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Purine nucleotides modulate proliferation of brown fat preadipocytes

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Abstract. The hypothesis that purine nucleotides and nucleosides affect brown fat preadipocyte proliferation was tested using isolated rat interscapular brown fat preadipocytes in culture. Daily addition of 100 μM adenosine triphosphate (ATP) ($n = 4$) to cultures enhanced the relative DNA content by 1.5-fold compared to control cultures ($P < 0.05$) measured using CyQUANT-GR fluorescence. Higher concentrations of ATP inhibited growth and 500 ($n = 2$) or 1000 μM ATP ($n = 3$) almost completely inhibited growth. ATP (100 μM) did not affect while 250–1000 μM ATP decreased protein content relative to control cultures. Adenosine (100 μM; $n = 3$) did not affect DNA or protein content, but 500 μM and 1000 μM adenosine suppressed brown adipocyte proliferation and inhibited protein synthesis. Cultured brown adipocytes quickly removed or degraded ATP in the culture media as determined by luciferin–luciferase bioluminescence, suggesting that the inhibitory effects of high ATP concentrations may result from its breakdown to adenosine. The results support the conclusion that ATP promotes and adenosine inhibits brown adipocyte proliferation.

INTRODUCTION

Sympathetic nerve stimulation *in vivo* promotes brown adipocyte proliferation (Bukowiecki, Geloen & Collet 1986, Geloen *et al.* 1988, Geloen, Collet & Bukowiecki 1992). Cold exposure or overeating enhance the thermogenic capacity of brown fat by increasing the number and energy-wasting capacity of brown adipocytes Norepinephrine or isoproterenol injections, which mimic cold exposure, induce increases in brown adipocyte DNA synthesis *in vivo* (Geloen *et al* 1988), and β-adrenergic stimulation promotes brown adipocyte mitogenesis *in vitro* (Bronnikov, Houstek & Nedergaard 1992). However, the effects of adrenergic stimulation *in vitro* do not fully explain the effects that cold exposure or overfeeding have on brown adipose tissue *in vivo* (Geloen *et al* 1988, Yamashita *et al* 1994, Yamashita *et al* 1995), suggesting that additional agents may contribute to sympathetic actions Norepinephrine and adenosine triphosphate (ATP) are often co-localized in sympathetic nerve terminals and may be released simultaneously by sympathetic neuronal activity (Lagercrantz 1976, Westfall *et al.* 1990). Moreover, brown adipocytes respond to extracellular ATP as well as adenosine, its parent nucleoside. P2 purinergic stimulation by extracellular

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ATP or adenosine diphosphate (ADP) increases cytosolic Ca^{2+} and activates membrane trafficking and membrane conductance, but does not alter norepinephrine-elicited thermogenesis (Pappone & Lee 1996, Lee & Pappone 1997). Adenosine, acting at A_1 purinergic receptors, inhibits norepinephrine-induced increases in cyclic adenosine monophosphate (cAMP), lipolysis, and cellular respiration (Szillat & Bukowiecki 1983, Schimmel, Elliott & Dehmel 1987, Schimmel & Elliott 1988). The experiments presented here tested the hypothesis that ATP and adenosine modulate the proliferation of cultured preadipocytes from brown adipose tissue. The results show that daily exposure to ATP or adenosine profoundly alters preadipocyte growth.

