

The P2X7 receptor is a key modulator of aerobic glycolysis

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Ability to adapt to conditions of limited nutrient supply requires a reorganization of the metabolic pathways to balance energy generation and production of biosynthetic intermediates. Several fast-growing cells overexpress the P2X7 receptor (P2X7R) for extracellular ATP. A feature of this receptor is to allow growth in the absence of serum. We show here that transfection of P2X7R allows proliferation of P2X7R-transfected HEK293 (HEK293-P2X7) cells not only in the absence of serum but also in low (4 mM) glucose, and increases lactate output compared with mock-transfected HEK293 (HEK293-mock) cells. In HEK293-P2X7, lactate output is further stimulated upon addition of exogenous ATP or the mitochondrial uncoupler carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP). In the human neuroblastoma cell line ACN, lactate output is also dependent on P2X7R function. P2X7R-expressing cells upregulate (a) the glucose transporter Glut1, (b) the glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase (G3PDH), (c) phosphofructokinase (PFK), (d) pyruvate kinase M2 (PKM2) and (e) pyruvate dehydrogenase kinase 1 (PDHK1); furthermore, P2X7R expression (a) inhibits pyruvate dehydrogenase (PDH) activity, (b) increases phosphorylated Akt/PKB and hypoxia-inducible factor 1 α (HIF-1 α) expression and (c) enhances intracellular glycogen stores. In HEK293-P2X7 cells, glucose deprivation increases lactate production, expression of glycolytic enzymes and p-Akt/PKB level. These data show that the P2X7R has an intrinsic ability to reprogram cell metabolism to meet the needs imposed by adverse environmental conditions.

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Adaptation to limited nutrient supply is a key survival factor for normal and transformed cells.¹ This phenomenon involves complex biochemical and metabolic changes that affect ATP synthesis and production of intermediates needed for the synthesis of basic cell constituents (lipids and nucleic acid). A key aspect of cell adaptation to unfavorable ambient conditions is the modulation of energy metabolism. The most important substrate used for generation of ATP or building blocks for nucleic-acid, amino-acid or lipid synthesis is glucose. Glucose deprivation triggers a complex adaptation response referred to as unfolded protein response (UPR) that involves mainly, but not exclusively, the endoplasmic reticulum (ER). UPR allows a profound reshaping of cancer cell metabolism that increases survival, progression and resistance to chemotherapies.² The molecular basis of the better adaptation of tumors to low glucose is not clear, albeit several mechanisms have been invoked, such as a reprogramming of mitochondria, repression of the CC3/TIP30 gene or down-regulation of specific micro RNA (e.g. miR-451).^{3–5} Tumor adaptation to low glucose intersects with another burning aspect of cancer cell biology, the rediscovered Warburg effect, that is, the persistence of high glycolytic rates in the presence of near-physiological oxygen levels.⁶ In fact, it has

been known for over 70 years that tumors have a high glycolytic metabolism that preferentially generates lactate rather than funneling pyruvate to Krebs cycle.⁷ More recently, it has become clear that the Warburg effect is not an exclusive feature of cancer cells, as rapidly proliferating cells also may exhibit a strong glycolytic metabolism in the presence of oxygen.⁸ Aerobic glycolysis involves, and might be modulated by, changes in the expression of several enzymes of the glycolytic pathway, for example, hexokinase, phosphofructokinase (PFK), glyceraldehyde 3-phosphate dehydrogenase (G3PDH), pyruvate kinase M2 (PKM2), as well as enzymes specifically controlling pyruvate entry into the Krebs cycle, such as pyruvate dehydrogenase kinase1 (PDHK1).⁹ If and how these enzymes are also modulated under conditions of limited glucose availability is not known.

In the past, we have investigated the metabolic effects of the P2X7 receptor (P2X7R), a member of the P2 receptor family. P2X7R is an ATP-gated plasma-membrane ion channel that traditionally has been associated to cytotoxicity,¹⁰ however, growing evidence now suggest that its tonic, as opposed to sustained, activity exerts a strong trophic effect.^{11–13} We and others have shown that P2X7R expression is necessary for proliferation of T and B lymphocytes and

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Abbreviations: ER, endoplasmic reticulum; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; HEK293-mock, mock-transfected HEK293; HEK293-P2X7, P2X7R-transfected HEK293; HIF-1 α , hypoxia-inducible factor 1 α ; NFATc1, nuclear factor of activated T-cell cytoplasmic 1; P2X7R, P2X7 receptor; PAS, periodic acid-Schiff; PFK, phosphofructokinase; PDH, pyruvate dehydrogenase; PDHK1, pyruvate dehydrogenase kinase 1; PKM2, pyruvate kinase M2; TMRM, tetramethyl rhodamine methyl ester; UPR, unfolded protein response

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microglial cells.^{14–17} Furthermore, P2X7R is also involved in T-cell differentiation and Ag-specific dendritic cell activation.^{18–20} We have extensively investigated the growth-promoting activity of P2X7R, both in cells expressing the native receptor and in heterologous expression cellular models, for example, HEK293 or Hela cells.¹² HEK293 cells are a good model to investigate P2X7R function as they on one hand have no endogenous P2X7R and on the other can be easily transfected.

