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Airway Smooth Muscle Proliferation and Mechanics: Effects of AMP Kinase Agonists

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Abstract: Obesity is a risk factor for asthma. The purpose of this study was to determine whether metformin, an agent used in the treatment of an obesity-related condition (type II diabetes), might have therapeutic potential for modifying the effects of obesity on airway smooth muscle (ASM) function. Metformin acts via activation of AMP-activated protein kinase (AMPK), a cellular sensor of energy status. In cultured murine ASM cells, metformin (0.2–2 mM) caused a dose-dependent inhibition of cell proliferation induced by PDGF (10^{-8} M) and serotonin (10^{-4} M). Another AMPK activator, 5-aminoimidazole-4-carboxamide-1-ß-D-riboruranoside (AICAR), also inhibited PDGF-induced proliferation. Furthermore, cells treated with metformin or AICAR, also exhibited an attenuation in the rate of cytoskeletal remodeling, as quantified by spontaneous nanoscale motions of microbeads tightly anchored to the cytoskeleton (CSK) of the ASM cell. ASM cells treated with metformin or AICAR, however, exhibited no appreciable differences in stiffness as measured by optical magnetic twisting cytometry (OMTC) or their abilities to stiffen in response to contractile agonist serotonin. Taken together, these findings suggest that metformin, probably through activation of AMPK, reduces the rate of ongoing reorganization of the CSK and inhibits ASM cell proliferation.

Keyword: obesity, airway smooth muscle, cytoskeleton remodeling, contractility, proliferation

1 Introduction

Obesity is a major public health problem that is a known risk factor for type II diabetes, hypertension, atherosclerosis, and some forms of cancer [1]. Recent data indicate that obesity is also a risk factor for asthma [1-6]. In asthma, the key end-effector of acute airway narrowing is the airway smooth muscle (ASM) cell [7, 8], and the ability of the ASM cell to remodel its internal cytoskeleton (CSK) is now thought to play an important role in the disease presentation, including airway hyperresponsiveness [9-13]. As such, agents that can modify ASM function, particularly in obesity, could be potential tools for managing airway dysfunction in this population.

In the management of type II diabetes, metformin has anti-hyperglycemic effects via activating AMP-activated protein kinase (AMPK) [14, 15]. AMPK is a sensor of cellular energy status, and among its many targets are enzymes involved in fatty acid and glucose metabolism [16, 17]. AMPK is typically activated by events that increase the cellular AMP:ATP ratio, including heat shock, metabolic inhibitors, hypoxia, ischemia, glucose deprivation, and exercise; AMPK can also be activated in a manner independent of such AMP:ATP imbalance by agents such as hyperosmotic stress [18-20]. Accordingly, AMPK activation turns on metabolic pathways that produce ATP and, at the same time, turns off pathways that consume ATP.

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Might AMPK agonists, such as metformin, also be useful in modifying the effects of obesity on the airways? Our rationale for posing this question is as follows. Among the many changes observed in the asthmatic airway, the foremost is increased airway smooth muscle mass [21-23]. Agents that activate AMPK, including metformin, have anti-proliferative effects in human aortic smooth muscle cells [24, 25] and both anti-proliferation and pro-apoptotic effects in various cancer cells [26]. AMPK regulates cyclin expression and mTOR activation, and these effects likely contribute to the anti-proliferative effects of AMPK activation [19]. Moreover, data from our laboratory have recently established that remodeling dynamics of the ASM cell are highly sensitive to the cellular energy status [27] and hence could be affected by agents such as metformin that modulate AMPK activation.

Here we measured the effects of AMPK agonists on proliferation, cytoskeletal (CSK) remodeling, and contractility of the cultured murine ASM cell. In particular, we examined the effects of metformin and another agent that activates AMPK, 5-aminoimidazole-4-carboxamide-1-ß-D-riboruranoside (AICAR). AICAR activates AMPK via its monophosphorylated form, ZMP, which mimics the allosteric effects of 5'-AMP [19, 20, 28, 29].

2 Methods

Materials and reagents: Tissue culture reagents were obtained from Sigma (St. Louis, MO) with the exception of Dulbecco's modified Eagles's medium (DMEM)-Ham's F-12 (1:1) which was purchased from Gibco (Grand Island, NY). The synthetic Arg-Gly-Asp (RGD) peptide (Peptite 2000; Integra Life Sciences) was provided by Dr. Juerg Tschopp. Recombinant murine platelet-derived growth factor–BB (PDGF-BB) was obtained from Biosource (Camarillo, CA) and reconstituted in 100 mM acetic acid with 0.1% bovine serum albumin according to the manufacturer's recommendations. PDGF-BB was aliquoted, frozen, and diluted in serum-free media on the day of use. All other reagents and drugs were obtained from Sigma (St. Louis, MO). Serotonin (5-hydroxytryptamine; 5-HT) was diluted in sterile distilled water, frozen in aliquots, and diluted appropriately in serum-free media on the day of use.

