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# Osteoarthritis and Cartilage

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## Chondrocytes respond to adenosine via A<sub>2</sub> receptors and activity is potentiated by an adenosine deaminase inhibitor and a phosphodiesterase inhibitor

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### Summary

**Objective:** To test the mechanisms by which adenosine and adenosine analogues stimulate adenylate cyclase and suppress lipopolysaccharide (LPS)-induced production of nitric oxide (NO) by chondrocytes.

**Methods:** Primary chondrocytes isolated from equine articular cartilage were plated in monolayer. Intracellular cyclic-AMP (cAMP) accumulation was measured following exposure to medium containing adenosine, the non-hydrolyzable adenosine analogue N<sup>6</sup>-methyladenosine, the A<sub>2A</sub> specific agonist N<sup>6</sup>-(dimethoxyphenyl)-ethyladenosine (DPMA), the adenosine deaminase inhibitor erythro-9-(2-Hydroxy-3-nonyl)adenine hydrochloride (EHNA), or forskolin, a potent stimulator of adenylate cyclase. Regulation of NO production by LPS-stimulated chondrocytes, as determined by nitrite concentration, was assessed in the presence of adenosine, N<sup>6</sup>-methyladenosine, DPMA, the broad agonist 5'-N-ethylcarboxamidoadenosine (NECA), or forskolin. Alternatively, LPS-stimulated chondrocytes were exposed to EHNA or the phosphodiesterase inhibitor rolipram in the presence or absence of supplemental adenosine.

**Results:** Adenosine, N<sup>6</sup>-methyladenosine, DPMA, and forskolin each increased intracellular cAMP accumulation in a concentration-dependent manner and suppressed NO production by LPS-stimulated chondrocytes. NECA also decreased NO production by chondrocytes stimulated with LPS. Incubation with EHNA, to protect endogenously produced adenosine, or rolipram, which prevents the degradation of cAMP, similarly suppressed LPS-stimulated NO production. The addition of exogenous adenosine with EHNA or rolipram further suppressed NO production.

**Conclusions:** This study documents functional responses to adenosine by articular chondrocytes. These responses are mimicked by the A<sub>2A</sub> receptor agonist, DPMA. Effects were enhanced by protecting adenosine using an adenosine deaminase inhibitor or by potentiating the cAMP response with rolipram. These experiments suggest that adenosine may play a physiological role in regulation of chondrocytes and that adenosine pathways could represent a novel target for therapeutic intervention. © 2002 Osteoarthritis Research Society International

**Key words:** Adenosine, Chondrocytes, Cyclic-AMP (cAMP), Nitric oxide (NO).

### Introduction

Chronic inflammation is a significant factor in the pathophysiology of many forms of joint disease. Both rheumatoid arthritis (RA) and osteoarthritis (OA), the most commonly diagnosed arthritic conditions<sup>1</sup>, are inflammatory diseases of the joint<sup>1–3</sup>. The purine base adenosine has been reported to reduce inflammation and swelling in several *in vivo* models of inflammation<sup>4–6</sup>. Additionally, daily systemic treatment with adenosine reduced the severity of adjuvant-induced arthritis in a rat model<sup>7</sup>. These findings suggest the potential value of adenosine as a therapeutic mediator of inflammatory joint disease. However, very little work has been done documenting the effects of purines on chondrocytes and additional documentation of the role of adenosine in the joint is warranted.

Adenosine has been shown to limit systemic inflammatory responses through receptor-mediated regulation of a wide variety of cell types. For example, adenosine suppresses monocyte phagocytosis<sup>8</sup> and inhibits neutrophil functions including adhesion, degranulation and production of reactive oxygen species<sup>9–11</sup>. In addition, extracellular adenosine restricts T cell receptor signaling preventing T cell activation and growth<sup>12,13</sup>. Inflammatory-mediated changes in vascular permeability are also blocked by adenosine, thereby reducing edema formation and restricting movement of immune cells from the vasculature<sup>14,15</sup>. One of the best documented antiinflammatory functions of adenosine is its ability to regulate cytokine synthesis. The production of proinflammatory cytokines including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )<sup>16,17</sup>, interleukin-8 (IL-8)<sup>18</sup>, macrophage inflammatory protein-1 $\alpha$ , IL-2 and IL-6<sup>19</sup> are suppressed by exposure to adenosine or adenosine analogs. Conversely, exposure to adenosine increases synthesis of IL-10<sup>20</sup>, a potent regulator of macrophage function.

In addition to its effects on the immune system, adenosine is also recognized as a critical intercellular mediator in other organ systems. For example, adenosine is an important neuromodulator in the central and peripheral nervous

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systems. Adenosine release has been measured from dorsal spinal cord<sup>21</sup> and is known to have profound anti-convulsant and analgesic effects<sup>21-23</sup>. Adenosine also regulates cardiovascular function, influencing both heart rate and blood flow<sup>24-27</sup>. Stimulation of adenosine receptors has also been shown to reduce renal injury in an ischemia-reperfusion model<sup>28</sup>. Although many cell types have been shown to respond to extracellular adenosine, the effects of adenosine on chondrocytes have not been investigated in detail.

Extracellular purine receptors are divided into two classes: P<sub>1</sub> receptors, which preferentially bind adenosine, and P<sub>2</sub> receptors, which bind ATP and ADP with greater affinity<sup>29</sup>. Both P<sub>1</sub> and P<sub>2</sub> receptors have been identified on the surface of human chondrocytes<sup>30</sup> and P<sub>2</sub> receptors on the surface of human synovial cells<sup>31</sup>. Adenosine receptors of the P<sub>1</sub> class have been further classified into four subtypes (A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, A<sub>3</sub>) based on pharmacological analysis and molecular cloning<sup>32</sup>. The A<sub>1</sub> and A<sub>3</sub> receptor subclasses are negatively coupled to adenylate cyclase and exert an inhibitory effect on cyclic-AMP (cAMP) production. The A<sub>2</sub> receptors, which stimulate adenylate cyclase and increase cAMP, have been subdivided into high affinity receptors (A<sub>2A</sub>) and low affinity receptors (A<sub>2B</sub>)<sup>32</sup>. The possible coexistence of multiple subclasses of adenosine receptors on the surface of articular chondrocytes complicates elucidation of the mechanisms involved with cellular responses to adenosine. Previous work has already documented the expression of adenosine receptor gene transcripts by articular chondrocytes<sup>30</sup>, but chondrocyte responses to adenosine receptor stimulation have not been documented.

