Chondroprotective effects of 4-methylumbelliferone and hyaluronan synthase-2 overexpression involve changes in chondrocyte energy metabolism

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Hyaluronan is a critical component of articular cartilage and partially helps retain aggrecan within the extracellular matrix of this tissue. During osteoarthritis, hyaluronan and aggrecan loss are an early sign of tissue damage. However, our recent attempts to mimic hyaluronan loss with the hyaluronan inhibitor 4-methylumbelliferone (4MU) did not exacerbate arthritis-like features of in vitro models of arthritis, but surprisingly, caused the reverse (i.e. provided potent chondroprotection). Moreover, the protective effects of 4MU did not depend on its role as a hyaluronan inhibitor. To understand the molecular mechanism in 4MU-mediated chondroprotection, we considered recent studies suggesting that shifts in intracellular UDP-hexose pools promote changes in metabolism. To determine whether such metabolic shifts are associated with the mechanism of 4MU-mediated pro-catabolic inhibition, using molecular and metabolomics approaches, we examined whether bovine and human chondrocytes exhibit changes in the contribution of glycolysis and mitochondrial respiration to ATP production rates as well as in other factors that respond to or might drive these changes. Overexpression of either HA synthase-2 or 4MU effectively reduced dependence on glycolysis in chondrocytes, especially enhancing glycolysis use by interleukin-1β (IL1β)-activated chondrocytes. The reduction in glycolysis secondarily enhanced mitochondrial respiration in chondrocytes, which, in turn, rescued phospho-AMP–activated protein kinase (AMPK) levels in the activated chondrocytes. Other glycolysis inhibitors, unrelated to hyaluronan biosynthesis, namely 2-deoxyglucose and dichloroacetate, caused metabolic changes in chondrocytes equivalent to those elicited by 4MU and similarly protected both chondrocytes and cartilage explants. These results suggest that fluxes in UDP-hexoses alter metabolic energy pathways in cartilage.

Previously, we found that the hyaluronan (HA)2 inhibitor 4-methylumbelliferone (4MU) blocked the transcriptional expression of MMP13, ADAMTS4, TSG6, and the HA synthase-2 (HAS2) enzyme in pro-catabolically activated chondrocytes (1). Moreover, 4MU protected intact cartilage explants from matrix loss in an in vitro model of osteoarthritis (OA). There were two interesting and unexpected aspects of this observation. First, 4MU blocked MMP production even in the absence of HA biosynthesis, suggesting that its effects on chondrocytes/cartilage were not related to its role as an HA inhibitor. Second, adenovirus-mediated overexpression of the HAS2 enzyme (HAS2-OE), conditions that resulted in large excesses in HA production, was also effective at blocking pro-catabolic markers and MMPs in activated chondrocytes (2). Surprisingly, the HAS2-OE inhibitory effects were not due to signaling events associated with enhanced extracellular HA. Combined treatment with 4MU and HAS2-OE was complementary. How could HA inhibition and HA overproduction both block the OA-like phenotype associated with activated chondrocytes? Both of these two methods appeared to be exerting unexpected off-target effects.

The canonical pathway of 4MU action involves the formation of 4MU-glucuronic acid (GlCUA) glycoconjugates (3), limiting the availability of UDP-GlcUA for the synthesis of HA (4, 5). Because high-affinity transporters pump UDP-GlcUA into the Golgi (5, 6), 4MU exerts less effect on glycosaminoglycan substitution of proteoglycans and likely more effect on enzymes that require access to cytoplasmic pools of UDP-GlcUA. In this

2 The abbreviations used are: HA, hyaluronan; Dox, doxycycline; ECAR, extracellular acidification rate; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; HAS, hyaluronan synthase; HAS2-OE, hyaluronan synthase-2 viral overexpression; MMP, matrix metalloproteinase; 4MU, 4-methylumbelliferone; OA, osteoarthritis; OCR, oxygen consumption rate; TSG6, TNF-stimulated gene 6 protein; GlCUA, glucuronic acid; AICAR, 5-aminimidazole-4-carboxamide ribonucleotide; TCA, tricarboxylic acid; mPH, milli-pH units; 2DG, 2-deoxyglucose; sGAG, sulfated glycosaminoglycan; TNF, tumor necrosis factor; LPS, lipopolysaccharide; DAMP, damage-associated molecular pattern; HoloAg, HA oligosaccharide; FN-fr, N-terminal fibronectin fragment preparation; DCA, dichloroacetate; DBA, dimethyl-bisphenol A; AMPK, AMP–activated protein kinase; mTOR, mechanistic target of rapamycin; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; qRT-PCR, real-time quantitative RT-PCR.
**Rescue of energy metabolic changes in chondrocytes**

In articular chondrocytes, the inhibition of HA biosynthesis (via 4MU) as well as the overexpression of HA biosynthesis (HAS2-OE) inhibit the expression of MMPs and provide overall chondroprotective effects (1, 2). To determine potential properties the two protocols have in common, chondrocyte energy metabolism was examined. As shown in Fig. 1, bovine chondrocytes were tested live, in real time, using Seahorse flux analyzer technology to determine rate changes in medium accumulation of H⁺ protons (indicative of lactic acid accumulation) and, simultaneously, for O₂ consumption (indicative of mitochondrial respiration). After establishing baseline metabolic values (open squares), different metabolic mediators were injected to better characterize the contribution of glycolysis and TCA pathways. Fig. 1A shows a representative cell energy phenotype test wherein averaged values for the extracellular acidification rate (ECAR, mpH/min) are plotted versus oxygen consumption rate (OCR, pmol/min). Chondrocytes under all conditions responded to stress testing, wherein the cells were forced to maximal glycolysis and maximal mitochondrial O₂ consumption (closed squares). Little difference in metabolic potential was observed (all values trended toward the right by ~90°). IL1β-treated chondrocytes (red squares) were more glycolytic as compared with control chondrocytes (blue squares). Interestingly, both HAS2-OE– and 4MU–treated control chondrocytes exhibited reduced glycolytic activity (orange and teal squares, respectively), but of more importance, co-treatment with IL1β and HAS2-OE or with IL1β and 4MU (purple or green squares) resulted in reducing the ECAR metabolism of IL1β-activated chondrocytes, with values moving back toward that of control cells. These changes in proton release were quantified and more readily compared in Fig. 1B, showing a statistically significant rescue of IL1β-enhanced ECAR by both 4MU and HAS2-OE.