Cell isolation and culture of murine ASM cells: Mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were 7-8 weeks of age. Mice were euthanized with an overdose of sodium pentobarbital (175 mg/kg), in accordance with a protocol approved by the Harvard Medical Area Standing Committee on Animal Resources and Comparative Medicine. The trachea was aseptically excised and placed in Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution (HBSS) of the following composition (mM): 5 KCl, 0.3 KH_2PO_4, 138 NaCl, 4 $NaHCO_3$, 0.3 Na_2HPO_4 and 1.0 glucose. ASM cells were then prepared from the tracheas as described previously [8, 9, 30, 31]. Briefly, the tracheae were cleaned of connective tissues, cut longitudinally through the cartilage, and enzymatically dissociated with HBSS containing 0.05 % elastase type-III and 0.2 % collagenase type-IV for 45 min in a shaking water bath at 37˚C. Dissociated cells in suspension were centrifuged and re-suspended in Dulbecco's modified Eagles's medium (DMEM)-Ham's F-12 medium (1:1) supplemented with 10 % fetal bovine serum (FBS) and the antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin-β). Cells were plated on culture flasks and grew until confluence at 37˚C in humidified air containing 5 % CO_2. The media was changed every 3-4 days, and confluent cells were passaged with 0.25 % trypsin-0.02 % ethylenediaminetetraacetic acid (EDTA) solution every 10-14 days. ASM cells in culture were elongated and spindle shaped, grew with the typical hill-and-valley appearance, and showed positive staining for the smooth muscle-specific proteins α-actin and calponin. In the present study, we used cells in passages 2-8.

Measurement of ASM cell proliferation: Two methods were used to assess cell proliferation: direct cell counting and crystal violet DNA staining. For experiments assessing the effects of

metformin on cell proliferation, cultured murine ASM cells were serum deprived and supplemented with 10 μg/ml insulin, 5.5 μg/ml transferrin, and 6.7 μg/ml sodium selenite (ITS media) for 24 h. Cells were then seeded into 24 well plates at a density of 5 x 10^3 cells/cm^2. After an additional 24 h in serum-free medium, cells were treated with metformin (0.2 – 2 mM). These doses of metformin are those that have been previously shown to be effective in activating AMPK in other cell types [17, 32]. For control, cells were treated with the buffer only (100 mM acetic acid with 0.1% bovine serum albumin for rmPDGF-BB and sterile distilled water for serotonin). Three hours later PDGF-BB (10^{-8} M) or serotonin (10^{-4} M) was added to the wells and, subsequently, harvested and counted after indicated time (0, 3, or 5 days later).

For experiments assessing the effects of AICAR on cell proliferation, cells were subcultured into 96-well U-bottom plates at 7000 cells/well in a low serum, growth arrest media consisting of DMEM/F-12 50:50 with 1% FBS and Insulin-Transferrin-Selenium X (Invitrogen, Carlsbad, CA). Separate wells were seeded with cell numbers from 1000-10,000 cells/well to generate a standard curve. Cells were maintained in this media for 48 hours before rmPDGF-BB (10^{-8} M) stimulation in the presence or absence of AICAR. Proliferation was measured 72 hours later using DNA staining by crystal violet (Sigma, St. Louis, MO) as described by others [33]. Cells were washed once with Hank's Balanced Salt Solution (HBSS) supplemented with 2 mM $CaCl_2$ and 10 mM HEPES, fixed for 20 minutes with 70% ethanol at –20˚ C, incubated for 15 min in dilute crystal violet stain (1% w/v) at room temperature, and washed 6 times with distilled water. The fixed and stained adherent cells were then dissolved in the wells with 100 uL of acetic acid (33%) per well. Optical density (OD) was determined at 550 nm with an ELISA plate reader.

Characterization of spontaneous bead motion: The rate of cytoskeletal remodeling was evaluated by measuring spontaneous motions of beads bound to ASM cells through integrin receptors, as previously described [8, 27]. Confluent cells were serum deprived and hormone supplemented 24 h before use, as described above. The cells were then harvested and plated at 35,000 cells/cm^2 on plastic wells (96-well Removawell, Immulon II: Dynetech) previously coated with type I collagen (Vitrogen 100; Cohesion, Palo Alto, CA) at 5 μg/ml and allowed to adhere for 24 h at 37^{o}C in humidified air containing 5% CO_2. These conditions have been optimized for seeding cultured cells on collagen matrix and for assessing their mechanical properties [8, 30, 34]

To evaluate the effect of metformin and AICAR, cells were treated with metformin (0.2 – 2 mM for 3 h), with AICAR (0.2 – 2 mM for 2 h), or with an equal volume of serum-free media (control cells). The cells were then incubated for 15 min with ferrimagnetic microbeads (Fe3O4, 4.5 μm diameter) coated with a synthetic RGD (Arg-Gly-Asp)-containing peptide (50 μg peptide/mg beads) in serum-free medium. This allowed a specific binding of the beads to cell surface integrin receptors on adherent cells. Unbound beads were removed by washing cells with serum free medium [30].