The potential antiinflammatory effects of adenosine in the joint can be evaluated by examining the production of inflammatory mediators, including nitric oxide (NO). The enzyme nitric oxide synthase (NOS) exists in two constitutive forms (cNOS) as well as an inducible form (iNOS)<sup>33</sup>. Cells expressing cNOS regulate physiological functions by transiently producing small amounts of NO while those expressing iNOS produce larger quantities of NO for a more prolonged period of time<sup>33</sup>. In diarthrodial joints, chondrocytes express iNOS and are the major source of intraarticular NO production<sup>34,35</sup>. The synthesis of NO by articular cells is increased *in vitro* in response to inflammatory stimuli such as bacterial lipopolysaccharide (LPS) or IL-1<sup>33,36</sup>. Several reports indicate that a large increase of NO in the joint can be linked to inflammation resulting in decreased chondrocyte proliferation and decreased proteoglycan and collagen synthesis<sup>37-40</sup>. Altering chondrocyte production of NO could be key in retarding the joint destruction that results from inflammatory disease.

The purpose of the current investigation was to document functional roles for adenosine in articular chondrocytes and to determine if the A<sub>2</sub> receptor subtype was involved in that response. The study also examines the effects of prolonging adenosine and cAMP exposure times using the non-hydrolyzable adenosine analogue N<sup>6</sup>-methyladenosine, the adenosine deaminase inhibitor EHNA and the cAMP-specific phosphodiesterase (Type IV) inhibitor rolipram.

## Materials and methods

### MATERIALS

Cyclic-AMP enzyme immunoassay (EIA) kits were purchased from Amersham Pharmacia (Piscataway, NJ,

Table I  
Pharmacological effects of purinergic reagents

Drug	Action
Adenosine	Extracellular purine receptor agonist
N <sup>6</sup> -methyladenosine	Non-hydrolyzable analogue of adenosine
DPMA	Adenosine A <sub>2A</sub> receptor agonist
NECA	Adenosine A <sub>2</sub> and A <sub>1</sub> receptor agonist
Forskolin	Direct adenylate cyclase activator
EHNA	Adenosine deaminase inhibitor
Rolipram	cAMP-specific phosphodiesterase inhibitor

U.S.A.). The fluorescent DNA quantitation kit was obtained from Biorad (Hercules, CA, U.S.A.). Gentamicin sulfate was produced by Schering-Plough Animal Health Corp. (Madison, NJ, U.S.A.). Amphotericin B, fetal calf serum (FCS) and Dulbecco's modified Eagle's Medium-Ham's F-12 nutrient medium (DMEM F-12) were purchased from Gibco-BRL (Grand Island, NY, U.S.A.). Type-II collagenase was purchased from Worthington Biochemical (Freehold, NJ, U.S.A.). All other chemicals, including adenosine and adenosine receptor agonists, were purchased from Sigma (St. Louis, MO, U.S.A.) or Sigma-RBI (Natick, MA, U.S.A.).

Of the tested reagents, N<sup>6</sup>-methyladenosine is a substituted adenosine derivative that is resistant to breakdown by adenosine deaminase<sup>41</sup> (Table I). Synthetic adenosine analogs used in this study also include N<sup>6</sup>-[2-(3,5-dimethoxyphenyl)-ethyl]adenosine (DPMA), an A<sub>2A</sub> specific receptor agonist<sup>42</sup>, and the nonselective A<sub>2</sub> and A<sub>1</sub> receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA)<sup>43</sup>. The adenine analogue erythro-9-(2-Hydroxy-3-nonyl)adenine hydrochloride (EHNA) is a potent inhibitor of adenosine deaminase and was used to extend the half-life of both supplemental and endogenous adenosine<sup>44</sup>. Forskolin is a plant derivative that binds the stimulatory subunit of adenylate cyclase directly stimulating cAMP production<sup>45</sup>. This compound was used in this study as a positive control for cAMP production by chondrocytes. The cAMP-specific phosphodiesterase (Type IV) inhibitor rolipram was used to extend the half-life of cAMP<sup>46</sup> so that it could be measured and to enhance the potential cAMP-mediated effects of adenosine on NO production. The bacterial lipopolysaccharide (LPS) used in this study was obtained from *Escherichia coli* serotype 0111:B4.

### CELL ISOLATION AND PLATING

Full-thickness equine articular cartilage was aseptically dissected from the metacarpophalangeal joints of fresh equine cadavers as previously described<sup>47,48</sup>. Cartilage samples were harvested from nine horses ranging from 2 to 10 years of age. Cartilage was collected in a sterile mixture of DMEM F-12 containing 5% FCS, 2.5 µg/ml amphotericin B, 50 µg/ml ascorbate, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamicin sulfate.

Following dissection, cartilage samples were rinsed once with fresh DMEM F-12 medium and subsequently exposed to a three-step enzymatic digestion to free the chondrocytes from the extracellular matrix. Cartilage was incubated at 37°C for 15 min with 0.1% (w/v) type-I-S hyaluronidase followed by incubation for 1 h with 0.1% (w/v) type-XIV bacterial protease. Cartilage samples were then incubated overnight at 37°C in 0.1% (w/v) type-II collagenase. Following digestion, the medium was passed through a 70 µm cell strainer to remove undigested debris.

Released chondrocytes were collected by centrifugation at 230 *g* for 6 min, resuspended in fresh medium and counted using a hemacytometer. Freshly isolated chondrocytes were plated in 24- and 48-well plates at a density of  $5 \times 10^5$  cells/cm<sup>2</sup>. Tissue culture plates were incubated in a humidified atmosphere of 95% air/5% CO<sub>2</sub> at 37°C.