A second assay (mitochondrial stress test) examined effects on mitochondrial respiration more closely, as shown in Fig. 1C. IL1β-treated chondrocytes (dark gray bars) also displayed a statistically significant reduction in both basal and maximal mitochondrial respiration as compared with control cells (labeled Ctrl) in addition to enhancement of ECAR. This assay includes cell responses to oligomycin followed by FCCP injections (as stressed conditions) to convert these results into changes in mitochondrial contribution to ATP production (mito ATP). IL1β-treated chondrocytes showed a substantial deficit in mitochondrial ATP production (dark gray bars) as compared with controls (white bars). However, co-treatment of chondrocytes with 4MU and IL1β rescued both overall mitochondrial respiration and the mitochondrial contribution to ATP production. These data suggest that an overall shift in energy metabolism occurred following IL1β treatment—a shift that was rescued by 4MU or HAS2-OE. The next question is whether this metabolic change is associated with the chondroprotective effects of these agents.

The effect of 2-deoxyglucose on chondrocyte metabolism

To address whether a change in metabolism is the driving force behind the inhibitory effects of 4MU or HAS2-OE, the use of an alternative agent was explored, one that had no direct association with HA production. 2-Deoxyglucose (2DG), a validated inhibitor of glycolysis (a glucose hexokinase inhibitor), can be used, especially at low doses (8–10), to reduce the contribution of glycolysis needed to provide for cellular energy needs. Like 4MU and HAS2-OE, 2DG (2.0 mM) reduced ECAR production when applied to control chondrocytes alone (Fig. 1D) and, when used in combination with IL1β, reduced the proton efflux to rates similar to untreated, control chondrocytes. In addition, 2DG readily rescued IL1β-induced changes in basal and maximal mitochondrial respiration as well as mitochondrial contribution to ATP production as effectively as 4MU (Fig. 1C).

The Seahorse ATP rate assay provides a means to simultaneously view the contributions of glycolysis and mitochondrial respiration to chondrocyte ATP production (Fig. 1, E and F). In control chondrocytes, the use of glycolysis contributes to the majority of ATP produced (gray bars), about one-fifth from the TCA cycle (red bars). IL1β-activated chondrocytes display a reciprocal increase in glycolysis and decrease in mitochondrial contributions. These changes are reversed by co-treatment with 4MU or HAS2-OE as well as 2DG. Interestingly, 4MU and 2DG provide for enhanced mitochondrial activity slightly above that of control chondrocytes. In other experiments, when agents were added alone to control chondrocytes, only minor changes were noted (Fig. 1F). These results demonstrate that 2DG can mimic the metabolic effects of both 4MU and HAS2-OE.
Rescue of energy metabolic changes in chondrocytes

A. Energy Phenotype Test

B. Baseline ECAR

C. Mitochondrial Respiration

D. Baseline ECAR

E. Contribution to ATP Production

F. Contribution to ATP Production
Rescue of energy metabolic changes in chondrocytes

**The effect of 2-deoxyglucose on a pro-catabolic chondrocyte phenotype**

2DG was next used as a select inhibitor of the glycolysis pathway and to explore its effects on the pro-catabolic phenotype of activated chondrocytes. As shown in Fig. 2 (A and B), 2DG at concentrations of 2.0 or 20.0 mM reversed the IL1β-induced increases in MMP13 and TSG6 in human OA chondrocytes. A significant reduction in IL1β-stimulated bovine MMP13 and TSG6 was observed in bovine chondrocytes co-treated with 0.2, 2.0, or 20.0 mM 2DG (Fig. 2, C and D). These experiments indicated the minimum 2DG concentrations to generate these reductions. Similar 2DG reversal of IL1β-stimulated MMP3 was also observed in bovine chondrocytes (data not shown). When examined by Western blot analysis, 2DG at 2 and 20 mM was effective at reducing the accumulation of MMP13 protein in both the cell-associated and medium compartments (Fig. 2, E and F). Interestingly, although background levels of MMP13 differed between the two patient samples (seen in the medium fraction), both were reduced in the presence of 2DG (no IL1β, lanes 1 and 2). HIF1α protein, also stimulated by IL1β (Fig. 2F), was similarly reversed by co-incubation with 2.0 or 20.0 mM 2DG. Thus, 2DG, a potent blocker of the glycolysis pathway in chondrocytes, also blocks the expression of pro-catabolic markers associated with activated chondrocytes, in a fashion similar to 4MU or HAS2-OE.

**The biological effect of 2-deoxyglucose on intact human or bovine cartilage**

We have previously shown that the addition of 4MU can block the release of proteoglycan from intact bovine or human explants treated with IL1β or HA oligosaccharides (1), two in vitro models of osteoarthritis. To determine whether this was due to changes in chondrocyte energy metabolism, we repeated these experiments using the glycolysis pathway inhibitor, 2DG. As shown in Fig. 3 (A and B), untreated control explant cores from human OA patient tissue exhibited substantial surface damage and loss of safranin O staining (indicative of aggregan proteoglycan loss) occurring in the upper layers of the cartilage. Bovine cartilage explants (Fig. 3, C and D), representative of normal, non-OA tissue, exhibit safranin O staining throughout most layers. Nonetheless, after 7 days of exposure to IL1β, substantial amounts of proteoglycan were lost from the explants of both human and bovine samples. However, co-incubation of the explants in IL1β plus 2DG provided a nearly complete block of the loss of proteoglycan. It should also be noted that 2DG provided no repair or rescue to the surface fibrillation damage in human OA cartilage. This chondroprotective effect of 2DG was also quantified by a colorimetric assay of sulfated glycosaminoglycan (sGAG) released into the medium. Each human OA cartilage core explant sample exhibited different baseline values of released sGAG that accumulated in the culture medium (black bars); nonetheless, the addition of IL1β enhanced this level of release (Fig. 3E, gray bars). The inclusion of 2DG together with IL1β returned the values to levels close to untreated controls (green bars). In the bovine cartilage explants, the inclusion of 2DG brought the levels of sGAG release below those of controls (Fig. 3F), suggesting that normal turnover was also likely reduced.

In Fig. 2, the inclusion of 2DG correlated with a reduction of MMP13 mRNA and protein production by chondrocytes. In explant cultures of human OA cartilage, we also observed the release of the ~54-kDa MMP13 protein into the medium, with each patient explant exhibiting varying background levels of MMP13 in untreated cultures (Fig. 3G). Nonetheless, all of the background levels of MMP13 were enhanced when the explants were cultured in the presence of IL1β (lanes denoted as 2). Moreover, the inclusion of 2DG (lanes denoted as 3) reduced the release of MMP13 in each sample of human OA cartilage (due potentially to inhibition of transcription, translation, or release). These data demonstrate that 2DG is effective at reducing the multifaceted catabolic events associated with OA models involving intact cartilage as well as chondrocyte monolayers.