The wells were then individually placed under an inverted microscopic (approximately 50 to 150 beads per field of view), and the spontaneous displacements of individual microbeads were recorded every 83 ms [35]. The spontaneous movements of individual microbeads coated with RGD can occur only if the microstructures to which the beads are attached rearrange [7, 36]. The paths of bead motions in two dimensions were defined by computing the mean square displacements (MSDs) of all beads as a function of time t, MSD(t), (nm^2)

$$MSD(\Delta t) = \left\langle r^2(\Delta t) \right\rangle = \left\langle (r(t+\Delta t) - r(t))^2 \right\rangle \quad (1)$$

where $r(t)$ is the bead position at the time t, Δt is the time lag, and the brackets indicate an average over many starting times t and over all beads [27]. As was shown previously the MSDs of microbeads increased with time according to a power law relationship

$$MSD(\Delta t) = D^*(t/t_0)^{\alpha} \quad (2)$$

where D^* and α were estimated from a least squares fit of a power law, t_0 was taken as 1 s and

D^* is expressed in unit of nm^2. When the exponent α is smaller than unity the random motions are subdiffusive, and when the exponent is larger than unity the random motion are superdiffusive [8, 27]. Spontaneous displacements of individual microbeads were recorded for 5 min. During this time, spontaneous motions of each microbead were only a small fraction of bead diameter ($\sim$ 4.5 μm) and an even smaller fraction of cell size. Thus, the cell could be considered as being of infinite lateral extent and local spontaneous bead motions were not constrained by distant cell boundaries.

D^* and α were calculated from the best fit of the power law relationship for cells in each well. MSDs varied from well-to-well and from day-to-day. For the D^* coefficient the averaged standard deviation between the wells was 78 $\pm$7nm^2 for cells pretreated with AICAR and 74 $\pm$ 3.4 nm^2 for cells pretreated with metformin. Therefore, for comparison purposes we scaled the coefficient D^* of each well by the average D^* of the control cells measured on the same experimental day.

Optical magnetic twisting cytometry (OMTC): The effects of metformin (2 mM) and AICAR (2 mM) on the mechanical properties of cultured mouse ASM cells were measured using OMTC as previously described [10, 35, 37, 38]. In brief, RGD-coated ferromagnetic beads bound on the cell surface were first magnetized horizontally with a brief 1000-G pulse. This first pulse causes the magnetic moments of the microbeads to align in the direction parallel to the well surface. Next, an external magnetic field (the "twisting" field) of 20 G that was varying sinusoidally in time at 0.75 Hz was applied vertically. The twisting magnetic field imposes upon each microbead a mechanical torque tending to align the bead magnetic moment with this twisting field, but forces developed within the cell impede bead rotation. A CCD camera mounted on the inverted microscope detected optically (in spatial resolution of $\sim$10 nm) the lateral microbead displacements that accompany bead rotation. An automated bead-tracking algorithm was used to exclude beads that move more than 3.5 μm [35]. The ratio of the specific torque to the component of bead displacement that is in-phase with the applied torque was computed and expressed as the cell stiffness, g', in units of Pa/nm; this stiffness was measured for 300 s, baseline stiffness was assessed for the first 60 s. Changes in cell stiffness in response to the contractile agonist serotonin (5-hydroxytyrptamine, 5-HT) were recorded for the next 60-300 s. Serotonin has been shown to increase $[Ca^{2+}]_i$in rodent ASM cells [31] and cause acute airway narrowing in mice [39]. Changes in cell stiffness in response to serotonin were normalized to the baseline stiffness of each individual cell.

Statistics: Data are presented as means $\pm$SE; n represents the number of the wells. The difference between the D^* coefficients obtaining in the different treatment were determined by analysis of variance (ANOVA) for comparison of more than two sample means ($P < 0.05$ was considered statistically significant). In determining the effects of AICAR or metformin on contractile responses to serotonin, we first computed the average stiffness measured in the last 150 sec (time range 150–300s) (i.e. the plateau value after serotonin) for each well. Then, at each dose of serotonin, statistical differences in the response to addition of contractile agonist between the control and the treated wells were obtained by t-test. AICAR and metformin were studied on separate experimental days. Because of this, and because there was day to day variability in the response to serotonin, separate controls were run for each of these AMPK activators.

3 Results

ASM Proliferation: PDGF-BB (10^{-8} M) caused proliferation of murine ASM cells, as indicated by marked increases in cell number over the course of 5 days after addition of PDGF-BB to the culture medium. Metformin, which activates AMPK, caused a dose dependent reduction in PDGF-induced proliferation with approximately a 50% reduction in proliferation at 0.2 mM, and almost complete inhibition at 2 mM (Fig. 1A). Serotonin (10^{-4} M) also caused