#### CAMP ASSAY

Chondrocytes from three horses ( $N=3$ ) were used to evaluate intracellular cAMP accumulation in response to adenosine and its synthetic analogues. Cells were exposed to either adenosine, DPMA or N<sup>6</sup>-methyladenosine, at concentrations ranging from 0.1 to 100  $\mu$ M. In other wells, chondrocytes were exposed to the adenosine deaminase inhibitor EHNA at concentrations ranging from 0.01 to 10  $\mu$ M both alone and in the presence of 100  $\mu$ M adenosine. Chondrocytes were also incubated with concentrations of forskolin ranging from 0.01 to 10  $\mu$ M as a positive control for cAMP production. Negative control wells were incubated with medium without added reagents.

Plated cells were allowed to stabilize in medium containing 5% FCS for 48 h prior to exposure to purinergic reagents. Following the stabilization period, cells were allowed to equilibrate in fresh serum-free medium for 20 min and subsequently incubated for 20 min in the presence of rolipram at a final concentration of 100  $\mu$ M. The test reagent was then added to the test wells containing rolipram and the plate was incubated at 37°C for 9 min before the supernatant was removed and discarded. The reaction was terminated at 10 min by the addition of 1.0 ml of lysis buffer (0.25% dodecyltrimethylammonium bromide) to each dry well.

Intracellular production of cAMP was subsequently measured using a commercial EIA kit. Tissue culture plates were placed on an orbital shaker at room temperature at 150 rpm for 10 min. An aliquot of cell lysate from each well was then diluted 1:50 in lysis buffer and 100  $\mu$ l of each sample was pipetted in duplicate to the 96-well EIA plate. The tissue culture plate with the remaining lysate was stored at -20°C until DNA content was measured.

The EIA plate was incubated at 4°C for 2 h with the primary antibody and incubated for an additional 1 h at the same temperature after the addition of cAMP-horseradish peroxidase conjugate. The plate was rinsed four times with wash buffer (0.01 M phosphate buffer, pH 7.5 containing 0.05% Tween 20) and 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide in 20% (v/v) dimethylformamide was added as an enzyme substrate. The plate was placed on an orbital shaker at 100 rpm for 1 h at room temperature. Following the addition of 1.0 M sulfuric acid, the optical density was determined spectrophotometrically within 30 minutes using a microplate reader at 450 nm.

#### NO ASSAY

Chondrocytes from three horses ( $N=3$ ) were used to evaluate the effects of adenosine and its synthetic analogues on NO production. The purine reagents were tested alone to determine their effect on baseline NO production by unstimulated chondrocytes. In addition, the reagents were tested in medium containing 100  $\mu$ g/ml LPS to determine the effects of adenosine and its analogs on chondrocytes exposed to a known stimulus of NO production. For the purpose of this report, chondrocytes exposed

to LPS will be referred to as LPS-stimulated chondrocytes while those cultured without LPS will be termed unstimulated. Both adenosine and N<sup>6</sup>-methyladenosine were tested at 100  $\mu$ M, while DPMA and NECA were tested at a concentration of 10  $\mu$ M. Additionally, forskolin, a positive control for cAMP production, was tested at a concentration of 1  $\mu$ M to determine whether elevation in intracellular cAMP levels were responsible for observed purinergic effects on NO synthesis. These concentrations were selected based on reagent concentrations that were shown to significantly increase cAMP accumulation in the first phase of this study. Cells stimulated with LPS but not exposed to any other reagent served as a positive control for NO production. Cells maintained in tissue culture medium without LPS or any other reagents served as negative controls.

Using cells obtained from three horses ( $N=3$ ), chondrocytes in tissue culture medium without LPS and chondrocytes activated with LPS were exposed to either EHNA or rolipram at concentrations ranging from 1 to 100 nM. Both EHNA and rolipram were tested alone and with the coaddition of 100  $\mu$ M adenosine. The positive and negative controls included were identical to those chosen for the first series of NO experiments.

Nitric oxide has a short half-life in biological fluids or culture medium. However, nitrite, a stable end product of NO, can be measured using a spectrophotometric method based on the Griess reaction<sup>49</sup>. The ratio of nitrite concentration to total NO generated is stable in chondrocyte cultures, and nitrite levels can be used to accurately estimate NO concentration<sup>50,51</sup>.

Cells were plated onto 48-well plates in media containing the selected purinergic reagent and were allowed to attach for 24 h prior to the addition of LPS. The plating medium was then aspirated and replaced with fresh medium containing the same purinergic reagents. Half of the wells from each sample set were also treated with 100  $\mu$ g/ml LPS. After an additional 24 h incubation period, medium was removed and 200  $\mu$ l of 0.5 M NaOH was added to each well. The supernatant media and tissue culture plates containing lysed cells were stored individually at -20°C until samples were analysed for NO and DNA content, respectively.

Media aliquots of 50  $\mu$ l were pipetted into a clear-bottomed 96-well plate and 50  $\mu$ l of freshly mixed Griess reagent was added. After 5 min of agitation at low speed on an orbital shaker, NO concentration was determined by measuring optical density using a 96-well microplate reader at a wavelength of 570 nm.