**The effect of 2-deoxyglucose on a pro-catabolic chondrocyte phenotype following activation by other pro-inflammatory agents**

Other pro-inflammatory molecules (TNFα and LPS) as well as certain extracellular matrix degradation products (termed DAMPs) are also known to activate chondrocytes or cartilage, resulting in an OA-like phenotype similar to a response to IL1β. For example, we have shown that LPS and HA oligosaccharides (HAoligos) stimulate MMP13 production in chondrocytes as well as enhanced sGAG release explant cultures (1). This phenotype was blocked by co-treatment with 4MU. To determine whether 2DG could also block pro-catabolic markers stimulated via these alternative pathways, bovine chondrocytes were co-treated with 2DG in the presence of proinflammatory reagents, LPS or TNFα, as well as two different examples of DAMPS: a 30-kDa, N-terminal fibronectin fragment preparation (FN-fr) or HAAoligos. As shown in Fig. 4 (A–D), chondrocytes treated with each of these pro-inflammatory reagents/DAMPs exhibited a substantial up-regulation of MMP13 expression (dark gray bars). Co-treatment of 2DG with each agent significantly reduced the expression of MMP13, similar to the knockdown exerted by 4MU. HAAoligos also affected a stimulation of CD44 (Fig. 4E) and TSG6 (Fig. 4F); both were reduced by co-treatment with 4MU as well as 2DG. That these agents activate chondrocytes via receptors different from IL1βR (e.g. TLR4) implies that the mechanism for 2DG or 4MU...
reduction is not specific for a particular receptor pathway but blocks at some common point further downstream.

The effect of hypoxia on the chondroprotective effects of 4MU and 2DG

All of the experiments shown before (1, 2) and thus far in this study have occurred under normoxic culture conditions (20% oxygen), an environment that may differ significantly from cartilage of the OA joint in vivo, especially if we are considering altering mechanisms related to energy production. To explore these effects, bovine chondrocytes were cultured with or without IL1β in the presence or absence of 4MU or 2DG but with one set of cells placed under hypoxic (1% oxygen) conditions and another set under normoxic conditions. After 24 h, the

Figure 2. Effect of 2DG on chondrocytes activated with IL-β. Shaded bars in A–D depict representative qRT-PCR analyses of lysates from human or bovine chondrocytes examined for relative changes in MMP13 and TSG-6 mRNA as labeled. Bars are compared with controls set to 1.0; the first two bars represent conditions with no added IL1β. Graphs with open bars represent summaries of data (average ± S.D. (error bars)) from three independent experiments normalized to the respective positive control value (+IL1β) set to 100%, to which the effect of 0.2, 2.0, or 20 mM 2DG conditions was compared (all experiments with 2 and 20 mM values fall within 95% confidence levels below 100%). E and F, two independent experiments with human OA chondrocytes treated with or without 2 ng/ml IL1β in the absence or presence of 0.2, 2.0, or 20 mM 2DG for 24 h. Equivalent protein aliquots of lysates were probed by Western blot analysis for MMP13, HIF1α, or β-actin as labeled. Equivalent volumes of concentrated conditioned media were also analyzed to show changes in MMP13 present in the culture medium.
conditioned medium of these cells was examined for accumulation of l-lactate. As shown in Fig. 4G, IL1β alone (dark gray bars) resulted in a significant increase in lactic acid accumulation in cell cultures grown under both normoxic and hypoxic conditions. Co-incubations of chondrocytes with IL1β and either 4MU or 2DG both blocked the enhanced accumulation of lactic acid. The profiles of these results are similar to the ECAR data shown in Fig. 1 (B and D).
The effect of dichloroacetate on chondrocyte metabolism and pro-catabolic phenotype

To verify that the chondroprotective effects of 2DG were due to glycolytic shifts in energy metabolism, another validated inhibitor of glycolysis was investigated. Dichloroacetate (DCA) blocks glycolysis by inhibiting pyruvate dehydrogenase kinase, enhancing pyruvate dehydrogenase, resulting in the enhanced movement of pyruvate into the mitochondria for glucose oxidation over glycolysis. The addition of 25 mM DCA to chondrocytes reversed the IL1β-elevated dependence on glycolysis (detected as an enhanced proton efflux rate) in a fashion similar to that of 2DG (Fig. 5, A and B). When DCA was assayed for its biological inhibitor activity in chondrocytes, concentrations above 12 mM significantly blocked IL1β-stimulated expression of MMP13 (Fig. 5C) and TSG6 (Fig. 5D). Interestingly, no inhibition of IL1β-stimulated CD44 was observed (Fig. 5E). To ascertain whether these changes in OA markers represented a functional chondro-protection, DCA was added to intact cartilage explant cultures activated by either IL1β (Fig. 5F) or LPS (Fig. 5G). In both conditions, 25 mM DCA reduced the release of sGAG from the cartilage tissue similar to the blockage by 2DG, run for comparison. Thus, DCA appeared to exert the same general characteristics as 2DG, 4MU, and HAS2-OE. However, there was one difference that we noted that was surprising. As shown in Fig. 1E, although DCA was quite effective in blocking IL1β-enhanced usage of glycolysis, there was no rescue enhancement of ATP production due to mitochondrial respiration (red bars), as was observed for 4MU and especially 4MU.