MATERIALS AND METHODS

Cell culture

Brown fat preadipocytes were isolated from 1-week-old Sprague–Dawley rat pups (Simonson, Livermore, CA, USA) using a procedure based on Bronnikov *et al.* (1992). Rat pups were cold anaesthetized for approximately 30 min prior to decapitation, as approved by the University of California Animal Care and Use Committee. Interscapular brown fat pads were dissected and placed into phosphate-buffered balanced salt solution (PBS) (Life Technologies, Grand Island, NY, USA). Fat pads were minced and dissociated for 30 min with 2.8 mg/ml type I collagenase (Worthington Biochemical, NJ, USA) with 0.2 mg/ml DNAase I (Boehringer Mannheim, Indianapolis, IN, USA). Cell suspensions were triturated several times and serially filtered through 250, 70, and 40 μm filters (Falcon, Franklin Lakes, NJ, USA). The resulting single cell suspensions were pelleted by centrifugation at 700 ***g*** for 10 min. The supernatants were removed and the cell pellets resuspended in 10 ml complete Dulbecco's Modified Eagle's Medium (DMEM), containing glutamine (Life Technologies, Grand Island, NY, USA), 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 0.016 mg/ml insulin (Life Technologies), and 1% antibiotic-antimycotic solution (Sigma, St Louis, MO, USA) consisting of 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25 μg/ml amphotericin B. The cells were counted with a haemocytometer and suspended at $1–2 \times 10^5$ cells/ml in complete DMEM. Twenty-four well tissue culture plates (Falcon, Franklin Lakes, NJ, USA), previously coated with type I collagen (Calbiochem, La Jolla, CA, USA), were inoculated with 1 ml of the cell suspension per well. Cells were maintained in a 37°C incubator with 5% CO_2. Twenty-four hours following inoculation, the cells were rinsed twice with PBS and fed with complete DMEM or complete DMEM containing the experimental agonist. Each condition was performed on triplicate wells. Aseptic stock solutions of 100 mM ATP were prepared ahead of time, adjusted to pH 7.4 with NaOH, and added to the complete DMEM immediately prior to feeding. Adenosine was mixed into incomplete DMEM, adjusted to pH 7.4, and sterilely filtered; FBS, antibiotic and insulin were added immediately prior to feeding. Cells were re-fed daily. Plates removed for later analysis were rinsed with PBS , air dried, and stored at −20°C.

DNA and protein quantification

Tissue culture plates used for DNA and protein analysis were removed from the freezer and allowed to thaw. Cell lysis buffer (100 μl) (Molecular Probes, Eugene, OR, USA) with 0.1 mg/ml DNAase-free RNAase (Sigma) and 1 mM EDTA was added to each well. Cells were scraped off the dish with a plastic scraper and the resultant suspension sonicated on ice. Cell lysates were incubated for 1 h at 25°C to ensure RNA degradation. Some samples were

diluted with lysis buffer Ten microlitres of each sample were placed into wells of a 96-well plate (Falcon) Triplicate measurements were made on each sample

DNA content of the cultures was assayed using fluorescence with a procedure based on Roberts *et al* (1991) (CyQUANT™ Cell Proliferation Assay Kit, Molecular Probes) A concentrated bacteriophage DNA standard (Molecular Probes) was serially diluted and added to a separate 96-well plate Two hundred microlitres of a 1/400 dilution of CyQUANT-GR dye (Molecular Probes) was placed into each well Samples exposed to the CyQUANT-GR dye were incubated at 25°C in the dark for several minutes before reading Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm on a microplate fluorometer (Packard Instruments Co , Meriden, CT, USA) Two separate readings were made of each plate and the fluorescence of the DNA standards was measured both at the beginning and end of the experimental readings

Protein content of the cultures was assayed by measuring bicinchoninic acid–cuprous ion absorbance with a procedure based on Smith *et al* (1985) (BCA Protein Assay Reagent Kit, Pierce, Rockford, IL, USA) A 1 mg/ml stock bovine serum albumin (BSA) standard (Sigma) was serially diluted and added to a separate 96-well plate BCA working reagent (200 μl) (Pierce) was placed into each well Samples exposed to the BCA working stock were incubated at 25°C for 8–12 h before reading Absorbance was measured at a wavelength of 540 nm on a microplate reader (Bio-kinetics reader EL340, Bio-Tec Instruments Inc , Winooski, VT, USA) Two separate readings were made of each plate and the absorbance of the BSA standards was measured both before and after reading the experimental samples

ATP content

The ATP concentration in the culture medium was assayed by measuring luciferase bioluminescence with a procedure based on DeLuca & McElroy 1978 (ATP Bioluminescence Assay Kit CLS II, Boehringer Mannheim) Media samples (100 μL) were drawn from the culture wells and immediately stored at −80°C until analysis Experimental cultures were placed into a 37°C incubator with 5% CO_2 between sampling times Samples were thawed and diluted with distilled H_2O just prior to assay Diluted samples and luciferin–luciferase reagent were added to disposable reading cuvettes Luminescence was measured in a Turner TD-20e luminometer (Turner Designs, Sunnyvale, CA, USA)