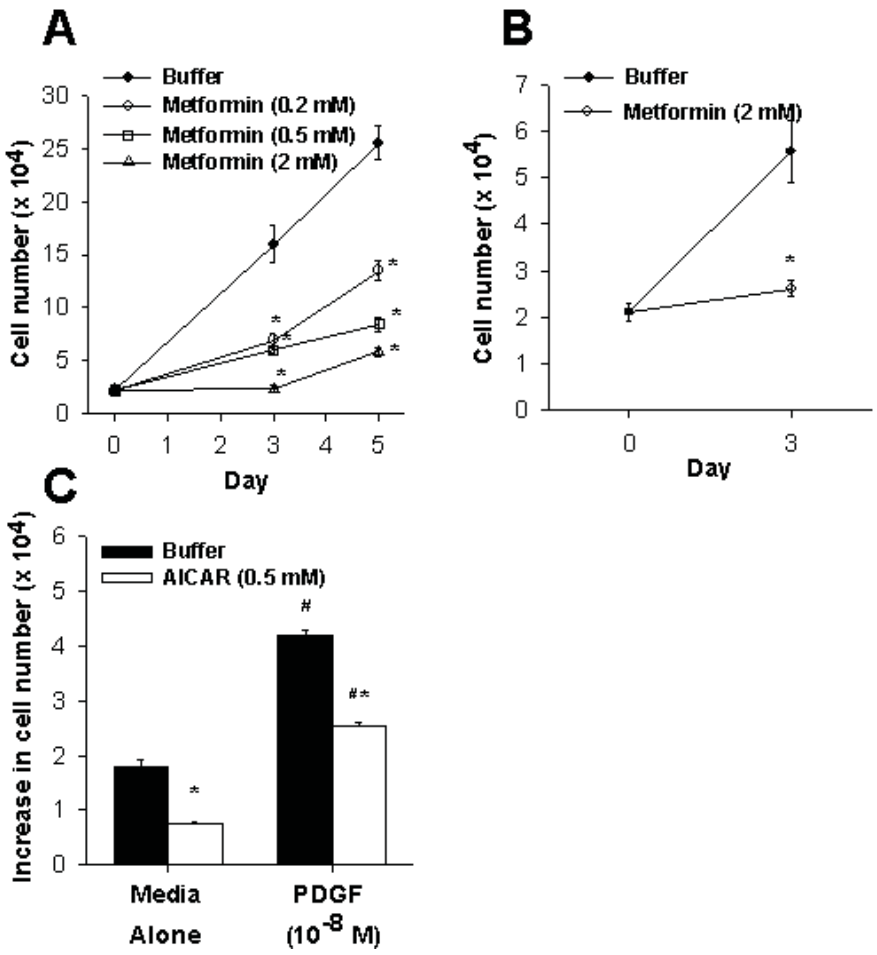

Figure 1: Effect of metformin on murine ASM proliferation induced by PDGF-BB (10^{-8} M) (A) or serotonin (10^{-4} M) (B). Metformin was administered 3 hours prior to the mitogen. Mitogen or control buffer (100 mM acetic acid with 0.1% bovine serum albumin for recombinant murine PDGF-BB and sterile distilled water for serotonin) was added on day 0. Results are mean ± SE of data from 4-8 cell wells. (C) Effects of the AMPK agonist, AICAR (0.5 mM) on increases in ASM cell number (over day 0 control) induced by culture for 3 days in media alone or media plus PDGF-BB (10^{-8} M). Note that the media contained 1% FBS. * $p < 0.05$ compared to cells treated with buffer alone; # $p < 0.05$ compared to cells treated with media alone.

proliferation of murine ASM cells, although the proliferative response was not nearly as great as that which occurred in response to PDGF-BB. Metformin (2 mM) also virtually abolished serotonin-induced proliferation (Fig. 1B). Metformin did not alter cell viability, as assessed by Trypan blue exclusion.

We also examined the effects of another AMPK activator, AICAR, on ASM cell proliferation induced by PDGF-BB (10^{-8} M) (Fig. 1C). Compared to cells cultured in media alone, 3 days of treatment with PDGF-BB caused an increase in cell number both in cells treated with buffer and in cells treated with AICAR (0.5 mM) ($p < 0.05$ in both cases). However, both in cells cultured in media alone and in cells cultured in media plus PDGF-BB, the presence of AICAR resulted in a significant reduction in cell number ($p < 0.05$ in both cases). The fact that regardless of treatment, there was an increase in cell number (compared to day 0) in cells cultured in media alone likely reflects the effects of the small amount (1%) of

FBS, a known mitogen, in the culture media. To assess the dose effects of AICAR, we also examined the effects of 1.0 mM AICAR on PDGF-induced proliferation. Whereas treatment with 0.5 mM AICAR resulted in an approximate 40% inhibition of cell proliferation induced by PDGF-BB (Fig. 1C), this effect was even greater in cells treated with 1.0 mM AICAR (approximately 95% inhibition, $p < 0.05$).

Spontaneous bead motions. *MSD*s were found to vary in time as a power law (Figs. 2A(a), 2B (a)), consistent with previous reports [8, 27, 36]. The coefficient D^* and the exponent α of the bead motion were estimated from a least-square fit. Increasing concentrations of metformin (Fig.2A) or of another AMPK agonist, AICAR (Fig. 2B), resulted in a significant decrease in D^*, indicating a decrease in the rate of cytoskeletal remodeling. At the highest concentrations used, metformin (2 mM) and AICAR (2 mM) caused 19$\pm$6.9 % and 38.9$\pm$6.48 % reductions in D^* respectively. At 1mM of metformin, MSD values were slightly greater than control whereas the D^* coefficient was lower; this was a result of the slightly higher value of the exponent α obtained at 1mM compared to control. For comparison purposes, 2-deoxy-D-glucose (10 mM), which depletes cellular ATP [27], resulted in 95$\pm$1.15 % decrease in D^*. The exponent α was always larger than unity (Figs. 2A and 2B), indicating superdiffusive beads motions, but α did not change significantly either with metformin or AICAR. Control values for the exponent α were 1.49+0.019 for cells incubated in ITs for the metformin studies and 1.47+0.018 for cells incubated in ITs for the AICAR studies.

