cutaneous immune variations in the contact hypersensitivity reaction that normally occur with exposure to acute and chronic perceived stress. This effect by the A₁ receptor appears to be independent of the stress-induced responses by the HPA axis and SNS as defined by corticosterone and catecholamine levels, but may involve the process by which circulating neutrophil granulocytes respond to stress conditions. Further investigations based on these preliminary results will be necessary to determine the mechanism by which adenosine receptors influence the complex process of stress-altered contact hypersensitivity.

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Conflict of Interest Statement Stephen J. Oliver: Nothing to disclose
Sneha Matthew: Nothing to disclose
Tuere Wilder: Nothing to disclose

Bruce Cronstein: Intellectual property: patents held or pending on the use of adenosine A_{2A}R agonists to promote wound healing and use of A_{2A}R antagonists to inhibit fibrosis; use of adenosine A₁R antagonists to treat osteoporosis and other diseases of bone; use of adenosine A₁R and A_{2B}R antagonists to treat fatty liver; and use of adenosine A_{2A}R agonists to prevent prosthesis loosening. Consultancies (within past 2 year): King Pharmaceutical (licensee of patents on wound healing and fibrosis above), CanFite Biopharmaceuticals, Savient Pharmaceuticals, Bristol-Myers Squibb, Roche Pharmaceuticals, Cellzome, Tap (Takeda) Pharmaceuticals, Prometheus Laboratories, Regeneron (Westat, DSMB), Sepracor, Amgen, Endocyte, Protalex, Allos, Inc., Combinatorx, Kyowa Hakka. Honoraria/speaker bureaus: Tap (Takeda) Pharmaceuticals. Stock: CanFite Biopharmaceuticals, received for membership on Scientific Advisory Board.

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