Histology

Mature brown adipocytes were stained with Oil-Red-O to visualize lipid and Harris' haematoxylin to observe nuclei (Taboas & Ceremsak 1967, Kasturi & Joshi 1982) Cells were fixed with a 10% formalin solution, sealed with parafilm, and stored at 25°C for staining A 1% stock solution of Sudan III (Oil-Red-O) (Sigma) was prepared in isopropanol (3 parts of 1% stock mixed with 2 parts distilled H_2O (dH_2O)) and filtered through Whatman #4 filter paper (Whatman, Fairfield, NJ, USA) prior to cell staining Filtered Oil-Red-O was applied to fixed cells for approximately 1 h The cells were then washed with 70% ethanol Harris's haematoxylin (5 g haematoxylin crystals, 50 ml 95% ethanol, 100 g $AlK(SO_4)_2$, 2 5 g HgO, 1000 ml dH_20) was filtered through a Whatman #4 filter prior to use Culture wells were treated for 15 s–1 min with Harris' haematoxylin Cells were rinsed with dH_2O Finally, a cover slip was placed over glycerol jelly to store the stained cells Stained cells were visualized and photographed using phase contrast microscopy

RESULTS

Figure 1 shows a light micrograph of cells grown in culture and stained for lipids with Oil-Red-O Undifferentiated cells predominated in one-day-old cultures, while there was a mixture of undifferentiated cells and differentiated brown adipocytes in 10-day-old cultures With further time in culture, mature brown adipocytes coalesced into a central mass, which in many cases detached from the dish and floated, and stained positive for lipids (data not shown)

Feeding cultures daily with nucleotide-containing medium affected the proliferation of brown fat preadipocytes Table 1 shows DNA content of cultures from one set of

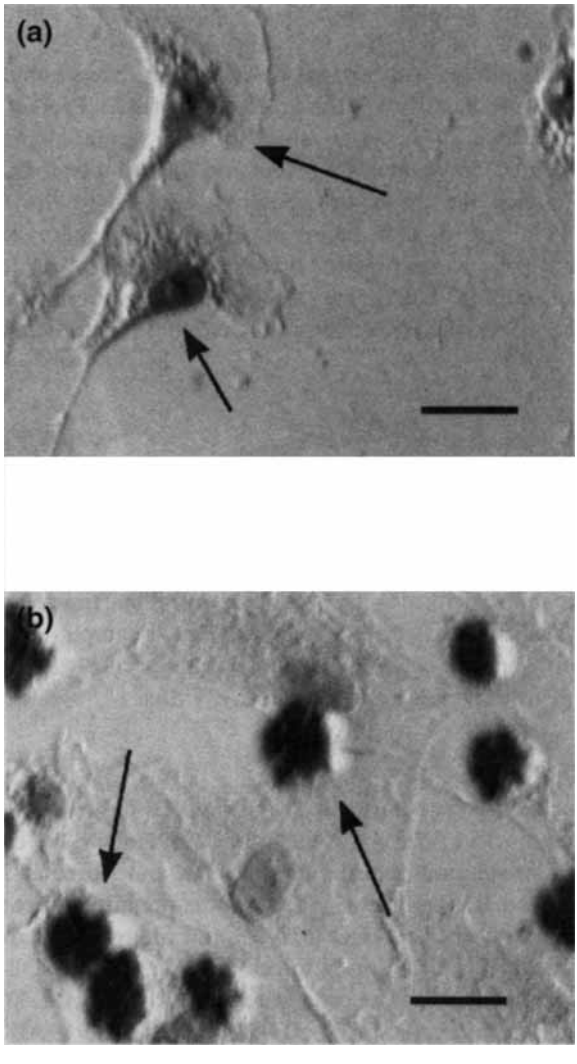

Figure 1 Oil Red O and haematoxylin stained cultured brown fat preadipocytes (a) Light micrograph of cells 1 day following isolation Arrows show examples of preadipocytes (b) Cells grown for 10 days in culture Arrows show examples of mature brown adipocytes that stained darkly for lipids Scale bar is 50 μm