Upon P2X7R transfection, HEK293 cells acquire the ability to grow in the absence of serum. This growth advantage depends on a strong increase in the efficiency of mitochondrial oxidative phosphorylation, an increased cellular ATP content, an improved ER Ca^{2+} handling and an increased activation of the transcription factor 'nuclear factor of activated T-cell cytoplasmic 1' (NFATc1).^{12,21} P2X7R-transfected cells have also an increased motility and a better ability to infiltrate soft agar and undergo metastatic dissemination *in vivo*.^{21,22}

In the present study, we report that P2X7R expression allows proliferation of HEK293 cells during the combined serum and glucose deprivation. P2X7R-transfected HEK293 (HEK293-P2X7) cells have a higher lactate output, over-express several of the key glycolytic enzymes and the ubiquitous glucose transporter Glut1, have larger glycogen depots and show an increased level of phosphorylated Akt/PKB (ph-Akt/PKB) and hypoxia-inducible factor 1 α (HIF-1 α). In low glucose, expression of glycolytic enzymes is strongly upregulated in HEK293-P2X7, much less in wild-type or mock-transfected HEK293 (HEK293-mock) cells. These results show that P2X7R expression allows better adaptability to unfavorable ambient conditions via upregulation of glycolytic enzymes and by increasing intracellular glycogen stores, and may help to better understand cancer cell energy metabolism and tumor progression and dissemination.

Results

We have previously shown that under conditions of limited nutrient supply (i.e., serum starvation), expression of the P2X7R confers a strong growth advantage to various human cell lines.^{11,12} The trophic effect of P2X7R is at least partially mediated by an increased efficiency of Ca^{2+} handling by the ER, an improved oxidative phosphorylation and a strong activation of the transcription factor NFATc1.^{12,21} This suggests that P2X7R increases cell adaptation to unfavorable environmental conditions. Glucose starvation, an additional condition of reduced nutrient supply often occurring in several physiological or pathological states, triggers a complex array of adaptive responses involving a thorough reprogramming of the metabolic pathways, and often culminating in the UPR.²³ Figure 1a shows that growth of HEK293-P2X7 cells proceeded unimpeded in serum-free, low-glucose medium, whereas on the contrary, proliferation of HEK293-mock cells was fully abrogated. The mechanistic basis of the growth-promoting effect of the P2X7R are not fully understood, however, we have shown that an improved energy metabolism is likely to have an important role, as P2X7R expression increases mitochondrial potential and improves mitochondrial ATP synthesis.¹² Figure 1b shows