#### DNA ASSAY

The total DNA content of chondrocytes per well was determined using the Hoechst dye assay modified for use with a 96-well fluorescent plate reader<sup>52</sup>. Plates were thawed at room temperature for approximately 45 min on an orbital shaker. Aliquots of cell lysate from the cAMP and NO assays were mixed with a solution of 2  $\mu$ g/ml Hoechst dye. After a five minute incubation at room temperature, fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

#### STATISTICAL ANALYSIS

All values are expressed as mean  $\pm$  standard error of the mean (S.E.M.). Values were reported as significant when

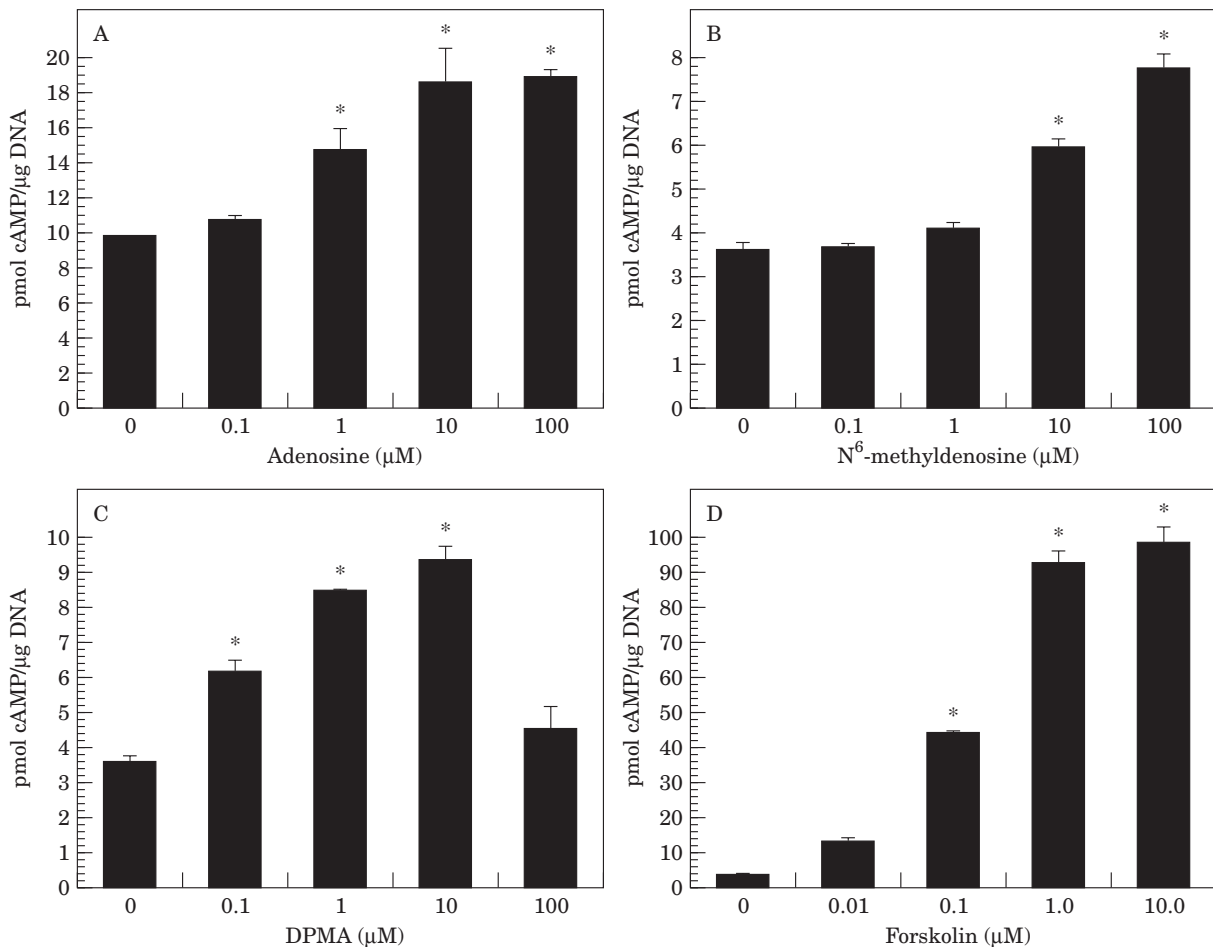


Fig. 1. Intracellular cAMP accumulation by equine chondrocytes cultured in monolayer and exposed to selected reagent for 10 min prior to cell lysis. Data were collected from chondrocytes obtained from a single horse, but are representative of results from three horses. A significant increase in cAMP accumulation (\*) occurred with exposure to (a) adenosine at concentrations of 1 to 100  $\mu\text{M}$  ( $P < 0.05$ ) (b)  $\text{N}^6$ -methyladenosine at concentrations of 10 and 100  $\mu\text{M}$  ( $P < 0.05$ ) (c) DPMA at concentrations of 0.1 to 10  $\mu\text{M}$  ( $P < 0.05$ ) and (d) forskolin at concentrations of 0.01 to 10  $\mu\text{M}$  ( $P < 0.05$ ). All cells were incubated in the presence of 100  $\mu\text{M}$  rolipram, a type IV phosphodiesterase inhibitor.

$P < 0.05$ . Data were analysed using a two-way analysis of variance (ANOVA) that was blocked by horse to allow comparisons between reagents. When the ANOVA indicated that the model was significant, *post-hoc* means comparisons were performed to determine differences between individual groups. For the cAMP experiments in which reagents were added individually, the differences between control measurements and treatment measurements were performed using a Dunnett's test ( $P < 0.05$ ). Alternatively, for the experiments in which reagents were added alone and in combination, data pairs were compared using both the Student's *t*-test with statistical significance accepted at  $P < 0.05$  and the more stringent Bonferroni's procedure. The Bonferroni adjustment was used to protect against type I errors, and the level of statistical acceptance was based on the number of means comparisons performed. All analysis was performed using SAS 8<sup>™</sup> statistical software package (SAS Institute, Cary, NC, U.S.A.).

## Results

### EFFECTS OF ADENOSINE ANALOGUES ON CAMP ACCUMULATION

Chondrocytes accumulated elevated levels of cAMP when exposed to adenosine at concentrations ranging from

1 to 100  $\mu\text{M}$ , but not at the lowest concentration tested [Fig. 1(a)]. The magnitude of the response to adenosine was concentration dependent. The non-hydrolyzable adenosine analogue  $\text{N}^6$ -methyladenosine significantly increased cAMP accumulation by chondrocytes at concentrations of 10 and 100  $\mu\text{M}$  but not at 0.1 or 1  $\mu\text{M}$  [Fig. 1(b)]. In a similar manner, the  $\text{A}_{2A}$  specific agonist DPMA significantly increased cAMP accumulation in chondrocytes when cells were exposed to concentrations from 0.1 to 10  $\mu\text{M}$  but not at 100  $\mu\text{M}$  [Fig. 1(c)]. Forskolin, which directly stimulates adenylate cyclase, strongly increased cAMP accumulation when added at concentrations from 0.01 to 10  $\mu\text{M}$  [Fig. 1(d)]. Levels of cAMP detected in the presence of forskolin were approximately 20 times higher than those detected in response to purine receptor agonists.

