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Annulus Cells Release ATP in Response to Vibratory Loading In Vitro

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Abstract Mechanical forces regulate the developmental path and phenotype of a variety of tissues and cultured cells. Vibratory loading as a mechanical stimulus occurs in connective tissues due to energy returned from ground reaction forces, as well as a mechanical input from use of motorized tools and vehicles. Structures in the spine may be particularly at risk when exposed to destructive vibratory stimuli. Cells from many tissues respond to mechanical stimuli, such as fluid flow, by increasing intracellular calcium concentration ($[Ca^{2+}]_{ic}$) and releasing adenosine 5′-triphosphate (ATP), extracellularly, as a mediator to activate signaling pathways. Therefore, we examined whether ATP is released from rabbit (rAN) and human (hAN) intervertebral disc annulus cells in response to vibratory loading. ATP release from annulus cells by vibratory stimulation as well as in control cells was quantitated using a firefly luciferin-luciferase assay. Cultured hAN and rAN cells had a basal level of extracellular ATP ($[ATP]_{ec}$) in the range of 1–1.5 nM. Vibratory loading of hAN cells stimulated ATP release, reaching a net maximum [ATP] within 10 min of continuous vibration, and shortly thereafter, [ATP] declined and returned to below baseline level. [ATP] in the supernatant fluid of hAN cells was significantly reduced compared to the control level when the cells received vibration for longer than 15 min. In rAN cells, [ATP] was increased in response to vibratory loading, attaining a level significantly greater than that of the control after 30 min of continuous vibration. Results of the current study show that resting annulus cells secrete ATP and maintain a basal $[ATP]_{ec}$. Annulus cells may use this nucleotide as a signaling messenger in an autocrine/paracrine fashion in response to vibratory loading. Rapid degradation of ATP to ADP may alternatively modulate cellular responses. It is hypothesized that exposure to repetitive, complex vibration regimens may activate signaling pathways that regulate matrix destruction in the disc. As in tendon cells, ATP may block subsequent responses to load and modulate the vibration response. Rabbit annulus cells were used as a readily obtainable source of cells in development of an animal model for testing effects of vibration on the disc. Human cells obtained from discarded surgical specimens were used to correlate responses of animal to human cells. J. Cell. Biochem. 90: 812–818, 2003. © 2003 Wiley-Liss, Inc.

Key words: ATP signaling; annulus cells; intervertebral disc; mechanical stimuli; vibration

Low back pain is a common problem in the United States and throughout the world with a lifetime prevalence estimated at 80–90% of the population [Frymoyer and Cats-Baril, 1991; Nerlich et al., 1997]. Pathological changes in the lesions are characterized as degeneration, in the nucleous pulposus and annulus fibrosus [Johnstone and Bayliss, 1995; Nerlich et al., 1997]. The annulus fibrosus shows coarsened and hyalinized fibers and progressive fissuring (both circumferential and radial) and loss of collagen fibers in the lamellae [Bernick et al.,

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1991]. There are also changes in the non-collagen extracellular matrix including proteoglycans [Bernick et al., 1991; Johnstone and Bayliss, 1995]. The net result of the degenerative process is fibrous replacement of the nucleus pulposus by the sixth or seventh decade of life [Holm, 1993; Nerlich et al., 1997]. Although intrinsic and extrinsic factors contribute to cell death and matrix protein degradation, the etiology of the disease is not clear.