ASM responses to a contractile agonist: Metformin (2 mM for 2 h) had no significant effect on g' (0.318 $\pm$ 0.023 Pa/nm in metformin treated cells versus 0.288 $\pm$ 0.01 Pa/nm in control cells). Likewise, AICAR (2 mM for 3 h) had no effect on g' (0.366 $\pm$ 0.022 Pa/nm in AICAR treated cells versus 0.389 $\pm$ 0.028 Pa/nm in control cells treated with ITS only). All cells exhibited dose dependent responses to serotonin (insets in Figs. 3b, 3d), consistent with previous results in cultured rat ASM cells. Compared to control, cells pretreated with metformin had the same responses to 1 μM of serotonin (average normalized value of 1.25$\pm$0.06 in metformin treated cells versus 1.26 $\pm$ 0.07 in control cells, $P > 0.05$, Fig 3a) and 10 μM of serotonin (average normalized values of 1.38$\pm$0.03 in metformin treated cells versus 1.34 $\pm$ 0.05 in control cells, $P > 0.05$, Fig 3b). Responses of cells pretreated with AICAR to 1 μM of serotonin did not reach statistical significance compared to control (average normalized values of 1.61$\pm$0.08 in AICAR treated cells versus 1.38 $\pm$ 0.08 in control cells, $P = 0.056$, Fig 3c) and had almost the same response to 10 μM of serotonin (average normalized values of 1.61$\pm$0.08 in AICAR treated cells versus 1.53 $\pm$ 0.08 in control cells, $P > 0.05$, Fig 3d). Addition of higher concentrations of serotonin did not cause increases in stiffness above those induced by 10^{-5} M serotonin.

4 Discussion

A prominent pathological feature of the airways from patients with chronic severe asthma is increased smooth muscle content of the airway wall, a change that appears to involve both ASM hypertrophy and hyperplasia [22, 23]. Our results indicate that a drug used in the treatment of type II diabetes, metformin, reduces proliferation of ASM cells in culture, and suggest thereby a plausible rationale for questioning whether metformin might be useful in the treatment of ASM hyperplasia in asthma. Metformin caused a marked decrease in the ability of PDGF and serotonin to induce proliferation of murine ASM cells (Fig.1). These results are consistent with the ability of metformin to inhibit human vascular smooth muscle cell proliferation [24, 25, 40]. The ability of metformin to alter proliferative responses to both PDGF-BB, a classical growth factor that activates receptors with intrinsic tyrosine kinase activity, and serotonin, which acts via G-protein coupled receptors, suggests that metformin is mediating its effects downstream of where these signal transduction pathways converge [22]. Indeed, since metformin attenuated proliferative (Fig. 1), but not