### EFFECTS OF EHNA AND ROLIPRAM ON CAMP ACCUMULATION

Chondrocytes exposed to EHNA, an enzyme inhibitor that prevents the breakdown of adenosine, did not accumulate increased levels of cAMP. However, exposure to EHNA at concentrations ranging from 0.1 to 10  $\mu\text{M}$  increased cAMP accumulation in chondrocytes that were simultaneously exposed to 100  $\mu\text{M}$  adenosine (Fig. 2). This

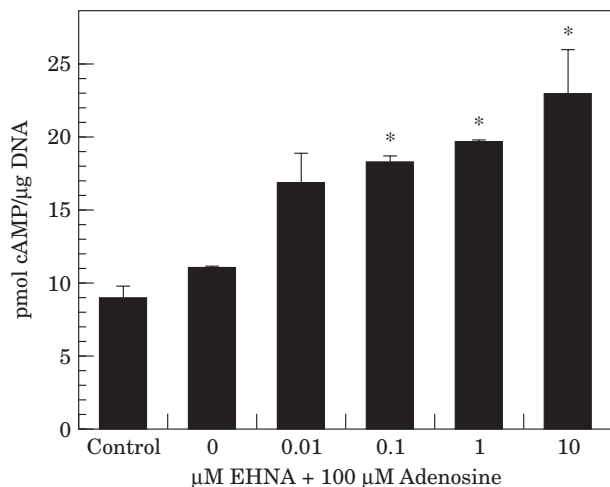


Fig. 2. Intracellular cAMP accumulation by equine chondrocytes cultured in monolayer and exposed simultaneously to EHNA and adenosine for 10 min prior to cell lysis. Data were collected from chondrocytes obtained from a single horse, but are representative of results from three horses. Control wells had no reagents added. A significant increase in cAMP accumulation (\*) occurred with simultaneous exposure to 100 μM adenosine and EHNA at concentrations of 0.1 to 10 μM when compared to cAMP accumulation seen in response to adenosine alone ( $P < 0.0024$ ). All cells were incubated in the presence of 100 μM rolipram, a type IV phosphodiesterase inhibitor.

increase in cAMP accumulation was significantly higher than the increase in intracellular cAMP accumulation measured in response to incubation with adenosine alone. In contrast, cells incubated with a lower concentration of EHNA (0.01 μM) did not significantly increase cAMP accumulation when exposed to 100 μM adenosine.

In all cases, supplementation with the type IV cAMP-specific phosphodiesterase inhibitor rolipram was required for experimental detection of cAMP accumulation. If rolipram was not added to the tissue culture wells during the incubation period, the accumulation of cAMP was below the detection threshold of the assay ( $< 1$  pmol/μg DNA) regardless of the reagent added. For example, cells exposed to 1 μM forskolin and treated with rolipram accumulated the highest measured levels of cAMP at  $17.6 \pm 0.4$  pmol of cAMP per μg DNA. However, when chondrocytes from the same horse were exposed to 1 μM forskolin in the absence of rolipram the intracellular levels of cAMP were below the detection threshold of the assay. In addition, chondrocytes that were not exposed to any exogenous purinergic reagents accumulated a small but measurable level of cAMP in the presence of rolipram alone.

#### EFFECTS OF ADENOSINE ANALOGUES ON LPS-STIMULATED NO PRODUCTION

Chondrocytes exposed to 100 μg/ml LPS for 24 h increased production of NO by approximately 10-fold over the baseline levels produced by unstimulated chondrocytes (Fig. 3). Nitric oxide production by unstimulated chondrocytes was minimal. Treatment of LPS-stimulated chondrocytes with 100 μM adenosine or N<sup>6</sup>-methyladenosine inhibited this increase in NO production (Fig. 3). In addition, 10 μM of either the non-selective adenosine receptor agonist NECA or the A<sub>2A</sub> specific agonist DPMA also

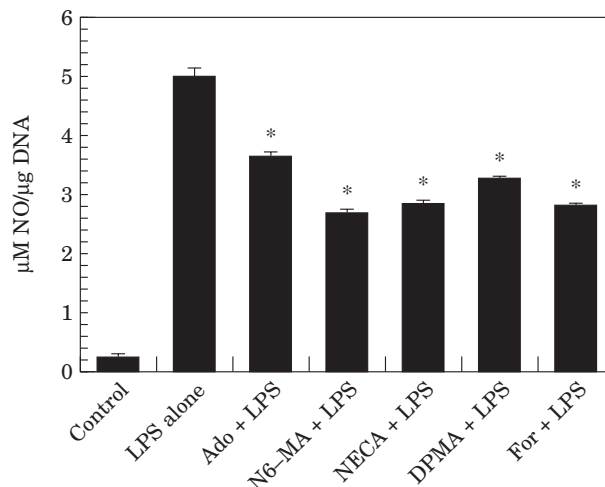


Fig. 3. Inhibition of LPS-stimulated NO production by adenosine and its synthetic analogues. Treatments are as follows: column (1) control—no reagents added; column (2) 100 μg/ml LPS alone; column (3) 100 μM adenosine+100 μg/ml LPS; column (4) 100 μM N<sup>6</sup>-methyladenosine+100 μg/ml LPS; column (5) 10 μM NECA+100 μg/ml LPS; column (6) 10 μM DPMA+100 μg/ml LPS; and column (7) 1 μM forskolin+100 μg/ml LPS. Data were collected from chondrocytes obtained from a single horse, but are representative of results from three horses. A significant decrease in LPS-mediated NO production (\*) occurred with exposure to all of the tested purinergic reagents ( $P < 0.0019$ ).

significantly decreased NO production by chondrocytes stimulated with LPS (Fig. 3). Exposure to 1 μM forskolin also suppressed chondrocyte NO release in response to LPS stimulation (Fig. 3). Adenosine, N<sup>6</sup>-methyladenosine, DPMA, NECA, and forskolin all inhibited LPS-stimulated NO synthesis to a similar degree. Exposure to N<sup>6</sup>-methyladenosine, NECA, DPMA, or forskolin alone did not significantly alter NO production by chondrocytes that were not cultured in the presence of LPS (data not shown). Cells cultured without LPS did, however, increase NO synthesis when exposed to 100 μM adenosine but NO levels were approximately 1:10 of the levels produced in the presence of LPS (Fig. 4).