Contribution of HIF1α to the shift in energy utilization due to 4MU or HAS2-OE

Channeme et al. (7) reported that changes in HIF1α and GFAT1 mediated a switch in energy usage by cancer cells grown under conditions of HAS2-OE. In their study, activation of HIF1α following HAS2-OE reduced usage of O2-dependent pathways for the supply of cellular energy needs. However, in chondrocytes, the effects of HAS2-OE are the opposite; enhanced usage of UDP-hexoses reduced reliance on glycolysis and promoted mitochondrial respiration (Fig. 1). Nonetheless, to determine whether changes in HIF1α participated in energy-shifting events in chondrocytes, mediating either IL1β-mediated increases in glycolysis or 4MU rescue of enhanced glycolysis, its expression was explored. In human OA chondrocytes (Fig. 6A), HIF1α protein was undetectable until the cells were treated with IL1β. Co-treatment of IL1β-stimulated chondrocytes with 4MU or HAS2-OE blocked the expression of HIF1α protein. In these experiments, HAS2-OE was initiated via AdmycHAS2 transduction using a high-expression cytomegalovirus promoter (versus Ad-LacZ control) or an inducible TET promoter with or without doxycycline. The mycHAS2 protein expression was observed as multimers of a 64-kDa monomer subunit (Fig. 6A). These results were in agreement with the data shown in Fig. 2E. Again, HIF1α protein was undetectable until the chondrocytes were treated with IL1β, and co-treatment with 2DG blocked the IL1β-induced accumulation of HIF1α in the same dose range as for reduction of MMP13. This suggests that HIF1α is clearly activated downstream by IL1β. Moreover, blocking IL1β-mediated increase in MMP13 protein expression using the selective NF-κB inhibitor, helenalin, also blocked IL1β-enhanced HIF1α accumulation by human OA chondrocytes (Fig. 6B). Nonetheless, a time course was run to determine whether HIF1α protein was upstream of MMP13. As shown in Fig. 6C, in chondrocytes isolated from cartilage samples from two patients, increases in HIF1α accumulation were maximal at 8 and 12 h following IL1β treatment, whereas MMP13 was maximal at 24 h. However, in a third chondrocyte preparation, maximal detection of both HIF1α and MMP13 was observed after 24 h of IL1β treatment.

To determine whether HIF1α played a role in the metabolic shifts in chondrocytes, its expression was reduced with siRNA. As shown in Fig. 7A, with siRNA knockdown of HIF1α protein in the presence of IL1β stimulation (fourth lanes), this knockdown did not affect or alter IL1β enhancement of MMP13. MMP13 in the cell and medium compartment was unchanged. Moreover, knockdown of HIF1α did not change or influence 2DG-mediated inhibition of MMP13 (sixth lanes). By real-time quantitative RT-PCR (qRT-PCR) analysis (Fig. 7B), although knockdown was not complete at the mRNA level, IL1β stimulation of MMP13 mRNA was again unchanged with HIF1α inhibition. HIF1α knockdown was also effective in bovine chondrocytes viewed via Western blotting (Fig. 7C) and qRT-PCR (Fig. 7D). Again, no change in IL1β stimulation of MMP13 mRNA was observed with HIF1α inhibition. Last, dimethyl-bisphenol A (DBA; molecular mass 256 Da) is often used as a potent chemical inhibitor of HIF1α (7). If HIF1α function mimicked HAS2-OE, 4MU, or 2DG downstream, we reasoned that its inhibition would result in a chondroprotective-like effect. To test this hypothesis, human OA cartilage explants were incubated without or with IL1β for 4 days in the absence or presence of DBA (Fig. 7E). However, blocking HIF1α activity in cartilage explants had no protective effect on IL1β-stimulated sGAG release or MMP13 protein release from the tissue. Additionally, no enhancement of release was observed. In sum, these results suggest that, although HIF1α is activated downstream of IL1β, it does not appear to play an important role in biological response of chondrocytes to 4MU or 2DG.

Figure 3. To determine the biological effects 2DG on cartilage, intact cartilage explants (6-mm cores) of human OA knee cartilage or bovine metacarpophalangeal joint cartilage were cultured for 7 days in medium with or without 2 ng/ml IL1β or with 2 ng/ml IL1β and 20.0 mM 2DG. A and B, two representative examples of human OA cartilage explant cores; C and D, two representative bovine cartilage cores fixed, sectioned, and stained for safranin O/fast green after these culture conditions as labeled. The red stain depicts sGAGs that were retained or lost from the tissue. A colorimetric was used to quantify sulfated glycosaminoglycan released or lost from the tissue and accumulated in the medium (after 2 or 7 days) of human (E) or bovine (F) explant cultures (errors bars, S.D.). x axes in E–G are labeled as control (1) (black bars), IL1β-treated (2) (gray bars), and IL1β + 2DG (3) (green bars). To the right of each bar graph is the average ± 95% confidence level (error bars) of the percentage of proteoglycan released, as compared with IL1β-treated positive controls (shown as 100%) due to the inclusion of 2DG (summary of 4–5 experiments). E, detection of MMP13 by Western blot analysis of equivalent-volume aliquots of conditioned medium from explant cultures of human OA cores from six different patient tissues.
The effect of 2DG on early signal transduction pathways initiated by IL1β treatment

As noted in Fig. 6B, IL1β-mediated stimulation of MMP13 and other markers is initiated in part by activation of NF-κB and blocked by helenalin. To determine whether the mechanism of 2DG-mediated inhibition of MMP13 production was associated with early signaling events, human OA chondrocytes were treated with IL1β in the absence or presence of 2DG.
As shown in Fig. 8, exposure of chondrocytes to IL1\(\beta\) for 5 and 15 min resulted in an enhancement in phospho-NF-\(\kappa\)B, phospho-p38 MAPK, and phospho-ERK1/2 as well as a minor response on phospho-AKT (Ser473). However, the inclusion of 2DG during this time period had no noticeable effect on the pattern of activation.

The role of GlcNAc transfer to proteins in 4MU–, HAS2-OE–, and 2DG–mediated inhibition of MMP13