Table 1. Changes in DNA content of brown fat preadipocytes cultures over time

	DNA content of brown fat preadipocyte cultures (μg/well)						
	Day 1	Day 3	Day 5	Day 7	Day 9	Day 12	Day 14
Control	0 13 ± 0 05	0 39 ± 0 15	0 94 ± 0 38	1 50 ± 0 33	5 16 ± 2 36	5 67 ± 1 07	6 62 ± 1 62
100 μM ATP	0 12 ± 0 01	0 44 ± 0 08	1 05 ± 0 32	1 04 ± 0 32	10 55 ± 1 26	9 71 ± 2 30	8 59 ± 1 85
500 μM ATP	0 15 ± 0 01	0 06 ± 0 01	0 10 ± 0 01	0 09 ± 0 01	0 26 ± 0 15	0 42 ± 0 04	0 55 ± 0 08
100 μM adenosine	0 13 ± 0 03	0 53 ± 0 04	1 14 ± 0 47	1 19 ± 0 47	5 51 ± 1 48	7 81 ± 0 25	5 32 ± 2 66
500 μM adenosine	0 10 ± 0 01	0 10 ± 0 01	0 06 ± 0 05	0 07 ± 0 05	0 70 ± 0 20	0 86 ± 0 06	0 75 ± 0 05

Results are μg DNA and are the mean ± SD of triplicate wells

experiments as a function of time in culture Up to 3 days after plating, none of the cultures increased in DNA content, indicating that they were not proliferating However, both control and nucleotide treated cells grew exponentially between days 7 and 9 before reaching a plateau Control cultures doubled approximately 2 5-times during the exponential growth phase Cultures treated daily with 100 μM ATP proliferated to a greater extent than control cultures, doubling approximately 3 5 times Cultures similarly treated with 100 μM adenosine were indistinguishable from controls In contrast, cultures exposed to 500 μM ATP or 500 μM adenosine did not proliferate as extensively as controls Culture protein content also increased with time, as shown in Table 2 However, cells treated with 100 μM ATP had a protein content similar to controls, suggesting that ATP-stimulated brown fat cells increased DNA synthesis more rapidly than protein synthesis Treating cultures with 500 μM ATP or adenosine inhibited protein synthesis

Daily exposure of cells to ATP affected the plateau density of brown fat preadipocytes Figure 2 shows the average relative DNA content of cultures grown for 11–14 days During the plateau phase DNA contents of control cultures ranged from 1 1 to 6 6 μg DNA per culture well Daily exposure to 100 μM ATP enhanced plateau phase DNA content by approximately 50% In contrast, cultures treated with higher concentrations of ATP (250 μM–1 mM ATP) showed decreased plateau DNA content Daily exposure to 100 μM

Table 2. Changes in protein content of brown fat preadipocyte cultures over time

	Protein content of brown fat preadipocyte cultures (μg/well)						
	Day 1	Day 3	Day 5	Day 7	Day 9	Day 12	Day 14
Control	25 ± 0 1	47 ± 1 3	186 ± 2 7	254 ± 6 2	412 ± 6 6	403 ± 9 5	501 ± 1 9
100 μM ATP	24 ± 0 4	41 ± 0 6	227 ± 6 2	272 ± 5 1	598 ± 9 4	516 ± 12 7	563 ± 16 5
500 μM ATP	25 ± 0 2	32 ± 0 3	42 ± 0 8	40 ± 1 7	37 6 ± 0 8	40 ± 3 7	53 ± 3 1
100 μM adenosine	25 ± 0 2	43 ± 1 2	221 ± 17 4	295 ± 33 8	475 ± 12 3	488 ± 7 6	510 ± 9 3
500 μM adenosine	22 ± 0 3	46 ± 0 6	33 ± 0 0	34 ± 0 7	38 3 ± 5 5	40 ± 1 5	59 ± 4 0