The intervertebral disc is subjected to biomechanical factors as well. The disc is under some load at all times due to the forces of the muscles, ligaments and hydrostatic pressures [Nachemson, 1966]. Mechanical load stimulates disc changes directly through cellular effects and indirectly through alterations of the local environment such as vascular circulation or innervation. Therefore, mechanical loading may result in structural and metabolic differences within the intervertebral disc. Physiological loading is beneficial to maintain metabolism and function of the disc just as it is in articular cartilage [Holm and Nachemson, 1983; Oshima et al., 1995; Ishihara et al., 1996]. However, excessive mechanical loading appears to be detrimental both experimentally and clinically. Epidemiological and cohort studies have shown a definite association between excessive loading of the spine and increased problems with low back pain, disc degeneration, and disc herniation [Frymoyer et al., 1980, 1983; Damkot et al., 1984; Hansson and Holm, 1991; Bovenzi and Zadini, 1992; Miyashita et al., 1992; Pope and Novotny, 1993; Seidal, 1993; Hales and Bernard, 1996; Adarsh et al., 1999]. As a mechanical risk factor, vibratory loading, particularly chronic exposure to whole body vibration, results in a significant increase in low back degenerative disorders [Hulshof and van Zanten, 1987; Hansson and Holm, 1991; Bovenzi and Zadini, 1992; Pope and Hansson, 1992; Wilder, 1993]. Excessive vibration in the 4–6 Hz range can be harmful [Adarsh et al., 1999]. The natural vibration frequency of the human body is 3–7 Hz [Haack, 1956; Hornick, 1961], 4–8 Hz for human trunk [Troup, 1978], and 4.4 Hz for lumbar vertebrae [Punjabi et al., 1986]. Haack and coworkers [1956] have also reported that vibration at lower frequency up to 6 Hz is transmitted undiminished to the human body. Operation of heavy equipment such as tractors exposes the operator to vibration in the range of 1–7 Hz [Haack, 1956; Morrison and Horrington, 1962; Pope and Hansson, 1992]. However, few studies have tested effects of vibratory loading on annulus cells.

Adenosine 5′-triphosphate (ATP) is a ubiquitous intracellular source of energy. However, many cell types secrete ATP in response to mechanical stimulation [Harden et al., 1997; Lazarowski et al., 1997; Watt et al., 1998]. Extracellular ATP binds to the specific purinoceptors in the plasma membrane and initiates intracellular signaling cascades [Dubyak and el-Moatassim, 1993]. Recent studies showed that apyrase (degrades ATP) or suramin (a purinoceptor antagonist) reduced the Ca^{2+} wave propagation across chondrocytes following a mechanical stimulation, suggesting that extracellular ATP may be involved in mechanotransduction [D'Andrea et al., 1998]. Secreted ATP may serve as an extracellular effector in an autocrine/paracrine function, signaling by increasing intracellular calcium concentration ($[Ca^{2+}]_{ic}$) [Minchew et al., 1999a,b]. In the present study, we hypothesized that vibratory loading would induce ATP release in cultured rabbit (rAN) and human annulus (hAN) cells that would feed back on purinoceptors and stimulate cell signaling. We report that human and rabbit annulus cells release endogenous ATP in response to vibration and that ATP may modulate the response to mechanical load.

MATERIALS AND METHODS

Isolation and Primary Culture of Human and Rabbit Annulus Cells

Human annulus fibrosus tissues were obtained from material discarded at surgery. Patient ages ranged from 14- to 50-years old (5 patients). Rabbit annulus fibrosus tissues were collected from adult New Zealand White Rabbits (approximately 3 kg each; 10–11-weeks old, five rabbits) immediately after being euthanized. Lumbar discs were obtained in an en bloc fashion using an osteotome via an anterior approach. Annulus fibrosus tissue was pooled from the harvested lumbar disc. Inner and outer segments of tissue were dissected free of nucleus pulposus and adherent connective tissue. To obtain annulus fibrosus cells, tissues were minced and digested with 0.5% collagenase for 20 min at 37°C with gentle agitation [Elfervig et al., 2001]. Cells were grown in Medium 199, containing 10% fetal bovine serum, 20 mM HEPES buffer (pH 7.2), and 1% penicillin/

streptomycin. At passages 2–6, cells were seeded at 25k cells/cm^2/well of 24-well plastic plates in complete medium and growth-arrested by halving the medium with serum-free medium on days 3 and 5. On day 6, the medium was changed to Eagle's Minimal Essential Medium without phenol red (chemicals used in cell culture were supplied from Gibco BRL, Grand Island, NY).

Vibratory Loading Device

A special vibratory jig was used to apply loads to the cells in culture [Weinhold et al., 2000]. This device was designed to produce a sinusoidal vibration with frequency range of 1–10 Hz at 0.1 G amplitude on cells attached at the bottom of the culture plate by altering the dimensions of the eccentric cam utilized in the follower/actuating rod/culture plate assembly (Fig. 1). The acceleration at the surface of the culture plate has been characterized by using cyanoacrylate glue to adhere a piezoelectric accelerometer to the plate surface. Therefore, the vibration measured at the culture surface was likely directly sensed by the cells. In our initial analysis of the vibration spectrum of the acceleration signal, it was confirmed that the acceleration at 6 Hz was the component with the greatest amplitude.