We initially posited that one trait in common between 4MU and HAS2-OE was a potential disruption in cytoplasmic pools of UDP-hexose. As such, one mechanism for the inhibition of MMP13 (and chondroprotection) might involve changes in the transfer of GlcNAc to select proteins synthesized by chondrocytes, a process termed O-GlcNAcylation—a posttranslational event with a regulatory potential similar to that of protein phosphorylation (11). However, using RL2, an antibody that detects all GlcNAcylated proteins in lysates (Fig. 8), 2DG did not alter the profile of GlcNAcylated proteins present in IL1\(\beta\)-treated human OA chondrocytes, at least up to the 60-min time point. As another approach to test this hypothesis, chondrocytes were incubated in the presence or absence of thiamet G, a potent
Figure 6. Relationship between HIF1α and MMP13 in IL1β-activated chondrocytes. A, human OA chondrocytes from two patients were transduced with Ad-ZsGreen-mycHAS2, Ad-ZsGreen-LacZ, or Ad-Tet-mycHAS2 and then incubated with IL1β (2 ng/ml) for 24 h in the presence of Dox (200 ng/ml) or 4MU (0.5 mM). Equivalent protein aliquots of lysates were probed by Western blot analysis for HIF1α, Myc (to detect mycHAS2), and β-actin as labeled. B, data from another two preparations of human OA chondrocytes pretreated with 0.5–2.0 μM helenalin for 30 min as labeled, followed by an additional 16 h in the presence of IL1β (2 ng/ml). Aliquots from lysates were then probed by Western blot analysis for HIF1α, MMP13, and β-actin. C, cultures of three preparations of human OA chondrocytes were treated with or without 2 ng/ml IL1β for varying times between 0 and 24 h. Protein lysates were collected at each time point and probed by Western blot analysis for HIF1α, MMP13, and β-actin. Numbers shown below the protein bands represent -fold change in pixel intensity of that band (normalized to pixel intensity of reprobed β-actin band), wherein the intensity of the band at the zero time point was set to 1.0.
O-GlcNAcase inhibitor (12). By blocking GlcNAc removal, thiamet G enhances overall GlcNAc modification of cellular proteins. As shown in Fig. 9, all human OA chondrocyte cultures exposed to thiamet G exhibited increased expression of GlcNAcylated proteins, as detected by the RL2 antibody. This occurred in both IL1β-treated and control, untreated cultures. HIF1α protein was stimulated by IL1β but not altered in the presence of thiamet G (Fig. 9, A and C). Moreover, both 4MU (Fig. 9A) and 2DG (Fig. 9C) blocked the expression of HIF1α protein even when GlcNAcylation was enhanced with thiamet G. A similar pattern was observed for MMP13 protein accumulation in cell-associated (Fig. 9, A and C) or medium compartments (Fig. 9A); namely, IL1β-enhanced MMP13 production and 4MU or 2DG inhibition of MMP13 production were unchanged with or without thiamet G. Fig. 9B depicts the effects on HAS2-OE inhibition of MMP13. Again, IL1β stimulation of MMP13 protein was unaffected by thiamet G, and HAS2-OE–mediated inhibition of MMP13 was unaffected by thiamet G treatment. These data suggest that generalized enhancement of protein modification with GlcNAc does not block or enhance inhibition of IL1β-stimulated MMP13.

Association of changes in pAMPK with 4MU, 2DG, and DCA effects on chondrocytes

Changes in the phosphorylation of AMPK are often associated with sensing and regulating energy homeostasis in cells,
Rescue of energy metabolic changes in chondrocytes

Figure 8. Effect of 2DG on early signaling events during IL1β stimulation of chondrocytes. Human OA chondrocytes were incubated with IL1β (2 ng/ml) in the presence or absence of 2DG (20 mM) for 0–60 min as labeled. Protein lysates were prepared at each time point, and equivalent protein aliquots were probed by Western blot analysis for phospho-NF-κB and NF-κB (total), p-p38 and p38 (total), p-ERK1/2 and total ERK1/2, p-AKT and total AKT, and β-actin. Data shown are from a representative analysis of two experiments with or without 2DG, both with a similar lack of change in the pattern of these initial signal transduction events.

including chondrocytes (13–15). Western blot analysis was used to examine the changes in the phosphorylation status of AMPKα with or without IL1β treatment. As shown in Fig 10, IL1β treatment of bovine chondrocytes for 24 h resulted in a clearly visible diminution of phospho-AMPKα as compared with control chondrocytes. Although the absolute decrease varied (measured as normalized pixel intensity), there was a consistent decrease due to IL1β treatment in every experiment. More importantly, co-treatment of these cells with IL1β together with 4MU (A), 2DG (B), or DCA (C) all resulted in a rescue of the phospho-AMPKα status, oftentimes stronger than that of control chondrocytes. Similarly, treatment of control chondrocytes with 4MU, 2DG, and DCA (without IL1β) also supported the accumulation of phospho-AMPK, in some experiments substantially more than control but not statistically significantly different overall. Interestingly, although the inclusion of DCA provided a rescue of phospho-AMPK status and chondroprotection of cartilage and chondrocytes (Figs. 3 and 5), there was little effect of DCA on mitochondrial OCR and ATP production (Fig. 1E).

Discussion

Some chronic diseases, including OA, have been considered the result of changes in cell phenotype and changes in metabolism—changes brought on by aging, trauma, malalignment, obesity, etc. (14–17). Our data on the HA biosynthesis inhibitor 4MU may provide unexpected insight into these changes in phenotype and metabolism as largely due to mechanisms that are completely unrelated to HA biosynthesis. In vitro, 4MU, 2DG, and DCA readily penetrate into and affect intact articular cartilage. It is possible that such agents as these, already being used clinically, in preclinical animal models or in clinical trials for other diseases including cancer, diabetes, mellitus, pulmonary arterial hypertension, biliary antispasmodic activity inflammation, and autoimmunity (8, 10, 18–23), will have similar effectiveness in limiting the metabolic changes and reversing the pro-catabolic phenotype associated with OA.

In this paper, we have addressed the mechanism by which 4MU as well as HAS2-OE block the pro-catabolic phenotype associated with cytokine- or DAMP-activated chondrocytes. Both 4MU and HAS2-OE as well as 2DG and DCA all reduce the usage of glycolysis as an important energy source by chondrocytes (Fig. 1). As shown in Fig. 1 (E and F), a large percentage of ATP synthesis in chondrocytes is supplied by glycolysis even in normal quiescent chondrocytes cultured in vitro under normoxia growth conditions. Indeed, this dependence on glycolysis is further amplified when chondrocytes are activated by IL1β (15, 16, 24). However, in the presence of co-treatments of IL1β with 4MU, HAS2-OE, 2DG, or DCA, the resultant reduction in glycolysis usage returns essentially back to the level of controls. Changes in a dependence on mitochondrial respiration for ATP production are also altered, inversely mirroring the changes in the glycolysis pathway. Basal and (potentially) maximal mitochondrial respiration were substantially reduced in IL1β-treated chondrocytes (Fig. 1, C–F). This reduction in the use of the TCA cycle, as measured by O2 consumption, was rescued by all glycolysis inhibitors with the exception of DCA. Reduced mitochondrial respiration was matched by a reduced pAMPK status of IL1β-treated chondrocytes (Fig. 10), and in these experiments, DCA provided a rescue of pAMPK (Fig. 10C), like that observed for 4MU and 2DG (Fig. 10, A and B). Perhaps the DCA-mediated rescue of mitochondria functions requires a longer time frame.