Results are μg protein and are the mean ± SD of triplicate wells

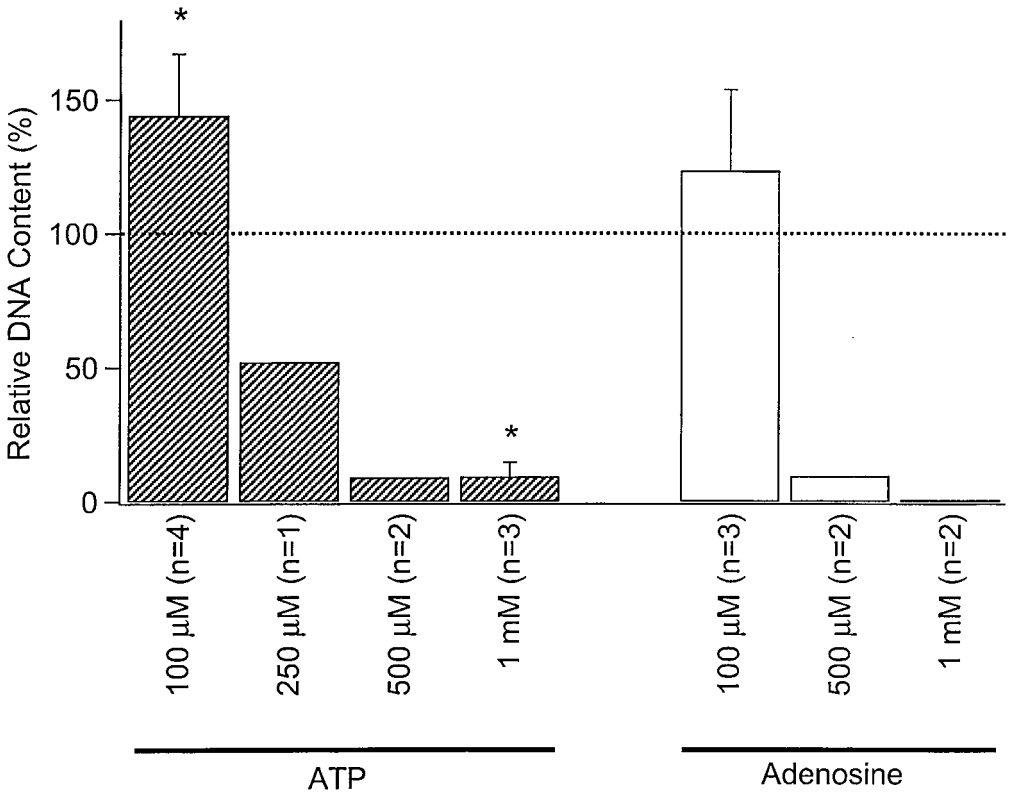

Figure 2. Effect of exogenous ATP and adenosine on plateau DNA content of ATP-exposed (hatched bars) and adenosine-exposed (open bars) cells. Culture DNA content is expressed as percentage relative to control, and given as mean ± SD where appropriate. Samples were drawn during the plateau phase of growth, after 11–14 days in culture. Cells were fed daily with complete DMEM without nucleotide (control) or with the concentration of agonist indicated. The number of independent experiments is also shown. *Denotes means significantly different from controls ($P < 0.05$).

adenosine did not significantly increase the plateau DNA content. However, higher concentrations of adenosine (250–500 μM) inhibited preadipocyte proliferation. Cells were not seen in cultures incubated with 1 mM adenosine, suggesting this concentration is cytotoxic.

Daily nucleotide and nucleoside exposure similarly affected protein synthesis. Figure 3 presents the average relative protein content compared to control cultures at confluence. During the plateau phase protein contents of control cultures ranged from 326 to 501 μg protein per culture well. One hundred micromolar ATP or adenosine did not increase relative plateau protein content, but higher concentrations of ATP or adenosine inhibited protein synthesis.

Brown adipocyte cultures degraded ATP. Figure 4 shows the ATP content of media as a function of time in the presence or absence of brown fat cells. ATP content decreased rapidly in the presence of cells, presumably as a result of breakdown or uptake. The time constant for the rate of ATP decline in the presence of brown fat cells was approximately 20–30 min, and approximately 120 min for ATP in serum-containing cell-free media. The ATP content of the media bathing cells without the addition of exogenous ATP was too low to be measured,