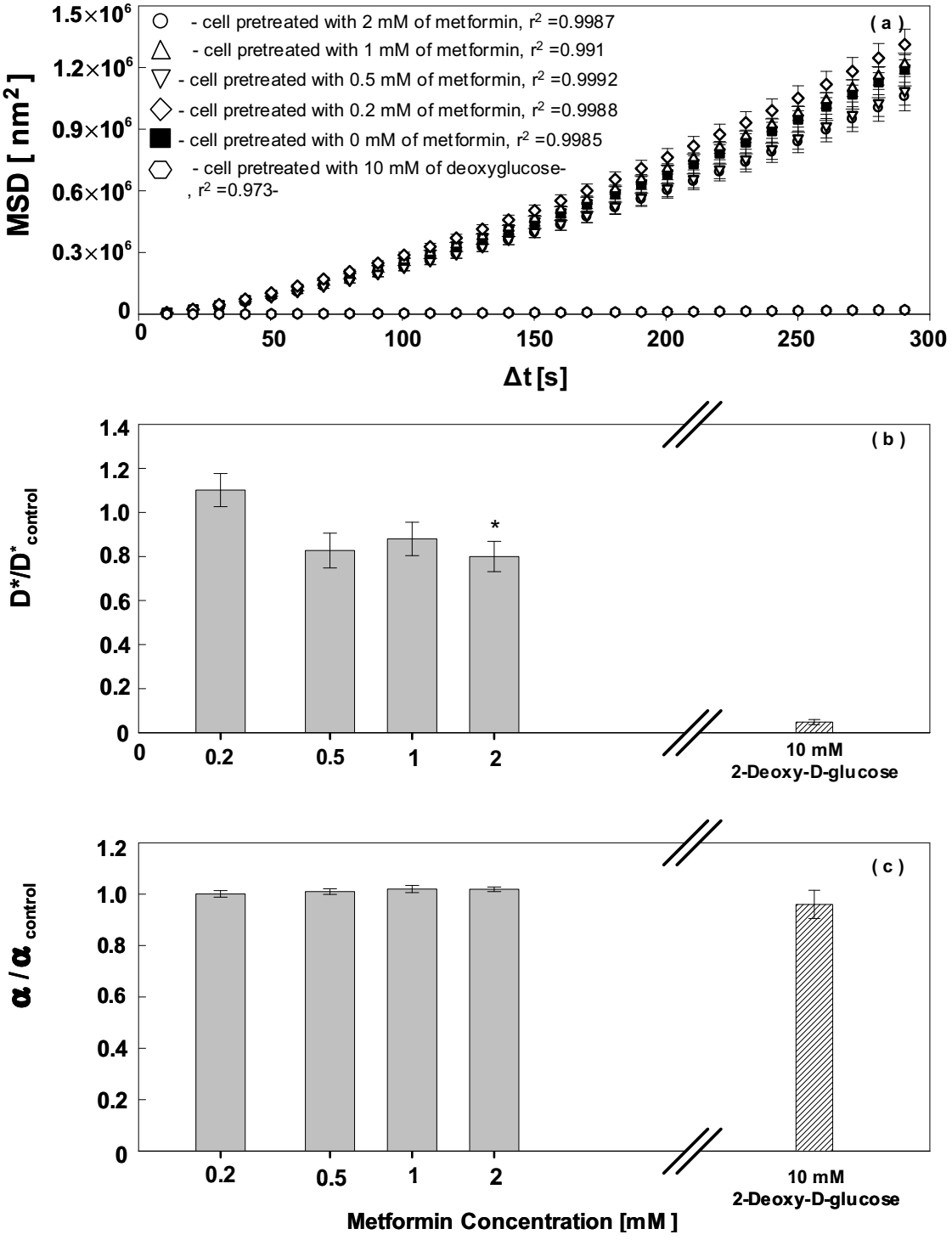
(a)
- cell pretreated with 2 mM of metformin, r2 =0.9987
- cell pretreated with 1 mM of metformin, r2 =0.991
- cell pretreated with 0.5 mM of metformin, r2 =0.9992
- cell pretreated with 0.2 mM of metformin, r2 =0.9988
- cell pretreated with 0 mM of metformin, r2 =0.9985
- cell pretreated with 10 mM of deoxyglucose-
, r2 =0.973-
MSD [nm2]
1.5×10^6
1.2×10^6
0.9×10^6
0.6×10^6
0.3×10^6
0
0
50
100
150
200
250
300
Δt [s]
(b)
D*/D*control
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
0.2
0.5
1
2
10 mM
2-Deoxy-D-glucose
(c)
α / αcontrol
1.2
1.0
0.8
0.6
0.4
0.2
0
0.2
0.5
1
2
10 mM
2-Deoxy-D-glucose
Metformin Concentration [mM]