#### EFFECTS OF EHNA AND ROLIPRAM ON LPS-STIMULATED NO PRODUCTION

The addition of 1 to 100 nM EHNA decreased the production of NO by chondrocytes that were stimulated with LPS (Fig. 5). Co-addition of 100 μM adenosine with EHNA resulted in a more profound inhibition of LPS-stimulated NO production at all concentrations tested. The levels of NO produced by chondrocytes exposed to all three reagents simultaneously (LPS, EHNA and adenosine) were significantly lower than those detected in the presence of LPS alone, LPS and adenosine alone, or LPS and EHNA alone (Fig. 5). This difference was significant at all concentrations of EHNA tested. However, there was not a statistically significant difference in NO levels between each concentration of EHNA tested. The inhibition of LPS-stimulated NO production detected in response to coaddition of both EHNA and adenosine was roughly equal to the combined responses observed with exposure to each reagent supplemented individually. Nitric oxide production by chondrocytes not exposed to LPS was not

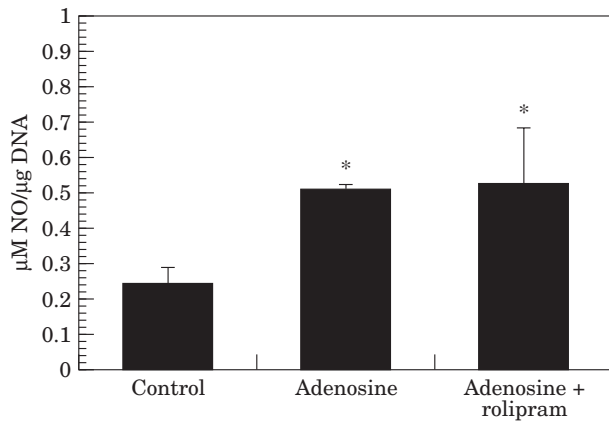


Fig. 4. Stimulation of NO production by adenosine. Data were collected from chondrocytes obtained from a single horse, but are representative of results from three horses. All samples were incubated in the absence of LPS. A small but statistically significant increase in NO production (\*) occurred with exposure to 100  $\mu$ M adenosine alone and with simultaneous exposure to 100  $\mu$ M adenosine and 100  $\mu$ M rolipram, a type IV phosphodiesterase inhibitor ( $P < 0.0012$ ).

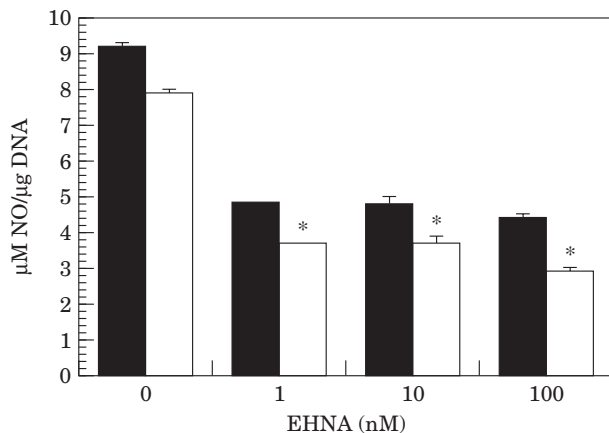


Fig. 5. Inhibition of LPS-stimulated NO production by EHNA. Data were collected from chondrocytes obtained from a single horse, but are representative of results from three horses. EHNA, at concentrations of 1 to 100 nM, inhibited production of NO by chondrocytes exposed to 100  $\mu$ g/ml LPS. Simultaneous exposure to EHNA and 100  $\mu$ M adenosine resulted in a significant decrease in (\*) LPS-mediated NO production when compared to the effect of adenosine alone or EHNA alone on LPS-induced NO at all concentrations of EHNA tested ( $P < 0.0012$ ). LPS, ■; LPS+adenosine, □.

significantly altered in the presence of 1 to 100 nM EHNA or by the coaddition of the same concentrations of EHNA and 100  $\mu$ M adenosine (Data not shown).

The addition of rolipram at concentrations of 1 to 100 nM inhibited LPS-stimulated NO production by chondrocytes (Fig. 6). Coaddition of 100  $\mu$ M adenosine with rolipram resulted in a more profound inhibition of LPS-stimulated NO production at all concentrations tested. The levels of NO produced by chondrocytes exposed to all three reagents simultaneously (LPS, rolipram and adenosine) were significantly lower than those detected in the presence of LPS alone, LPS and adenosine alone, or LPS and rolipram alone (Fig. 6). This difference was significant at all concentrations of rolipram tested. However, there was not a

