PC1

Regulation of Na^+-H^+ exchange in articular chondrocytes by O_2

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The avascular nature of articular cartilage means that chondrocytes are dependent on the diffusion of O_2 from the synovial fluid and subchondral bone. As a result, cartilage has a relatively low ambient O_2 tension, estimated at between 5% and 7% O_2. In addition, synovial, and cartilage, O_2 tensions can fall to very low levels in inflammatory conditions. Low O_2 tensions can affect ion homeostasis: cells acidify and Na^+-H^+ exchange (NHE) is inhibited (Milner *et al.* 2005). Reactive oxygen species (ROS) have been implicated in the response to altered O_2 tension (Chandeel & Schumacker, 2000). In the present study, we investigated their possible role in O_2 sensitivity of NHE in articular chondrocytes.

Cartilage slices were obtained postmortem from bovine and equine metacarpophalangeal joints. Chondrocytes, isolated overnight by collagenase digestion at ambient levels of O_2, were incubated for 3 h at 20% (termed normoxia) or 1% O_2 (termed hypoxia). Intracellular pH (pH_i) was then determined fluorimetrically using BCECF, using intracellular buffering to calculate proton efflux (J_H in mmol H^+ min^{-1}) following cell acidification (Wilkins & Hall, 1995). Production of reactive oxygen species (ROS), estimated using dichlorofluorescein fluorescence (Chandeel & Schumacker, 2000).

O_2 deprivation caused a significant acidification of steady-state pH_i and also slowed recovery of pH, through inhibition of amiloride-sensitive NHE. Under these conditions, production of ROS was reduced by 41 ± 6% (n = 7) cf values at 20% O_2 ($p < 0.05$). Following treatment with antimycin A (10-100 μM), ROS levels were increased from 59±9 to 84±7 (NS cf 20%). With antimycin A, J_H increased from 1.90±0.15 mmol min^{-1} ($p<0.05$ cf 20%) to 2.51±0.17 (N.S. cf 20%), compared with 2.42±0.12 at 20% O_2. Similar findings were observed following treatment with Co^{2+} (100 μM). NHE activity could therefore be correlated with ROS levels. Finally, when exposed to the protein phosphatase inhibitor calyculin A (100 nM), NHE activity (J_H) was 3.29±0.22 at 1% O_2, compared with 3.13±0.18 at 20% O_2. Calyculin A had no effect on ROS levels.

These findings suggest that NHE activity in articular chondrocytes is inhibited by low O_2 tension, with the putative signal being a reduction in ROS. The signalling cascade coupling ROS levels with NHE activity appears to act via protein phosphorylation. The importance of these results to the chondrocyte in vivo is discussed.

Chandel NS & Schumacker PT (2000). J Appl Physiol 88, 1880-1889.

Milner PI, Fairfax TPA, Tattersall AL, Browning JA, Wilkins RJ & Gibson JS (2005). J Physiol 565P, PC47.

Wilkins RJ & Hall AC (1995). J Cell Physiol 164, 474-481.

P.I.M. holds an Horserace Betting Levy Board Veterinary Training Scholarship.

Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

PC2

Mitochondrial reactive oxygen species and Na^+-H^+ exchange in articular chondrocytes

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Chondrocytes of articular cartilage experience a relatively low ambient O_2 tension, between 5% and 7% O_2. O_2 tensions may fall further to very low levels in joint disorders such as chronic inflammatory arthritis. Hypoxia therefore presents an important stress for articular chondrocytes. Low O_2 tension (1%) results in intracellular acidification and inhibition of membrane NHE (Milner *et al.* 2005) and reduces the levels of reactive oxygen species (ROS). Here we investigate the site of production of ROS. Cartilage slices were obtained postmortem from equine fetlock joints. Chondrocytes, isolated overnight by collagenase digestion at ambient levels of O_2, were incubated for 3 hours at 20% (termed normoxia) or 1% O_2 (termed hypoxia). Intracellular pH was determined using BCECF and intracellular buffering to calculate proton efflux (J_H) following cell acidification (Wilkins & Hall, 1995). Production of reactive oxygen species (ROS) was estimated using dichlorofluorescein (DCF) fluorescence (Chandeel & Schumacker, 2000).