Vibration on Annulus Cells and ATP Measurement in the Supernatant Fluid

After changing to serum-free medium, cells were rested in the system for 4 h, then stimulated with a sinusoidal vibration of 0.1 G amplitude at 6 Hz. Human annulus cells (hAN) were vibrated for 1, 5, 10, 15, 20, 30, or 60 min (one 24-well plate for one vibration period). Rabbit annulus cells (rAN) were vibrated for 1, 5, 15, or 30 min. These time points were chosen since release of ATP is usually rapid, included early times in which ATP could be measured, the duration after which ATP might down-regulate a response and lastly be degraded. Moreover, the maximum times for exposure to vibration (60 min) were considered consistent with moderate travel times in vehicles and exposure to vibration. For each vibration time point, the supernatant fluids were collected at 0, 1, 5, and 10 min post-vibration (hAN cells and rAN, 6 wells for each collecting time point, $n = 6$). The supernatant fluid samples were collected without contacting the cell layer, transferred to microfuge tubes, then boiled for

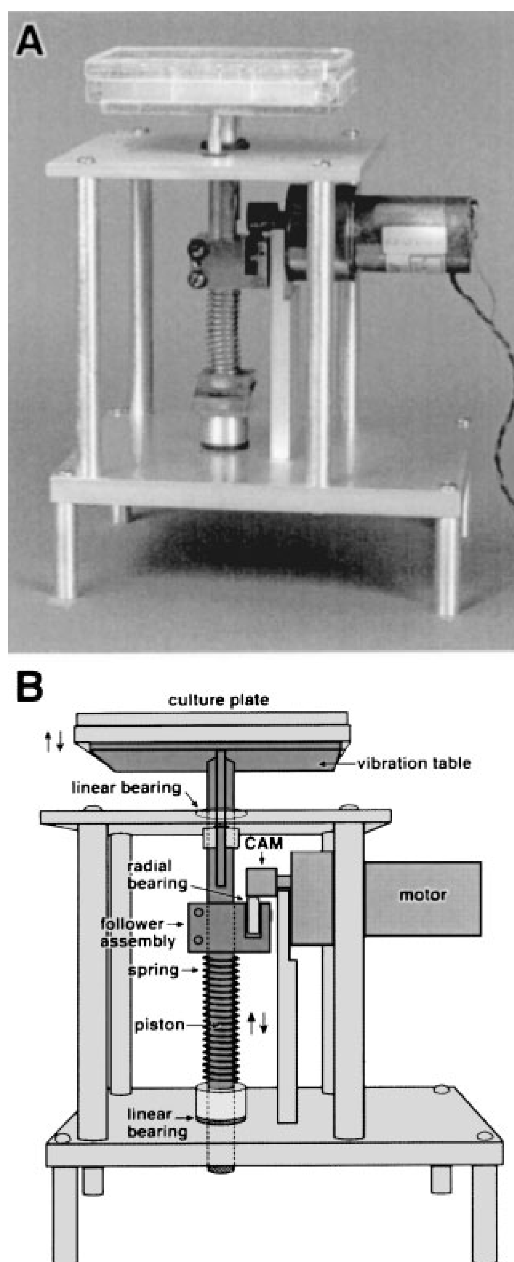

Fig. 1. Photograph (**A**) and drawing (**B**) of Vibratory Loading Device. A special vibratory jig was used to apply loads to the cells in culture. The vibratory loading device utilized an eccentric cam driven by a DC motor to produce the sinusoidal vibration of 0.1 G amplitude in the frequency range of 1–10 Hz. The cam rotated against a bearing interfaced to a piston delivering vibratory motion to the table where the culture plate was fixed. This loading device was designed by Dr. P.S. Weinhold and Dr. A.J. Banes.

1 min to inactivate ATPase. ATP concentration in the supernatant fluids ([ATP]) was measured by a firefly luciferase assay [Watt et al., 1998; Graff et al., 2000]. Samples were incubated with luciferin and luciferase (Analytical

Luminescence Laboratory, Ann Arbor, MI; distributed by PharMingen, San Diego, CA) in the reaction buffer and the emission was read in a 96-well plate using a luminometer with software (Labsystems, Helsinki, Finland, FLUOROSKAN ASCENT FL). The samples collected from the no vibration control plate were used as negative controls and samples from whole cell lysates served as positive controls.