Figure 2A

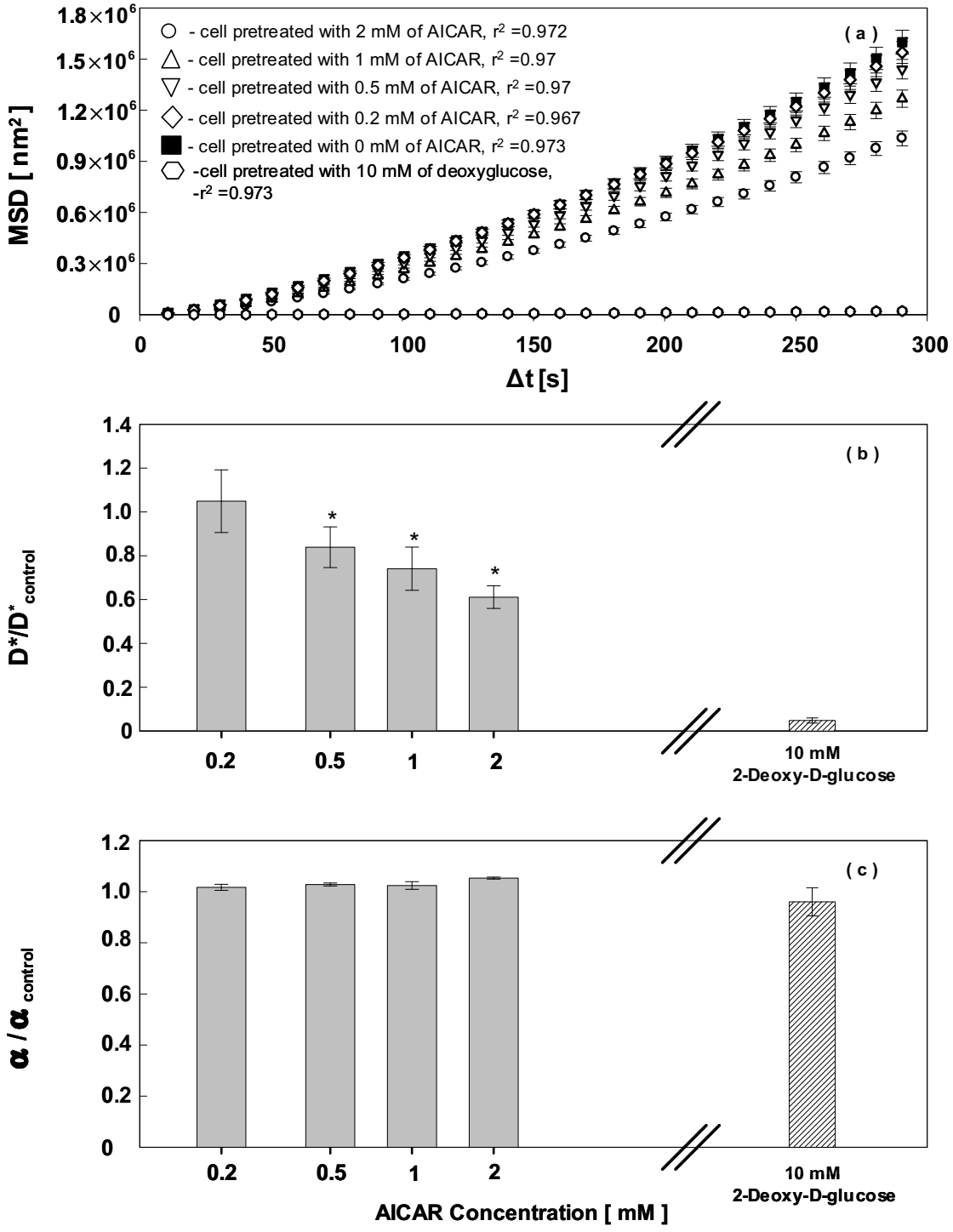

Figure 2B

Figure 2: Effect of metformin (A) and AICAR (B) on spontaneous bead motions quantified by their mean square displacements (MSDs) as a function of time (Eq. 2). The MSD increases with time according to a power law relationship. The data represent the average MSD's and the average α values of 1233-4755 cells measured in 10-14 wells per treatment on 3 experimental days (for treatment of 2 mM AICAR the data represent the average MSD's of cells measured in 40 wells on 7 experimental days). * indicates a statistically significant difference from the control ($p < 0.05$).

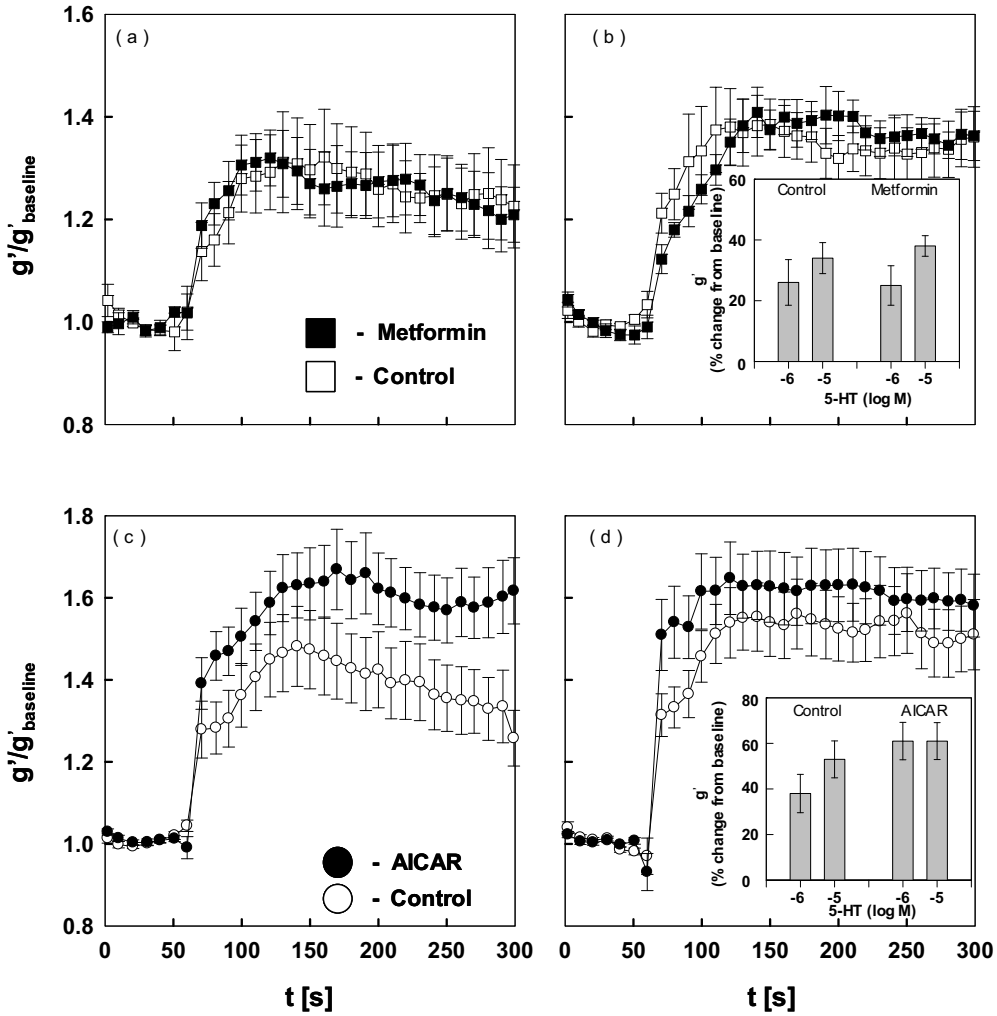

Figure 3: Normalized cell stiffness with respect to baseline stiffness in response to 1 μM (a and c) and 10 μM (b and d) of serotonin (5-hydroxytryptamine; 5-HT), opened circles and open squares represent the normalized stiffness of control cells (incubated for 2 h and 3 h in ITs, respectively). Filled circles and filled squared represent the normalized stiffness of cells pretreated with AICAR (2h, 2 mM) and metformin (3h, 2 mM), respectively. Insets bar graphs show dose-response relation of all cells to the contractile agonist serotonin. Results are mean $\pm$ standard error (n = 1428-1943 cells in 21-24 cell wells for cells pretreated with 2 mM of AICAR and for the control cells on 6 experimental days, n = 643-693 cells in 4-5 cell wells for cells pretreated with 2 mM of metformin and for the control cells on 1 experimental day.)