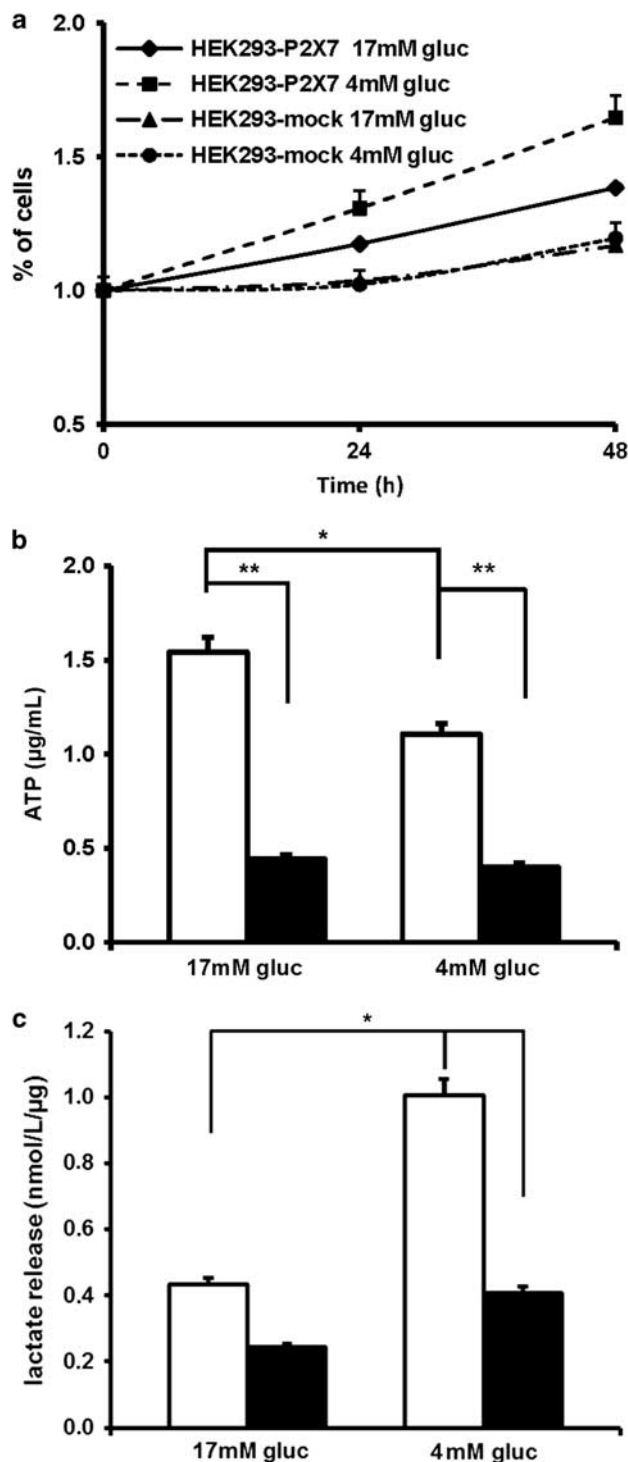


Figure 1 P2X7R expression stimulates cell metabolism. HEK293-P2X7 (open bars) and HEK293-mock cells (closed bars) were incubated in 17 or 4 mM glucose-supplemented medium, and cell proliferation (a), intracellular ATP content (b) and lactate output (c) were measured as described in Materials and Methods. Intracellular ATP and lactate output were assessed after a 24-h incubation under the different experimental conditions. Data are average \pm S.D. of multiple determinations ($n=9$ for ATP measurements; $n=12$ for lactate measurements). Statistical significance, * $P < 0.05$ and ** $P < 0.03$

that an improved energy metabolism is a feature of HEK293-P2X7 cells in low glucose, as total cellular ATP content under this condition of extreme nutrient deprivation is almost two-fold higher than in HEK293-mock cells. However, low glucose affected ATP synthesis of P2X7R-expressing cells to a larger extent than that of P2X7R-lacking cells, as high/low glucose ATP ratio was 1.45 in HEK293-P2X7 and 1.1 in HEK293-mock. This finding was surprising because, due to a more efficient mitochondrial metabolism of HEK293-P2X7 compared with HEK293-mock,¹² we anticipated that ATP synthesis in HEK293-P2X7 cells should be less sensitive to glucose deprivation. In order to assess the relative contributions of glycolysis and oxidative phosphorylation to ATP synthesis in the presence or absence of P2X7R, we measured lactate generation in high and low glucose. As shown in Figure 1c, in physiological glucose, HEK293-P2X7 cells released about twice as much lactate as HEK293-mock cells. Lowering glucose medium strongly increased lactate output in both HEK293-P2X7 (from 0.42 to 0.98 nmoles/l per μg protein) and HEK293-mock (from 0.24 to 0.42 nmoles/l per μg protein). Increase in lactate output caused by incubation in low glucose was larger in HEK293-P2X7 than in HEK293-mock as the low/high glucose lactate output ratio was 2.33 and 1.75 for HEK293-P2X7 and HEK293-mock, respectively. This experiment gives two relevant pieces of information: (a) basal P2X7R expression *per se* increases lactate output and (b) HEK293-P2X7 cells produce more lactate in response to nutrient starvation.

Mitochondrial uncoupling is one of the most potent stimuli for lactate production.²⁴ Pharmacological stimulation of P2X7R by exogenous ATP causes Ca^{2+} overload of mitochondria, mitochondrial potential collapse and uncoupling.¹² Thus, we tested the effect of stimulation with extracellular ATP on lactate output. Figure 2 shows that ATP is a powerful stimulus for lactate production, whether in physiological or low glucose, but only in HEK293-P2X7 cells,

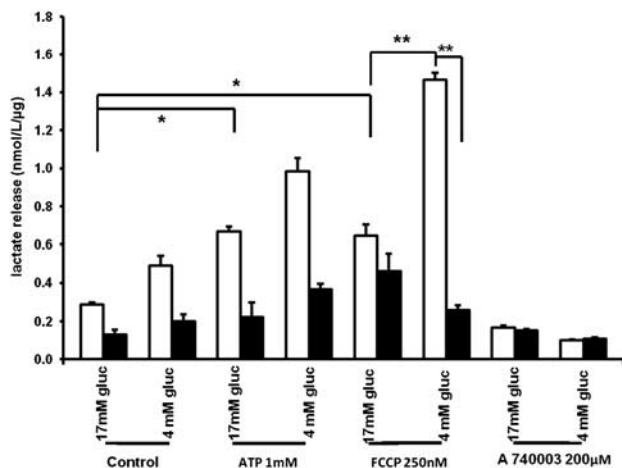


Figure 2 P2X7R expression is needed for ATP or FCCP-stimulated lactate output. HEK293-P2X7 (open bars) and HEK293-mock cells (closed bars) were incubated in 17 or 4 mM glucose-supplemented medium for 24 h in the absence or presence of ATP, FCCP or the P2X7R blocker A740003. Data are average \pm S.D. of multiple determinations ($n=3$). Statistical significance, * $P < 0.05$ and ** $P < 0.03$