Statistical Analysis

Unless otherwise indicated, the values are presented as the mean $\pm$ SD with the number of independent experiments. Statistical significance was tested by repeated measures ANOVA with Dunnett's or Tukey's tests. Significance levels were indicated with their P values. A result that was deemed significant is $P < 0.05$.

RESULTS

Results of ATP measurement from control culture supernatant fluids demonstrated that resting disc annulus cells, hAN and rAN, secrete ATP. After changing the medium, cells were incubated in the platform of the vibratory jig for 4 h to allow [ATP] to return to baseline levels before subjection to vibration. The baseline level of [ATP] was in the range of 1–1.5 nM in both hAN and rAN (1.36 $\pm$ 0.07 nM for hAN and 0.95 $\pm$ 0.17 nM for rAN) (Figs. 2 and 3), similar to published values for airway epithelial cells and chondrocytes [Watt et al., 1998; Graff et al., 2000]. Vibratory loading of hAN cells stimulated ATP release within 5 min, reaching a net maximum concentration by 10 min. The value was increased twofold when compared to that of the 1 min loading group. We measured [ATP] at different collection time points post-vibration after each vibration in order to test the hypothesis that signaling to vibration might result in an immediate–as well as a sustained rise in [ATP]. A transient rise of [ATP] followed by a subsequent decline of [ATP] was measured in the supernatant fluids collected from hAN cells immediately after the vibration stimulus. [ATP] at post-vibration time points (1, 5, or 10 min after each vibration) did not change significantly within 10 min after the bout of vibration. When hAN cells were vibrated for

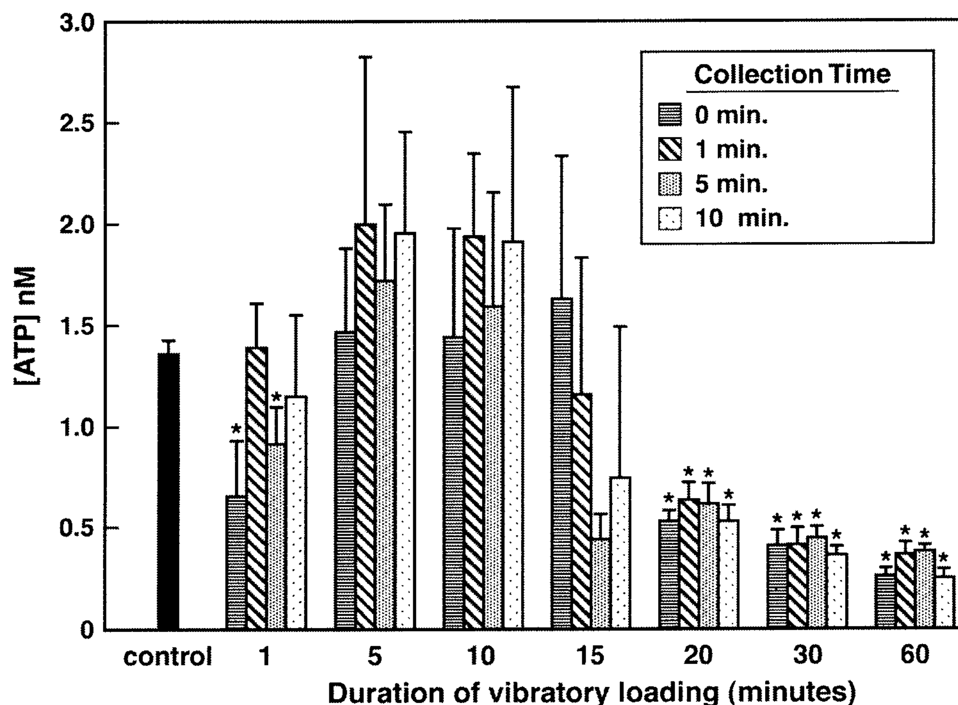

Fig. 2. ATP release by vibratory loading in human annulus cells. ATP release by human annulus (hAN) cells exposed to vibratory loading (6 Hz) was measured. Cultured hAN cells released ATP at a baseline concentration ([ATP] of the supernatant fluid) in the range of 1–1.5 nM. Vibratory loading of hAN cells stimulated ATP release within 5 min, reaching a net maximum [ATP] by 10 min. Shortly thereafter, [ATP] declined and returned to below baseline level. For vibration times longer than 15 min, [ATP] was significantly reduced compared to control levels (*significantly different from no vibration control: $P < 0.001$ for 1 min vibration, $P < 0.0001$ for 20, 30, and 60 min vibration).