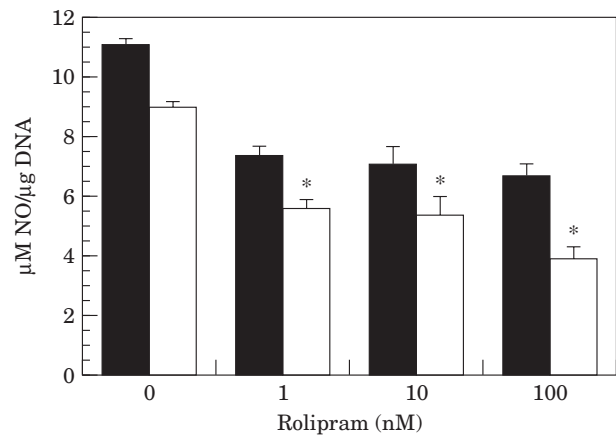


Fig. 6. Inhibition of LPS-stimulated NO production by rolipram. Data were collected from chondrocytes obtained from a single horse, but are representative of results from three horses. Rolipram, at concentrations of 1 to 100 nM, inhibited production of NO by chondrocytes exposed to 100  $\mu$ g/ml LPS. Simultaneous exposure to rolipram and 100  $\mu$ M adenosine resulted in a significant decrease in (\*) LPS-mediated NO production when compared to the effect of adenosine alone or rolipram alone on LPS-induced NO at all concentrations of EHNA tested ( $P < 0.05$ ). LPS, ■; LPS+adenosine, □.

statistically significant difference in NO levels between each concentration of rolipram tested. The inhibition of LPS-stimulated NO production detected in response to coaddition of both rolipram and adenosine was roughly equal to the combined responses observed with exposure to each reagent supplemented individually. Rolipram did not significantly alter NO production by unstimulated chondrocytes when tested at concentrations of 1 nM to 100 nM (data not shown). Unstimulated chondrocytes did increase NO synthesis when exposed to both 100  $\mu$ M adenosine and 100 nM rolipram simultaneously, but this increase was not statistically different than the increase detected in the presence of 100  $\mu$ M adenosine alone (Fig. 4).

## Discussion

Previous studies have documented the presence of adenosine receptor transcripts in articular chondrocytes<sup>30</sup>; however, functional responses by these cells to extracellular adenosine have not been described in the literature. The objective of this study was to establish whether adenosine and its analogs were capable of altering cAMP and NO production by articular chondrocytes, and to determine which subclass or subclasses of adenosine receptors were responsible for any observed biological responses. The results of this study show that chondrocytes respond to extracellular adenosine by increasing cAMP accumulation in a concentration-dependent manner. This study also demonstrated the ability of adenosine and adenosine analogues to significantly inhibit LPS-induced production of NO.

Measurement of intracellular cAMP levels was utilized here to monitor the functional response of articular chondrocytes to extracellular adenosine. An increase in cAMP accumulation in response to extracellular adenosine indicated the presence of active adenosine receptors on the surface of articular chondrocytes. This increase further

suggested involvement of the  $A_2$  adenosine receptor subclasses, which are known to activate adenylate cyclase<sup>32</sup>. In contrast, the presence of  $A_1$  and  $A_3$  receptor subclasses was not indicated by this response, as these subclasses are negatively coupled to adenylate cyclase and would not be expected to increase baseline cAMP accumulation. However, the presence of  $A_1$  and  $A_3$  receptors on chondrocytes cannot be ruled out, as they were not investigated further in this study. It is possible that all three subclasses of adenosine receptors are present on the surface of equine chondrocytes but that the  $A_2$  predominate, resulting in increased cAMP accumulation.

Forskolin, which directly activated adenylate cyclase, was utilized in this study as a positive control for cAMP production. The levels of intracellular cAMP accumulated in response to forskolin were much higher than the levels detected following exposure to adenosine receptor agonists, which was expected. Rolipram, a type IV phosphodiesterase inhibitor that prevents the catabolism of cAMP, was selected in this investigation for use in the cAMP assay because of its ability to prevent the rapid degradation of cAMP<sup>53,54</sup>, allowing the accurate detection of the cAMP levels being produced. In this study, rolipram was essential for the detection of any cAMP, even in the presence of forskolin, a potent stimulator of adenylate cyclase.

The  $A_2$  subclass of adenosine receptors have been further subdivided into the high affinity receptors, known as the  $A_{2A}$ , and the low affinity receptors, or  $A_{2B}$ <sup>32</sup>. The involvement of  $A_{2A}$  receptors in the chondrocyte response to extracellular adenosine demonstrated here is supported by the increase in cAMP accumulation shown in response to the  $A_{2A}$  specific agonist DPMA. However, an increase in cAMP accumulation was not detected with the highest concentration of DPMA tested. It may be that such a high concentration of this selective agonist is toxic or that DPMA loses receptor specificity when present at high concentrations. Although the positive response to DPMA indicates the presence of functioning  $A_{2A}$  receptors on equine chondrocytes, the presence of functioning  $A_{2B}$  receptors cannot be ruled out on the basis of this study. Distinguishing between the activation of  $A_{2A}$  and  $A_{2B}$  receptors in this study was complicated by the lack of a commercially available  $A_{2B}$  specific agonist.

It is well established that adenosine has a short half-life in biological systems<sup>24</sup> and this suggested that preventing the rapid degradation of adenosine could enhance its effects on chondrocytes. The adenosine analogue  $N^6$ -methyladenosine was chosen to investigate this hypothesis, as it is a poor substrate for adenosine deaminase<sup>41</sup>, which catalyses the breakdown of adenosine to inosine.  $N^6$ -methyladenosine increased the intracellular accumulation of cAMP by primary articular chondrocytes in a concentration-dependent manner but was not as effective as adenosine in promoting cAMP accumulation. This is consistent with previously published papers that report a reduced ability of  $N^6$ -methyladenosine to elicit physiological responses linked to  $A_2$  receptors when compared to the responses observed with adenosine<sup>55,56</sup>. It has also been reported that  $N^6$ -methyladenosine has a higher binding constant, and thus a lower affinity, for both  $A_1$  and  $A_2$  receptors than adenosine<sup>57</sup>.

The adenosine deaminase inhibitor EHNA was also selected for investigation in this study due to its ability to slow the breakdown of adenosine. Chondrocytes exposed to exogenous adenosine in the presence of EHNA demonstrated an increased accumulation of intracellular cAMP over cells exposed to adenosine alone. The results of this

study support the hypothesis that prolonged exposure to adenosine by preventing its degradation does indeed result in increased cAMP accumulation, as the adenosine deaminase inhibitor EHNA is able to enhance adenosine-mediated cAMP accumulation. The use of adenosine kinase inhibitors may similarly prove to be effective at protecting endogenously produced adenosine in the joint, providing antiinflammatory benefits.