Rotenone (1-100μM), an inhibitor of mitochondrial complex I, caused a reduction in ROS levels: 97±8, 59±3 and 36±3% cf control (means±SEM, n=3), at 1, 10 and 100μM. Myxothiazol (0.1-10μM), which inhibits mitochondrial complex III upstream of the ubisemiquinone, the site of production of ROS, was also tested. Results were similar to those with rotenone, e.g. at 10 μM myxothiazol decreased ROS to 45±10% (3). At both 20% and 1% O_2, both rotenone and myxothiazol also inhibited NHE activity (e.g. at 40μM, rotenone inhibited J_H at 1% O_2 from 1.59±0.09 mmol/min to 1.12±0.12, n=3). These mitochondrial inhibitors, therefore, behave differently to antimycin A, which blocks complex III downstream of ubisemiquinone, increases ROS production and also abolishes the hypoxic-induced reduction in NHE activity (see accompanying poster). Diphenyleneiodonium (DPI), which inhibits flavocytochromes, including the non-mitochondrial NADPH oxidase, as well as complex I NADH dehydrogenase, also inhibited ROS levels (at 1% O_2, ROS decreased from 77±7% to 41±2%, n=3). In addition, DPI abolished the protective effects of antimycin A on hypoxic inhibition of ROS and NHE activity when both inhibitors were added together (at 1% O_2 J_H declined further from 2.52±0.15 mmol/min with 50 μM antimycin A alone, to 1.72±0.22 mmol/min with both antimycin A and 10μM DPI, n=3).

These findings implicate the mitochondria, and, in particular complex III, as the site of production of ROS in articular chondrocytes. Inhibition of ROS production in hypoxia is associated with decrease in activity of NHE.

Chandel NS & Schumacker PT (2000). J Appl Physiol 88, 1880-1889.

Milner PI, Fairfax TPA, Tattersall AL, Browning JA, Wilkins RJ & Gibson JS (2005). J Physiol 565P, PC47.

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PC3

Effect of O_2 tension on Ca^{2+} homeostasis in bovine articular chondrocytes

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The avascular nature of articular cartilage means that chondrocytes are dependent on the diffusion of O_2 from the synovial fluid and subchondral bone. As a result, cartilage has a relatively low ambient O_2 tension estimated at between 5% and 7%. In addition, synovial O_2 tensions can fall to very low levels in inflammatory conditions, which can further lower O_2 levels within the matrix. These low O_2 tensions can affect ion homeostasis. For example, they alter pH homeostasis, with intracellular acidification associated with inhibition of Na^+-H^+ exchange (Milner et al. 2005). Intracellular Ca^{2+} also represents an important cell parameter, shown to be linked to pH levels (Fairfax et al. 2003). In the present study therefore, we investigated the effect of O_2 on Ca^{2+} homeostasis.

Cartilage slices were obtained postmortem from bovine metacarpophalangeal joints. Chondrocytes were isolated overnight by collagenase digestion at either 20% (termed normoxia) or 1% O_2 (termed hypoxia), and maintained at these levels during all subsequent procedures. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) was then determined fluorimetrically using fura-2 (5 μM; EM 510nm; EX 340nm/380nm). Ca^{2+} levels are given as the 340nm/380nm signal ratio (R) (Sanchez & Wilkins, 2004).

Ca^{2+} homeostasis was shown to depend on Ca^{2+} entry via the plasma membrane, thapsigargin-sensitive stores and Ca^{2+} efflux mediated predominantly by the Na^+-Ca^{2+} exchanger. When cells isolated overnight at 1% (hypoxia) were compared to those isolated at 20% O_2, it was found that $[Ca^{2+}]_i$ was elevated, with R typically rising from 1.27 to 1.51. By contrast, shorter term hypoxia (3 hours) had no effect on Ca^{2+} homeostasis, and nor was there a significant difference between cells isolated at 20% and 5% O_2. At 1% O_2, exposure of chondrocytes to either Co^{2+} (100 μM) or antimycin A (5 μM) caused a reduction in $[Ca^{2+}]_i$. For example, with Co^{2+}, R fell from 1.59 to 1.36. Hypoxia has been shown to decrease levels of reactive oxygen species (ROS) in chondrocytes, whilst exposure to either Co^{2+} or antimycin A increases them (see accompanying poster).