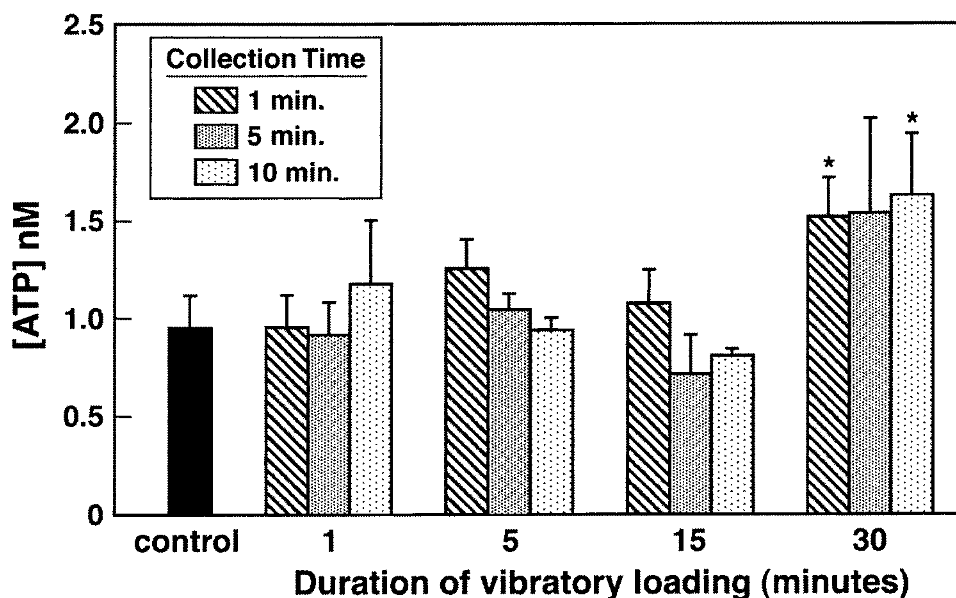

Fig. 3. ATP release by vibratory loading in rabbit annulus cells. ATP release by rabbit annulus (rAN) cells in response to vibratory loading (6 Hz) was measured. Cultured rAN cells released ATP at a baseline concentration in the range of 1–2 nM. [ATP] increased above control levels. However, the kinetic response was reduced and the rise of [ATP] was significant only when cells were vibrated for 30 min (*significantly different from no vibration control, $P < 0.05$).

longer than 15 min, [ATP] in the culture supernatant fluid was significantly lower than control levels at any post-vibration time point (as with 20 min vibration the ratios to control at 0.53 ± 0.05, 0.64 ± 0.10, 0.62 ± 0.12, 0.53 ± 0.08 at post-vibration 0, 1, 5, 10 min, respectively, $P < 0.001$) (Fig. 2). Levels of [ATP] continued to decline for up to 60 min in vibration, suggesting that vibration may modulate extracellular [ATP] through a balance between ATP release and ATP degradation/utilization (ATPase activity). In rAN cells, on the other hand, ATP was released by vibration and accumulated above the control level, but at a lower rate compared with the kinetics shown in hAN cells (Fig. 3). The increase in [ATP] was significant only when the cells were vibrated for 30 min (Fig. 3). [ATP] at post-vibration time points in rAN cell culture was not significantly changed within 10 min. Under the conditions used, cells remained attached at the culture plate substratum throughout the vibration period and thereafter.

DISCUSSION

Intervertebral disc cells receive complex mechanical forces through the load bearing surrounding tissues. It is thought that mechanical loading can cause disc changes as the result of direct cellular effects on tissues around the spine. Annulus cells sense and respond to fluid shear stress by increasing intracellular calcium levels ($[Ca^{2+}]_{ic}$) and opening calcium channels [Elfervig et al., 2001]. However, no study has addressed signaling responses of intervertebral disc cells to a major physiologic mechanical load: vibration. This is the first report indicating that resting annulus cells release ATP in vitro and that vibration can modulate ATP secretion and/or hydrolysis.