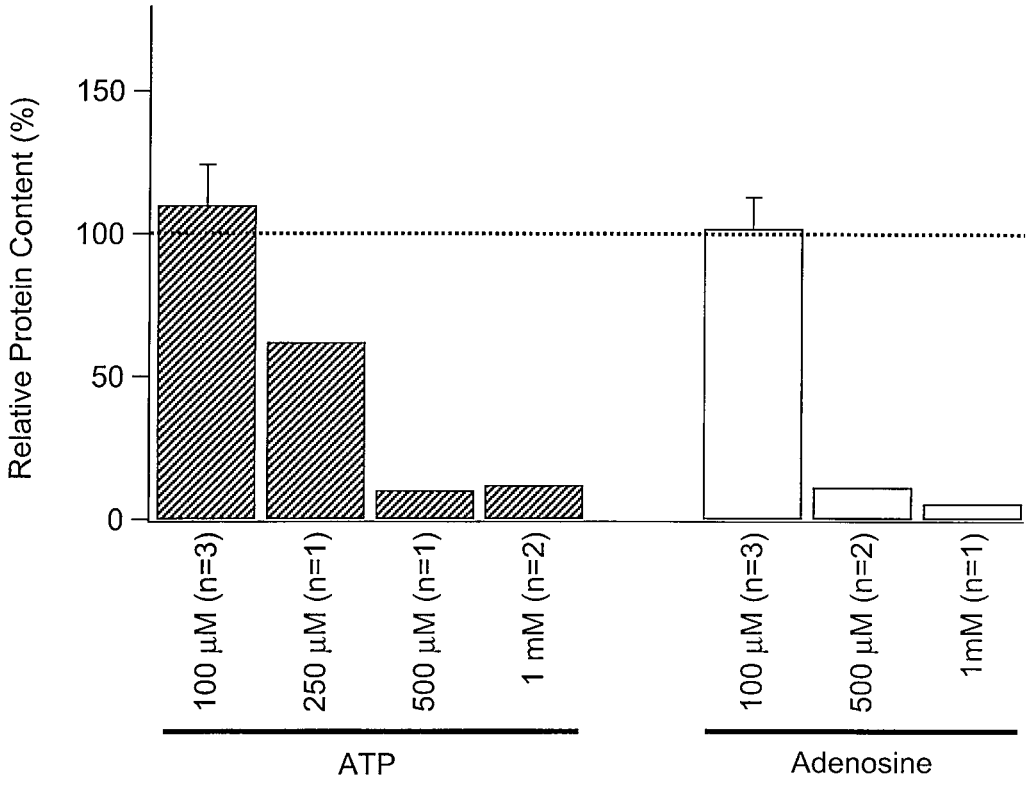

Figure 3 Effect of exogenous ATP and adenosine on plateau protein content of ATP-exposed (hatched bars) and adenosine-exposed (open bars) cells Culture protein content expressed as percentage relative to control and given as mean ± SD where appropriate Same cultures and experimental conditions as in Figure 2 Concentrations of agonists and number of independent experiments are indicated

even though these cells were exposed to the same mechanical stimuli, suggesting that preadipocytes do not release significant quantities of ATP under these conditions

DISCUSSION

Daily exposure of brown fat preadipocytes to purine nucleotides and nucleosides had significant positive and negative effects on their proliferation in culture Low concentrations of ATP stimulated DNA but not protein synthesis, while higher concentrations of ATP inhibited both DNA and protein synthesis Adenosine also modulated brown fat preadipocyte proliferation, but its effects were mainly inhibitory Low concentrations of adenosine did not affect either DNA or protein synthesis, while higher concentrations of adenosine clearly inhibited cell growth and proliferation Extracellular ATP was either rapidly degraded via the action of ectoenzymes (Pearson *et al* 1980) or removed through a ATP-binding cassette transporter (Abraham *et al* 1993, Cantiello 1997) by cultured brown adipocytes, suggesting that extracellular adenosine or other breakdown products of ATP may be responsible for the inhibitory effects of added ATP on cell proliferation Overall, these findings indicate that extracellular purine nucleotides and nucleosides can profoundly affect the proliferation of brown fat preadipocytes