contractile (Fig.3) responses to serotonin, it is unlikely that this drug is affecting either serotonin receptor expression or coupling of these receptors to G proteins.

It is likely that the observed effects of metformin were mediated via AMPK activation. Several investigators have reported that metformin activates AMPK [17, 32, 41, 42] and that metformin mediates its effects on glucose homeostasis via AMPK activation [14, 15]. Indeed, the effects of metformin on proliferation and ASM cytoskeletal remodeling were mimicked by another AMPK agonist, AICAR (Fig.2). In addition, other AMPK activators also inhibit proliferation of aortic smooth muscle cells and proliferation of various cancer cells [24-26, 43, 44]. In this context, it is interesting to note that AMPK activation results in increased degradation of mRNAs encoding cyclins

A and B1 [45]. Such effects may contribute to the effects of metformin on ASM cell proliferation. Activation of AMPK also reduces mTOR activity, which is required for protein synthesis, an important component of cellular proliferation [46]. Hence, it may be that metformin affects ASM proliferation via effects on mTOR. Nevertheless, we cannot rule out the possibility that the effects of metformin and AICAR observed are the result of other effects of these drugs, since AMPK-independent effects of both AICAR and metformin have been reported [14, 18].

As regards cytoskeletal remodeling, several laboratories including our own have shown that the myosin motor works within a CSK scaffolding that is in a continuous state of turnover and is dramatically malleable [11-13, 36, 47, 48]. Because this remodeling allows the airway smooth muscle cell to attain the same high level of active force over a wide range of muscle lengths, it is thought to play a major role in airways hyperresponsiveness in asthma [8, 49-51]. As a measure of the rate of structural rearrangements of the CSK, spontaneous molecular-scale displacements of a microbead tightly bound to the CSK have been shown to be useful. Data in Fig. 2 show that both metformin and AICAR attenuated this measure of the rate of ASM cytoskeletal remodeling. As discussed in Trepat et al [36], it is likely that such changes do indeed reflect the properties of the cytoskeleton rather than coupling of the magnetic beads to the cells. For example, magnetic twisting cytometry has been shown to be sensitive to manipulations of molecules such as actin, myosin, and HSP 27, but insensitive to disruption of membrane cholesterol. Moreover, these methods discriminate semi-flexible polymer dynamics of actin [52]. None of these observations is easily explained by ligation dynamics or membrane dynamics, and certainly not all of them.

In this connection, it is important to recognize that measures of spontaneous tracer displacements as described above versus measures of cytoskeletal material properties such as stiffness need not change in concert. For the case of systems at thermodynamic equilibrium the generalized Stokes-Einsten relationship (GSER) would predict that spontaneous mean square displacements of a tracer are entirely determined by and predictable from the stiffness and viscosity of the surrounding medium, and conversely. However, it is now well established in the case of the cytoskeleton of the living cell, which is a non-equilibrium system, that the GSER breaks down in dramatic fashion [27, 36]. For example, when ATP is depleted the mechanical properties of the cytoskeleton change only modestly whereas tracer mean square displacements decrease by about 2 orders of magnitude [27, 53, 54]. The rate of progression of cytoskeletal remodeling events are almost entirely ATP dependent [27, 36], whereas cytoskeletal material properties such as stiffness are far less so.

As expected, ASM cells in culture showed a brisk contractile response to serotonin (Fig. 3). However, maximum contractile responses were little influenced by pretreatment with metformin or AICAR. Therefore, while metformin was a potent inhibitor of proliferation, it appears not to exacerbate ASM contractility.

It is increasingly apparent that obesity is a risk factor for asthma. Both the prevalence and incidence of obesity are increased in obese and overweight individuals, and asthma symptoms and severity decline with weight loss in morbidly obese subjects [1-6, 55-58]. The mechanistic basis for this relationship remains to be established, but changes in adiponectin, a hormone synthesized in adipocytes, may play a role. Serum adiponectin is reduced in obesity [59, 60], and a recent report from our laboratory indicates that administration of exogenous adiponectin attenuates allergic airways responses in mice [61]. Importantly, in many cell types adiponectin mediates its effects via activation of AMPK [62, 63]. Our results suggest that it is conceivable that loss of adiponectin mediated AMPK activation in ASM contributes to airway dysfunction in obese asthmatics.

In summary we have shown that metformin, an activator of AMPK, stabilizes ASM cell by reducing the rate of ongoing microstructural reorganization and inhibits ASM proliferation, and that it does so without altering ASM contractility. These

data suggest a potential therapeutic value for metformin in the treatment of the obese asthmatic.

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