The suggested initiation of the pro-catabolic phenotype includes inflammation, injury, or aging that produce NO oxidative stress, leading to enhanced ER stress, reduced AMPK phosphorylation, more ROS oxidative stress, and enhanced catabolism (16, 24). It is already known that pAMPKα1 is reduced in OA as well as IL1β- or TNFα-activated chondrocytes (13). Moreover, acting through the pAMPKα1-SIRT1-PGC1α (peroxisome proliferator-activated receptor co-activator 1α) axis is one likely mechanistic pathway for both the activation of and rescue from a pro-catabolic phenotype in
chondrocytes (14). Pharmaceutical activation of pAMPK with AICAR results in suppression of pro-catabolic activity induced by the IL1 or TNF (13). Thus, it is possible that the chondroprotective effects of 4MU, 2DG, and DCA occur downstream of a pAMPK rescue.

Initially, we found that the link between the chondroprotective effects of 4MU and HAS2-OE and energy metabolism was due to their effects on reducing glycolysis, primarily because both biological and metabolic effects could be readily mimicked by the glycolysis inhibitors 2DG and DCA. However, 4MU, HAS2-OE, 2DG, and DCA also rescue deficits in mitochondrial respiration and AMPK phosphorylation in activated chondrocytes; thus, it is not clear which energy pathway switch is initially driving the process. The straightforward conclusion is

Figure 9. Contribution of GlcNAcylation to the inhibition of activated chondrocytes by HAS2-OE, 4MU, or 2DG. Human OA chondrocytes were incubated with or without IL1β (2 ng/ml) with or without thiamet G (TG; 100 μM) for 24 h as labeled. A, chondrocytes also were incubated with or without 4MU (0.5 mM). B, chondrocytes were first transduced with 10 infectious units/cell of Ad-Tet-mycHAS2 2 days before the start of the experiment. HAS2-OE was initiated by inclusion of Dox (HAS; 200 ng/ml). C, chondrocytes also were incubated with or without 2DG (20 mM). Protein lysates were prepared, and equivalent protein aliquots were probed by Western blot analysis for HIF1α, MMP13, GlcNAcylated protein (RL2), or mycHAS2 (anti-Myc). Following development, blots were stripped, washed, and reprobed for β-actin. Equivalent-volume aliquots of conditioned medium from some of these cultures were also probed by Western blot analysis (A) for MMP13 released into the medium.
that the energy pathways are cross-correcting. Whereas a pro-catabolic phenotype in chondrocytes may be initiated by stressed mitochondria followed by a secondary compensation and enhancement of glycolysis to support cellular ATP production, the reverse may also be possible. In other words, forcing a reduction in glycolysis may drive repair of stressed mitochondrial pathways.

At some point, before or after reductions in mitochondrial respiration, there is a shift to enhanced dependence on glycolysis. Yang et al. (25) have observed that OA chondrocytes exhibit high levels of the M2 isoform of pyruvate kinase (PKM2) and suggest that a switch between PKM1 to MKM2 isoforms is the mechanism responsible for enhanced glycolysis associated with OA. Other pathway or network axes that participate include the AMPK-to-mTOR-to-autophagy (15, 24) and HIF1α and HIF2α (26, 27), all with cross-talk links to other networks, such as NF-κB and autophagy.

Cross-correcting energy pathways may explain how 2DG and DCA exert influence over AMPK phosphorylation, resulting in changes in SIRT1 and PGC1α and a diminution of the pro-catabolic phenotype. However, it is less clear how 4MU and HAS2-OE tie into this mechanistic scheme. The majority of our mechanistic experiments were aimed at determining how fluxes in UDP-GlcUA (common to both 4MU and HAS2-OE) and/or fluxes in UDP-GlcNAc, affect energy metabolism in chondrocytes. Following the lead of Chanmee et al. (7), wherein HAS2-OE in their system activated a HIF1α-GFAT1 axis, we examined the role of HIF1α in our model. In their study, HAS2-OE induced a HIF1α activation—an activation that resulted in a switch to enhanced dependence of glycolysis. Moreover, HIF1α and GFAT1 were upstream of the metabolic shift and resultant change in cell phenotype. As such, knockdown of HIF1α reduced the expression of PDK1 and the percentage of cells that reverted to tumor stem cells (7). Reduced reversion to tumor stem cells could also be achieved by treatment with the HIF1α inhibitor DBA. Surprisingly, this series of events was opposite to what we observed in chondrocytes. In our studies, little HIF1α was observed in normal chondrocytes, but it was substantially enhanced following stimulation with IL1β and coordinate with increases in MMP13 and

Figure 10. Effect of 4MU, 2DG, and DCA on p-AMPK changes in IL1β-activated chondrocytes. Bovine chondrocytes were treated for 24 h without (Ctrl) or with IL1β (2 ng/ml) and with or without 4MU (0.5 mM) (A), with or without 2DG (2.0 mM) (B), or with or without DCA (25 mM) (C). Protein lysates were then prepared, and equivalent protein aliquots were probed by Western blot analysis for phospho-AMPKα (p-AMPK). Following development, blots were stripped, washed, and reprobed for total AMPK and β-actin. Bar graphs to the right, summary of fold change in pixel intensity of bands for p-AMPK (normalized to pixel intensity of its respective total AMPK band) wherein the intensity of the p-AMPK band of control, untreated chondrocytes was set to 100. Error bars, S.D.
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As such, the exact mechanism for how fluxes in UDP-hexoses via 4MU (or HAS2-OE) give rise to changes in chondrocyte metabolism remains unresolved. In our previous study (1), we determined that the mechanism of action of 4MU was not dependent on HA biosynthesis or signaling via CD44, but due to cell toxicity or alteration of GlcNAcylation, not due to changes in UDP-glucuronosyltransferases or upstream signal transduction events (as in Fig. 8), and finally, not due to changes in mRNA stability. In this study, 2DG also did not alter early signal transduction, including activation of NF-κB (Fig. 8). This lack of early effect is similar to what we observed previously for 4MU (1) and HAS2-OE (data not shown) (2). Thus, the metabolism-related inhibition by these agents likely occurs at a later stage. The activating receptor is also not specific as IL1β, TNFα, and AMPK or hyperglycemic conditions of excess glucose can be tempered or moderated by enhanced or reduced HA biosynthesis (5, 33). Endogenous HAS2 may provide the natural source of moderate and may be why chondrocytes up-regulate its synthesis after cytokine treatment. We propose that 4MU, HAS2-OE, 2DG, or DCA becomes useful when these endogenous responses are not enough to rescue cells from a pro-catabolic phenotype.