These findings suggest that Ca^{2+} homeostasis in articular chondrocytes is affected by low O_2 tension, with the putative signal being a reduction in ROS. We hypothesise that high levels of ROS mediates a reduction in $[Ca^{2+}]_i$ via stimulation of the Na^+-Ca^{2+} exchange.

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Milner PI, Fairfax TPA, Tattersall AL, Browning JA, Wilkins RJ & Gibson JS (2005). J Physiol 565P, PC47.

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PC4

Pharmacological activators of AMPK have differential effects on transepithelial Na^+ transport processes in human H441 lung epithelial cells

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We have previously shown that activation of AMP-activated protein kinase (AMPK) with phenformin or AICAR decreased transepithelial amiloride-sensitive Na^+ transport ($I_{amiloride}$), apical Na^+ conductance (G_{Na+}) and ouabain-sensitive Na^+-K^+-ATPase ($I_{ouabain}$) activity in H441 lung epithelial cells (1). The biguanide, phenformin, is thought to activate AMPK by inhibition of Complex I of the mitochondrial respiratory chain and increasing the intracellular AMP:ATP ratio (2,3). In contrast, the adenosine analogue 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) activates AMPK by mimicking the effect of AMP without affecting cellular ATP levels in intact cells. By measuring short circuit current (I_{sc}) across H441 cell monolayers, we have further explored the effect of these drugs and the activation of AMPK on ion transport processes across H441 cells. Phenformin (5nM) and AICAR (2mM) evoked similar ~2.0-fold increase in AMPK activity in H441 cells from 0.03 ± 0.002 to 0.05 ± 0.008 and 0.04 ± 0.004 to 0.07 ± 0.005 nmols/min/mg, respectively. Activity was significantly increased at 30 min ($p < 0.01$, $n = 3$, respectively) and was maintained over 24 hours. Phenformin and AICAR inhibited transepithelial $I_{amiloride}$ to minimal levels (~20% of control) at 4–8 hours with similar efficacy ($t_{1/2}$ 32.5 min and 33.5 min, respectively, $n = 3$). In contrast, 5 mM phenformin maximally inhibited $I_{ouabain}$ to 25% of control levels with a $t_{1/2}$ of 12 min ($n = 3$) but AICAR only inhibited $I_{ouabain}$ to 66% of control levels with a $t_{1/2}$ of 58 min. Measurement of NADPH autofluorecence by excitation at 405 nm wavelength (indicative of inhibition of mitochondrial function) showed that phenformin but not AICAR was associated with increased NADPH fluoresecence (35.6 ± 0.7 fluorecence units, $p < 0.01$, $n = 20$). Transepithelial amiloride-sensitive Na^+ transport is regulated by apical Na^+ entry through amiloride-sensitive Na^+ channels and extrusion via Na^+-K^+-ATPase. That phenformin and AICAR had similar effects on AMPK activity and $I_{amiloride}$ but AICAR had a greater effect on $I_{amiloride}$ than $I_{ouabain}$ indicate that activation of AMPK inhibits Na^+ entry as the rate-limiting step in transepithelial Na^+ transport. Furthermore, the more potent effect of phenformin compared to AICAR on $I_{ouabain}$ indicates that factors additional to AMPK activation, which could include

ATP availability, modulate Na^+K^+ATPase activity in H441 lung epithelial cells.