Recent studies have shown that annulus fibrosus cells transduce a mechanical stimulus to signaling pathways intracellularly in mechanisms such as ion channels, membrane receptors or cell-matrix interactions. Fluid flow and IL-1β act synergistically to increase and sustain high $[Ca^{2+}]_{ic}$ in human annulus cells [Elfervig et al., 2001]. Likewise, vibratory stimuli in annulus cells might modulate signaling responses particularly in conjunction with cytokines, using ATP as an immediate effector molecule. Vibration at 6 Hz at 0.1 G for 2–8 h decreased gene expression of extracellular matrix and matrix metalloproteinases in rabbit annulus cells in vitro [Yamazaki et al., 2003]. One effect of longer period of vibration (6 Hz, 0.1 G, 8 h/day for 3 days) is that DNA synthesis was upregulated in growth−arrested (G_0) human annulus cells [Weinhold et al., 2000]. It

was felt that this was a stimulus to the nondividing cells and not a signal to enter an apoptotic pathway. Therefore, vibratory loading may act by stimulating catabolic and/or anabolic pathways. The quantity and quality of the vibration stimulus may be critical in determining which pathway is activated. In particular, extracellular ATP may play an important role in cell- and tissue-maintenance.

Results of the present study demonstrated that resting annulus cells secrete ATP at levels that are approximately those of published values for airway epithelial cells and chondrocytes [Watt et al., 1998; Graff et al., 2000]. Vibratory loading at 6 Hz stimulated ATP release within 5 min in hAN cells and 30 min in rAN cells, compared with results of the static controls. The reason for the delay of ATP release from rAN in response to vibration is unclear. In a study of the effects of fluid shear stress on rabbit tendon cells $[Ca^{2+}]_{ic}$ did not rise unless calcium and serum were present in the medium [Archambault et al., 2002]. Therefore, cofactors, including extracellular calcium can regulate mechanical signals.

It is possible that ATP may be detectable earlier and that actual values are greater than those being measured in the supernatant fluid samples, given that extracellular NTPases are known to quickly degrade ATP [Graff et al., 2000; Yagutkin et al., 2000]. A rise in [ATP] was detected at 30 min vibration, but a decreased signal may have resulted from ATPase activity [Graff et al., 2000; Tsuzaki et al., 2003]. After 15 min of vibration, in particular in human cells, a decline in detectable [ATP] occurred during the subsequent vibration periods (20, 30, 60 min), indicating continuous destruction of ATP by ATPase. It is likely that vibration stimulates annulus cells to increase ATPase activity as well as ATP release.

Extracellular ATP may act in an autocrine/paracrine fashion as a signaling molecule when disc cells are mechanically loaded. We have demonstrated that intervertebral disc cells express cell surface purinoceptors and respond to 1 μM ATP by increasing intracellular $[Ca^{2+}]_{ic}$ [Elfervig et al., 2001]. Exogenous ATP has also been shown to transiently desensitize annulus cells to respond to subsequent mechanical stimulation [Russo et al., 2001]. ATP release during the initial vibration period may in turn block the subsequent recognition of the vibratory signal to annulus cells. ATP can induce phosphorylation of proteins such as ion channels and intracellular pathway kinases that may regulate cell responses to vibratory stimulation. ATP reduces load-inducible inflammatory gene expression in human tendon cells, suggesting that ATP may serve as a signal dampener to block excess load signals to connective tissue cells [Tsuzaki et al., 2003]. We hypothesize that annulus cells secrete ATP and utilize this nucleotide as a signaling messenger, perhaps to block subsequent responses to mechanical stimuli [Banes et al., 1995, 2001; Tsuzaki et al., 2003].

ATPase and ecto-nucleotidases hydrolyze ATP quickly to terminate the primary action, while purine metabolites such as ADP, AMP and adenosine, as by-products of ATP degradation, may contribute further to modulate the response. Therefore, the effects of vibration via ATP release may be more complex. Furthermore, in vivo, intervertebral discs may receive vibration concomitant with other mechanical forces including compression and shear that may modify the vibratory loading regimen to disc cells in vivo.

In conclusion, vibration stimulates release of ATP from intervertebral disc cells which feeds back on purinoceptors to increase $[Ca^{2+}]_{ic}$. Discs are subjected to complex vibration at all times during ambulation and work. ATP may modulate disc cell response to mechanical load and reduce potential deleterious effects from constant signal input.

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