Experimental procedures

Materials

Ham’s F-12 and DMEM were obtained from Mediatech; FBS was from HyClone; and IL1β and TNFα were from R&D Systems, Inc. 4MU was from Alfa Aesar (A10337); oligomycin was from MP Biomedicals; and DAB was from Abcam. Pronase (53702; EMD Millipore Calbiochem), collagenase P (11249002001; Roche Applied Science), and collagenase D (11088882001; Roche Applied Science) were used in dissociation of tissues. Cell lysis buffer was from Cell Signaling Technologies, and Clear Blue X-ray film was from Genesee Scientific. All other reagents were from Sigma-Aldrich, including the 30-kDa fragment N terminus fragment of fibronectin (catalog no. F9911) equivalent to the 39-kDa fibronectin fragment that we (34) and others (35) have previously used. HA oligos were prepared and purified as described previously (36) and derived from rooster comb HA (Sigma). The HA oligos are present as a mixture of HA hexa-, octa-, and decasaccharides. We recently reviewed information on the activity and comparative use of these and other HA oligo preparations (37).

Cell culture

Primary bovine articular chondrocytes were isolated from the articular cartilage of metacarpophalangeal joints of 18–24-month-old adult steers as described previously (1, 2). Primary
human articular chondrocytes were isolated from knee cartilage obtained following joint replacement surgery, within 24 h after surgery and with institutional approval. Human cartilage samples (n = 33) were from patients (~67% female, 33% male) with an average age of 62.5 ± 15.9 years. Bovine and human chondrocytes were liberated from full-thickness slices of articular cartilage by sequential Pronase/collagenase P digestion. Chondrocytes were plated as high-density monolayers (0.5–1.0 × 10⁶ cells/cm²) in medium containing 10% FBS. Typically, after 24–48 h in culture, the medium was changed to 1% FBS for 12 h, and chondrocytes were then incubated in serum-free medium for 1 h prior to the addition of IL-1β (2 ng/ml), LPS (0.1 μg/ml), TNFα (10 ng/ml), FN-fr (1.0 μg/ml), or 250 μg/ml HA oligos. Some experiments included co-treatment with 4MU (1.0 μM), 2DG (0.2–20 mM), DCA (6–50 mM), or doxycycline (Dox) (200 ng/ml) as labeled. Other agents tested were heparin (0.5–2.0 μM), DBA (0.5 mM), and thiamet G (100 μM). Time courses varied depending on the experiment as labeled. In experiments with 4MU, the reagent was dissolved in DMSO and then added to the culture medium with a final concentration of 0.1% DMSO; DMSO only at the same concentration was used as a control.

For hypoxia studies, bovine chondrocytes were cultured as above. At the time of addition of IL1β, 4MU, or 2DG, one set of cells were placed under hypoxic culture conditions (1% O₂) in a humidified, 37 °C, InviVo2 400 physiological cell culture work station from the Baker Company (Sanford, ME). Another set of chondrocytes (from the same preparation of cells) were simultaneously placed in a normoxic (20% O₂), humidified chamber at 37 °C. Chondrocytes in both environmental conditions were incubated for 24 h, at which time the conditioned media was removed and then equal volume aliquots of diluted (1:5) conditioned medium were assayed for lactate levels using a commercial colorimetric assay kit according to the manufacturer’s instructions (Eton Bioscience, Research Triangle, NC). L-lactate concentrations were determined by comparison with a standard curve, and data are presented as percentage change from control, untreated chondrocytes.

For HAS2-OE, bovine or human chondrocytes were transduced after harvest but before plating by incubating the suspended cells with Ad-HAS2 constructs (10 infectious units/cell) for 2 h at 37 °C with occasional gentle mixing, in a 1:1 mixture of DMEM/Ham’s F-12 medium, 50 units/ml penicillin, l-glutamate, and ascorbic acid but without serum. Ad-HAS2 viruses were either Ad-ZsGreen-myHas2 (38) wherein control chondrocytes were transfected with Ad-ZsGreen-LacZ or Ad-Tet-mycHas2 (1, 2) wherein cells were subsequently treated with or without Dox. Chondrocytes were then plated as high-density monolayers (0.5–1.0 × 10⁶ cells/cm²) in medium still containing virus and allowed to attach overnight at 37 °C. Medium was then changed to fresh media containing 10% FBS and Dox if the Ad-Tet-On virus was being used. After 24 h in culture, the medium was changed to 1% FBS with Dox if appropriate) for 12 h and then incubated in serum-free medium for 1 h prior to the addition of activating agents (i.e. IL-1β) and with or without varying concentrations of Dox in fresh serum-free culture medium treatment for varying times.

**Cartilage explant cultures**

Full-thickness 4- or 6-mm cores of bovine and human OA articular cartilage were cultured in 1.0 ml of DMEM/Ham’s F-12 medium containing 10% FBS for 48 h. The medium was then replaced with serum-free DMEM/Ham’s F-12, and the tissues were incubated for 4–7 days in the presence of various activators, including IL-1β (2 ng/ml) or LPS (10 ng/ml) with or without 4MU, 2DG, or DCA. For histology, the treated explants were fixed with 4% buffered paraformaldehyde overnight at 4 °C; rinsed in 30% sucrose, PBS; and embedded in paraffin. Sections (8 μm) were prepared and stained with safranin O for the detection of proteoglycans and counterstained with Fast Green (1). In other experiments, the culture medium was collected and concentrated, and equivalent-volume aliquots were processed for Western blotting to detect MMP13. In additional experiments, medium aliquots were analyzed by a colorimetric assay for released proteoglycan content by a dimethylmethylene blue assay for sGAG release (1, 39, 40).

**Knockdown of HIF-1α by siRNA**

For siRNA-mediated inhibition of HIF-1α, human or bovine chondrocyte cells were plated at 1.8 × 10⁶ cells/well in a 24-well plate. After attachment, the monolayers were transfected with 5 pmol of siRNA in a 1:1 solution of Lipofectamine RNAiMAX (Invitrogen) and Opti-MEM (Gibco) as per the manufacturer’s instructions. siRNAs used were Silencer® predesigned siRNA for human HIF1α (Ambion, catalog no. 16708; assay ID 144736)—an siRNA sequence that targets identical sequences in human and bovine HIF-1α—and a control siRNA from Santa Cruz Biotechnology, Inc. (sc-37007).