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PC5

Functional TGFβ type II receptors are required for D-glucose stimulation of L-arginine transport in human umbilical vein endothelium

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L-Arginine transport and nitric oxide (NO) synthesis are increased in human umbilical vein endothelium (HUVEC) exposed to high D-glucose (Vásquez *et al.* 2005). Hyperglycemia increases expression of Transforming Growth Factor β1 (TGF-β1)(McGinn *et al.* 2003), which stimulates endothelial NO synthase expression (Oyadomari *et al.* 2001). It is unclear whether TGF-β1 alters expression of L-arginine transporters, and whether TGF-β type II receptors (TβRII) are required for D-glucose stimulation on L-arginine transport. We have characterized TGF-β1 and TβRII roles on D-glucose stimulated L-arginine transport.

Confluent HUVEC (M199, 20% sera, 3.2 mM L-glutamine, 100 IU/ml penicillin-streptomycin, 37°C, 5% CO_2) were exposed (2 h) to 2% sera and then 0-24 h to 5 mM D-glucose, 5 mM D-glucose+TGF-β1 (2 ng/ml), 25 mM D-glucose, or 25 mM D-glucose+TGF-β1. L-[^{3}H]Arginine transport (15-1000 μM, 2 μCi/ml, 37°C, 1 min) was determined. Human Cationic Amino acid Transporter 1 (hCAT-1) mRNA was quantified by real time RT-PCR (28S rRNA was internal reference). Latent and active TGF-β1 levels were measured by ELISA. Phosphorylated and total Smad2 and p42/44^{mapk} protein levels were determined by Western blot. A replication-defective adenoviral vector expressing a truncated human TβRII receptor (Ad-TTβRII) was prepared (Yamamoto *et al.* 1996) and HUVEC were transfected (2% serum, 12 h).

Maximal velocity (V_{max}) of L-arginine transport (V_{max} 6.9±0.1 pmol/μg protein/min, K_m 113±5 μM, mean±SEM, n=23) is increased (P<0.05, unpaired Student's t test) by 25 mM D-glucose (V_{max} 17±1 pmol/μg protein/min, K_m 140±24 μM) or TGF-β1 (V_{max} 19±1 pmol/μg protein/min, K_m 123±25 μM). D-Glucose stimulation of L-arginine transport was unaltered (P>0.05) by TGF-β1 (V_{max} 15±0.8 pmol/μg protein/min, K_m 147±23 μM). L-Arginine (100 μM) transport was unaltered (P>0.05) in cells overexpressing TTβRII (Control 2.8±0.3, 25 mM D-glucose 4.2±0.6, TGF-β1 4.5±0.4 pmol/μg protein/min). hCAT-1 mRNA expression (3.1 x10^4 mRNA copies) was increased by high D-glucose (6.7-fold) and TGF-β1 (4.6-fold) in not transfected cells. Smad2 and p42/44^{mapk} phosphorylation were increased by high D-glucose (3.8-fold) and TGF-β1 (4.1-fold); however, total protein was unaltered. Smad2 and p42/44^{mapk} phosphorylation was blocked by PD-98059 and was negligible in cells overexpressing TTβRII. Latent (0.51±0.06 ng/ml/10^6 cells) and active (0.49±0.07 ng/ml/10^6 cells) TGF-β1 in 25 mM D-glucose were higher than control (latent 0.28±0.04, active 0.21±0.03 ng/ml/10^6 cells). Thus, high D-glucose–stimulated L-arginine transport could result from a mechanism involving TβRII stimulation by TGF-β1, involving activation of p42/44^{mapk} and Smad2.

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PC6

Compound mutations can dictate disease mechanism in congenital hyperinsulinism

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ATP-sensitive potassium channels (K_{ATP}) are present in the plasma membrane of a number of tissues and form a link between cellular metabolism and membrane excitability. This is particularly pertinent in the pancreatic β-cell where K_{ATP} channels play a major role in regulating insulin release (Ashcroft et al. 1984; Ashcroft & Ashcroft, 1990; Seino & Miki, 2003, 2004). SUR1 and Kir6.2 constitute the molecular counterparts of the channel present in pancreatic β-cells. Mutations in SUR1 and Kir6.2 occur in a hereditary disease, congenital hyperinsulinism (CHI). CHI is characterised by inappropriately high levels of insulin release and hypoglycaemia in children at birth often with disabling long-term neurological consequences. CHI is relatively rare and it is not clear how common are compound mutations and what their role is in disease pathogenesis.