The antiinflammatory potential of adenosine is being investigated in many other biological systems<sup>46,58</sup>. The study described here utilized an *in vitro* model of the joint to test the effects of adenosine on NO production by chondrocytes alone and in the presence of LPS, a proinflammatory stimulus. Chondrocytes incubated in the presence of LPS increased production of NO, as previously documented<sup>35</sup>. Incubation with adenosine and adenosine analogues significantly reduced production of NO by chondrocytes that were simultaneously exposed to LPS. This reduction was observed with adenosine, a broad adenosine receptor agonist, NECA, or with the  $A_{2A}$  specific agonist DPMA. The ability of DPMA to reduce LPS-stimulated NO production further supports the presence of functioning  $A_{2A}$  receptors, on equine chondrocytes.

In contrast to responses observed in the presence of LPS, adenosine receptor agonists did not significantly alter baseline NO production by chondrocytes that were not exposed to LPS. This result was expected, as unstimulated chondrocytes produce very low levels of baseline NO and express negligible levels of iNOS<sup>35</sup>. However, exposure to the highest concentration of adenosine tested did cause a small increase in NO production. The NO levels measured in response to the highest concentration of adenosine alone were 10- to 20-fold lower than those seen in response to LPS stimulation. The observed production of low levels of NO with the addition of exogenous adenosine in the absence of proinflammatory stimuli may be attributed to the presence of adenosine receptor subclasses that both stimulate and inhibit adenylate cyclase on the same cell type. A high concentration of adenosine may be able to saturate any  $A_1$ ,  $A_2$ , and  $A_3$  receptors on the surface of articular chondrocytes. Additional work will be required to investigate the significance of the observed response to high levels of adenosine.

The ability of adenosine to reduce LPS-induced NO production appears to be at least partially mediated by the accumulation of cAMP in response to adenosine receptor activation. For example, forskolin, a direct stimulator of adenylate cyclase, was able to decrease LPS-induced NO synthesis in the absence of adenosine. Additionally, rolipram, which inhibits the rapid degradation of cAMP by phosphodiesterases, enhanced the adenosine-mediated decrease in NO production by LPS-stimulated chondrocytes. The effects of cAMP accumulation in LPS-stimulated chondrocytes are consistent with prior reports demonstrating that the production of cAMP has antiinflammatory effects on neutrophils and that these effects were enhanced by exposure to rolipram<sup>46,59</sup>.

Although this study suggests that an increase in adenosine receptor-mediated cAMP is responsible for the observed decrease in LPS-induced NO, the mechanism for that association is not clear. Prior work in other cell types has shown that cAMP activates protein kinase A (PKA), which can signal through a variety of intermediate signaling proteins<sup>60</sup>. For cAMP to decrease LPS-induced NO, as demonstrated in this study, there is probably an effect of PKA-mediated pathways on the signaling pathway that is activated by LPS. Lipopolysaccharide is known to share a

number of signaling proteins with IL-1 and TNF, and the effect of cAMP may be on the transcription factor NF $\kappa$ B, which is activated by all three<sup>61</sup>. There is evidence that in some cell types intracellular cAMP accumulation can inhibit the activation of NF $\kappa$ B<sup>62,63</sup>. Further study is necessary to determine the specific mode of interaction that appears to be occurring between the two signaling pathways in the inhibition of NO production by chondrocytes.

In addition to the physiological effects mediated by exogenously supplemented adenosine, these experiments also suggest a possible role for endogenous adenosine on chondrocytes. The suppression of LPS-induced NO synthesis by EHNA alone, which prevents the normally rapid enzymatic breakdown of adenosine, suggests that chondrocytes may release physiologically significant levels of adenosine when stimulated with LPS. Exposure to EHNA alone did not significantly alter baseline production of NO by unstimulated chondrocytes, but was able to significantly inhibit the production of NO by chondrocytes that were stimulated with LPS. This suggests that endogenous adenosine may be released by chondrocytes exposed to inflammatory stimuli and that this adenosine is able to interact with cell surface receptors and subsequently reduce NO synthesis.

In this study, the phosphodiesterase inhibitor rolipram suppressed NO production by chondrocytes that were exposed to LPS but did not significantly alter the production of NO by unstimulated chondrocytes. These findings suggest that increased levels of cAMP are generated in the presence of LPS but that this cAMP would be degraded in the absence of rolipram. One explanation is that there may be increased release of adenosine in response to LPS. Released adenosine could then interact with cell surface receptors in an autocrine or paracrine fashion and mediate an increase in intracellular cAMP production. This supposition is supported by the inability of EHNA to increase the concentration of intracellular cAMP when present alone even though EHNA was shown to heighten cAMP accumulation in response to adenosine. Additional studies have been initiated to investigate the ability of chondrocytes to release adenosine, particularly when under the influence of pro-inflammatory stimuli such as IL-1 or LPS.

To consider the physiological relevance of this study, it is important to compare the levels of stimulation required to elicit a response to the concentration of adenosine known to be present in biological systems. Other studies have demonstrated adenosine release at sites of inflammation at concentrations up to 1  $\mu$ M *in vivo*<sup>64</sup>. The results of this study demonstrate increased cAMP accumulation by chondrocytes exposed to 1  $\mu$ M adenosine. It is certainly possible that local concentrations of adenosine at sites of joint inflammation may be even higher than those previously measured, as adenosine detection is complicated by its very short half-life in biological systems<sup>24</sup>.

The data reported in this paper demonstrate some of the functional effects of adenosine receptor stimulation on equine articular chondrocytes. The therapeutic potential of endogenous adenosine for down-regulating immune cell mediated damage is already under investigation in other systems<sup>11,15</sup>, but there is a critical need for more detailed investigation of the role of exogenous and endogenous adenosine in the joint. Additional studies are indicated to examine the mechanism of action and release of adenosine in the joint and to expand previous work to include other species.

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