Total protein was extracted using cell lysis buffer—containing protease and phosphatase inhibitor mixtures. Alternatively, radioimmune precipitation assay buffer with protease and phosphatase inhibitors was used when lysates were to be probed for HIF1α. Equivalent protein concentrations were loaded into 4–12% NuPAGE® Novex® Tris acetate gradient gels (Thermo Fisher Scientific). In some experiments, the conditioned culture medium was also collected and processed for Western blotting by loading aliquots of equivalent volume to minigels. Following electrophoresis, proteins within the acrylamide gel were transferred to a nitrocellulose membrane using a Criterion blotter apparatus (Bio-Rad), and the nitrocellulose membrane was then blocked in TBS containing 0.1% Tween 20 and 5% nonfat dry milk (TBS-T-NFDM) for 1 h. Immunoblots were incubated overnight with primary antibody in TBS-T-NFDM at 4 °C, rinsed three times in TBS-T, and incubated with secondary antibody in TBS-T-NFDM for 1 h at room temperature. Detection of immunoreactive bands was performed using chemiluminescence (Novex ECL, Invitrogen). In some cases, the blots were stripped using Restore Plus Western Stripping Buffer (Thermo Fisher Scientific) for 30 min at room temperature and reprobed using another primary antibody. Developed X-ray films were imaged and digitized using a Bio-Rad GelDoc with ImageLab software. Pixel intensities for MMP13 bands were used for quantification after normalization to loading control bands (β-actin or GAPDH). All other experimental details not mentioned here are described in the figure.
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For the use of TRIzol reagent (Thermo Fisher Scientific). Total RNA was isolated from the bovine and human chondrocyte cultures according to the manufacturer’s instructions for the use of TRIzol reagent (Thermo Fisher Scientific). Total RNA was reverse-transcribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was performed using Sso-Advanced SYBR Green Supermix (Bio-Rad) and amplified on a StepOnePlus Real-Time PCR System (Applied Biosystems) to obtain cycle threshold (Ct) values for target and internal reference cDNA levels. Specific primers for real-time RT-PCR were custom-made by Integrated DNA Technologies (Coralville, IA). RT^2 Real Time™ SYBR Green reagents were from SA Biosciences.

The human-specific primer sequences are as follows: GAPDH, forward (5'-GAA TTT GGC TAC AGC AAC AGG-3') and reverse (5'-AGT GAG GGT CTC TCT CCT CC-3'); HIF1α, forward (5'-CAG TCG ACA CAG CCT GGA TA-3') and reverse (5'-TGT CCT GTG AGT TGT CC-3'); MMP13, forward (5'-CAG TCG TGG TGA TGA AGA TGA TGA-3') and reverse (5'-CGC GAG ATT TGT AGG GTA G-3'); TSG6, forward (5'-GTG GGC TCT TTA CAG ATC CAA AGC-3') and reverse (5'-CAA CAT AAT CAG CCA AGC AAC-3'); 18s rRNA, forward (5'-TAG TAG CAG GGA CCC GGC GTG-3') and reverse (5'-CTT CAA CAT AGT CAG ACC T-3'); MMP3, forward (5'-CTC ACA GAC CTG ACT CGG TT-3') and reverse (5'-CAC GCC TGA AGG AGA AG-3'); MMP13, forward (5'-CCT GCT GGA ATC CTG AAG AAA-3') and reverse (5'-AGT CTG CCA GTC ACC TCT AA-3'); TSG6, forward (5'-GTG GTG GTG TGT TTA CAG ATC CAA AGA G-3') and reverse (5'-CTT CAA CAT AGT CAG CCA AGC AAG-3'); 18s rRNA, forward (5'-TGA CAC GGA CCC GGC GTG-3') and reverse (5'-CCA TCC AAT CAG TAG TAG CG-3'). Real-time RT-PCR efficiency (E) was calculated as $E = 10^{(-1/slope)}$ (41). The -fold increase in copy numbers of mRNA was calculated as a relative ratio of target gene to GAPDH (ΔΔCt), following the mathematical model introduced by Pfaff (42) as described previously (1, 2).

Metabolomic studies using Seahorse flux analyzer

Transduced bovine chondrocytes were plated at 8.0 × 10^4 cells/well into specially designed 96-well Seahorse XF cell culture microplates. The confluent monolayers were preincubated without or with 200 ng/ml Dox in 1% FBS for 12 h and then for 24 h with or without 2 ng/ml IL-1β and with or without the same level of Dox or 1 mM 4MU, 2 mM 2DG, or 25 mM DCA. The medium was changed to serum-free Seahorse XF Base Medium (without phenol red but with 10 mM glucose, 1.0 mM pyruvate, and 2.0 mM glutamine added) or Seahorse XF DMEM, pH 7.4, cells, depending on the assay. Assay medium also contained fresh IL1β. The cells were then mated with a sensor cartridge and analyzed in a Seahorse XFe 96 flux analyzer (Agilent Tech) for real-time detection of changes in proton accumulation and oxygen consumption following the manufacturer’s guidelines. Briefly, for the Agilent XF cell energy phenotype test, a combined injection of oligomycin (2 μM final) and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (0.25 μM final) were applied after the instrument completed measurement of basal values. When performing an Agilent XF mito stress test, timed sequential injections of oligomycin (2 μM final) followed by FCCP (0.25 μM final) and, last, a 1:1 mixture of antimycin A with rotenone (0.50 μM final) were applied after measurement of basal values. To perform an Agilent XF glyco rate assay, timed sequential injections of a 1:1 mixture of antimycin A with rotenone (0.50 μM final) and, last, 2DG (50 mM final) were applied after measurement of basal values. For the Agilent XF ATP rate assay, timed sequential injections of oligomycin (1.5 μM final) followed by a 1:1 mixture of antimycin A with rotenone (0.50 μM final) were applied after measurement of basal values. Algorithms provided in Agilent assay report generator Excel files were used to generate blots and bar graphs. Mitochondrial ATP production derived from the mito stress test is expressed as OCR (pmol of O2/min); in the software, ATP production in these OCR units is closely proportional to true ATP values. A measure of ATP production rate in terms of pmol/min ATP is provided by the ATP rate assay.

Statistical analysis

All data except as noted were obtained from at least three independent experiments performed in duplicate or triplicate. In some experiments, a two-tailed unpaired Student’s t test was used for direct comparison of treatment group with control. For multiple comparisons of groups, analyses of variance
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(ANOVA) was performed, followed by Tukey or Tukey–Kramer post hoc tests as indicated in the figure legends. A p value of <0.05 was considered significant. *, p < 0.05; **, p < 0.01; ***, p < 0.001.


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