We have studied two families with compound mutations in the sulphonylurea receptor (SUR1) with congenital hyperinsulinism. The first patient had diffuse disease and was homozygous for two mutations in SUR1, namely D1193V and R1436Q. In a second family, the patient had focal disease and was compound homozygous for two mutations from the father (G228D and D1471N) and one from his mother (V1572I). Mutations were introduced into hamster SUR1 with site-directed mutagenesis

using the QuickChange kit (Stratagene). Co-expression of mutants with Kir6.2-GFP or Kir6.2 was carried out in HEK293 cells and live cells were imaged using a Biorad Radiance 2100 laser scanning confocal Nikon TE300 microscope (Biorad, UK). $^{86}Rb^+$ was used as a congener for K^+ transport through the ATP-sensitive K channels in transiently transfected cells treated with or without channel stimulants and/or inhibitors. In addition, cells were studied with whole-cell patch clamp electrophysiology, under a physiological K^+ gradient with 0.85 mM ATP in the pipette solution.

Channel complexes containing the D1193V mutant were delivered to the plasma membrane and were functional and those containing R1436Q were also present at the plasma membrane but were non-funtional. Combining the two mutations (SUR1D1193V/R1436Q) led to intracellular retention of the channel complex. For SUR1D1193V, stimulating efflux using 100μM diazoxide or 2.5mM NaCN and 20mM 2-deoxy-D-glucose increased efflux 2-fold when compared to control ($P < 0.001$, one-way ANOVA with Bonferroni's post hoc test); 10μM glibenclamide inhibited stimulation and fluxes were 0.6- and 0.7-fold lower than control, respectively (see Table 1). SUR1 G228D and D1471N singly or in combination lead to intracellular retention of the channel complex and loss of function (except for D1471N which showed activity on patch clamp). In contrast V1572 was trafficked appropriately and was functional. In addition, the expression of SUR1V1572I with SUR1 SUR1G228D/D1471N and Kir6.2 led to a recovery of function as indicated by substantial Rb^+ fluxes. Our *in vitro* data are consistent with a mechanism in which the maternally inherited copy of SUR1 is inactivated.

Table 1. $^{86}Rb^+$ efflux from cells co-transfected with Kir6.2 and the indicated SUR1 mutants

	1	2	3	4	5
D1193V	10.77±0.36	21.11±2.16	6.59±0.37	23.17±1.99	7.67±0.38
D1193V/R1436Q	9.97±0.69	11.08±1.57	8.46±0.81	10.90±1.10	9.30±0.89
R1436Q	5.45±0.11	4.92±0.19	4.13±0.18	5.79±0.25	5.09±0.24
G228D	8.81±0.35	7.98±0.69	6.43±0.53	9.13±0.47	7.99±0.49
G228D/D1471N	9.52±0.78	9.30±0.97	8.84±1.06	9.89±0.94	8.95±1.01
D1471N	8.18±0.52	10.15±0.28	8.28±0.74	9.46±0.36	7.54±0.77
V1571I	11.39±0.80	35.90±2.94	7.55±0.22	34.50±2.82	7.68±0.30
V1571N+G228D/D1471N	11.04±0.06	25.23±0.29	6.38±0.19	23.56±0.52	6.94±0.15

Conditions: 1, control; 2, 100μM diazoxide; 3, 100μM diazoxide + 10μM glibenclamide; 4, 2.5mM NaCN + 20mM 2-deoxy-D-glucose; and 5, 2.5mM NaCN +2 0mM 2-deoxy-D-glucose + 10μM glibenclamide. $^{86}Rb^+$ efflux was determined in efflux media (composition (mM): 130 NaCl, 10 glucose, 10 Hepes, 7 KCl, 2 $CaCl_2$ and 1 $MgCl_2$) with or without stimulants and/or inhibitors. Data are given as means±S.E.M. for 3 experiments.

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