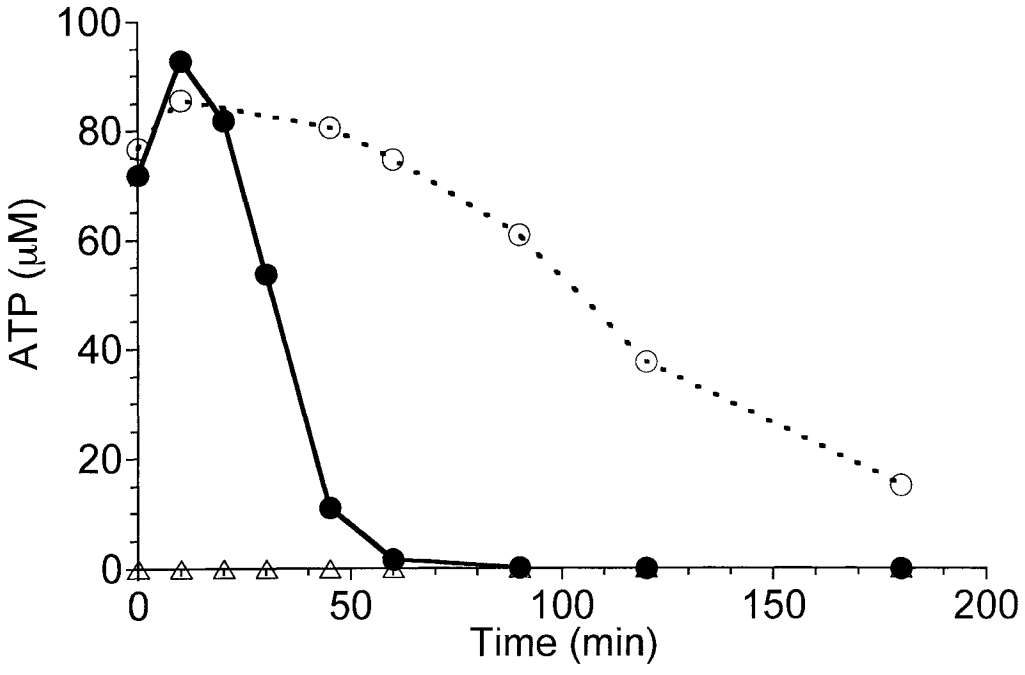

Figure 4. Degradation of extracellular ATP by cultured brown fat preadipocytes. ATP concentration of media as a function of time is shown for cultures incubated at 37°C. ATP (100 μM) in DMEM was added to either cultured cells (filled circles) or cell-free culture plates (open circles). Control cells (triangles) had their media exchanged and sampled, but did not have any ATP added. Results are from a single experiment.

ATP as a mitogenic factor

The finding that extracellular ATP is a mitogenic factor in brown fat preadipocyte cultures is novel but not unexpected. ATP acts as a mitogen for mesangial, smooth muscle, and several lineages of transformed cells (Huang, Wang & Heppel 1989, Schulze-Lohoff *et al.* 1992; Wang, Huang & Heppel 1992). ATP can also act synergistically with platelet derived growth factor (PDGF), epidermal growth factor (EGF), and insulin to enhance mitogenesis of 3T6 cells, and with adenosine to promote proliferation of smooth muscle and A431 cells (Huang *et al.* 1989, Wang *et al.* 1992). Since ATP was added to media containing serum and insulin in the experiments reported here, it is possible that ATP interacts with other mitogenic factors to regulate brown fat preadipocyte proliferation.

ATP can also be involved in regulation of cell hypertrophy as well as cell proliferation. For example, the hypertrophy of cardiac myocytes is regulated by both noradrenaline (norepinephrine) and ATP (Zheng *et al.* 1994, Zheng *et al.* 1996). Pre-exposure of cardiac myocytes to 100 μM ATP inhibits myocyte hypertrophy caused by noradrenaline, even though both agents increase the expression of immediate–early genes. The finding that extracellular ATP caused brown fat preadipocyte DNA to increase more than protein content raises the possibility that ATP promotes brown fat preadipocyte proliferation and not cellular hypertrophy.

Potential mechanism of purinergic modulation of proliferation

Purinergic stimulation may regulate brown adipocyte proliferation through modulation of ion conduction pathways. Voltage-gated K^+ currents (IKV) are known to be important for

cellular mitogenesis in brown adipocytes (Pappone & Ortiz-Miranda 1993), melanocytes (Nilius & Wohlrab 1992), lymphocytes (Lin *et al.* 1993, Verheugen *et al.* 1997), and glial cells (Pappas, Ullrich & Sontheimer 1994, Sontheimer 1994). Additionally, increases in cytosolic Ca^{2+} are commonly correlated to cellular mitogenesis and IKv activity in melanocytes and lymphocytes (Lin *et al.* 1993, Nilius, Schwarz & Droogmans 1993, Verheugen *et al.* 1997). In addition to showing that IKv is important for brown adipocyte proliferation, recent studies demonstrate that purinergic stimulation modulates brown adipocyte IKv activity (Wilson & Pappone 1999) and increases cytosolic Ca^{2+} (Lee & Pappone 1997). The finding that purinergic stimulation affects proliferation of brown fat preadipocytes complements these findings and suggests that IKv modulation, Ca^{2+} signalling, and proliferation may be interrelated events. The finding that purine nucleotides and nucleosides regulate brown fat preadipocyte proliferation *in vitro* may account for some of the differences observed between the environmental activation of brown adipocyte hypertrophy and hyperplasia and that induced artificially with adrenergic administration *in vivo* or *in vitro*.

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