as expected. To validate the effect of exogenous ATP, we tested the mitochondrial uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), which triggered a large lactate production, in physiological as well as low glucose. Very interestingly, stimulation of lactate production by FCCP was dependent on the presence of P2X7R. Finally, lactate production was inhibited by the selective P2X7R blocker A740003 in HEK293-P2X7, but not in HEK293-mock, cells. Experiments described so far were performed by expressing P2X7R in cells that do not normally express this receptor. To check if the native P2X7R has a similar effect on lactate output, we turned to the human neuroblastoma ACN, a cell line previously characterized for P2X7R expression in our laboratory.²⁵ As shown in Figure 3, lactate production by ACN cells was stimulated by exogenous ATP and abrogated by the selective P2X7R blocker A740003. In 4 mM glucose, stimulation of lactate release by ATP was minor and nonstatistically significant, very likely because under these conditions lactate production was already near maximally stimulated. These results suggest that P2X7R is able to modulate glycolytic metabolism under different conditions: (a) basal conditions, (b) upon stimulation with extracellular ATP and (c) in the presence of uncoupled mitochondria. We then set to identify the mechanisms involved.

A key enzyme controlling glycolysis is G3PDH, which catalyzes conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate. The *G3PDH* gene is upregulated following stimulation of glycolytic metabolism, especially in cancer cells.²⁶ Figure 4a shows that G3PDH levels are over three-fold higher in HEK293-P2X7 *versus* HEK293-mock cells in high glucose. In low glucose, G3PDH expression increases further in both cell types, but to a much larger extent in HEK293-P2X7 than in HEK293-mock cells. Three other key checkpoint enzymes in glycolysis, often upregulated in cancer cells, are PFK, PKM2 and PDHK1.⁸ Figure 4b shows that expression of PFK is higher in HEK293-P2X7 than in HEK293-mock cells, albeit level of expression is not significantly changed by glucose depletion. Figure 5A shows that

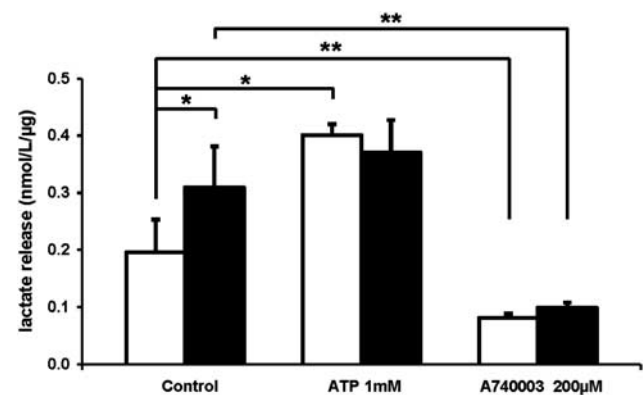


Figure 3 Lactate release is dependent upon P2X7R stimulation in the human neuroblastoma ACN cell line. 25×10^3 ACN cells per well were plated in 11 mM (open bar) or 4 mM (closed bar) glucose medium and lactate release was measured after a 24-h incubation in the absence or presence of either ATP or the selective P2X7R blocker A740003. Data are average \pm S.D. of multiple determinations ($n=3$). Statistical significance, * $P < 0.05$ and ** $P < 0.03$

in low glucose, level of expression of PKM2 is enhanced in HEK293-P2X7 compared with HEK293-mock cells. Under these conditions, PDHK1 is also overexpressed in HEK293-P2X7 cells (Figure 5B). PKM2 generates pyruvate from phosphoenol pyruvate and preferentially poises pyruvate for degradation to lactate, minimizing its transfer to the mitochondria. PDHK1 phosphorylates and thus inactivates pyruvate dehydrogenase (PDH), the key enzyme controlling pyruvate entry into Krebs cycle. This was confirmed by measuring PDH

activity, which was lower in HEK293-P2X7 compared with HEK293-mock cells (Figure 5C). However, contrary to our anticipation, we found that glucose deprivation enhanced PDH activity in both HEK293-P2X7 and HEK293-mock cells.

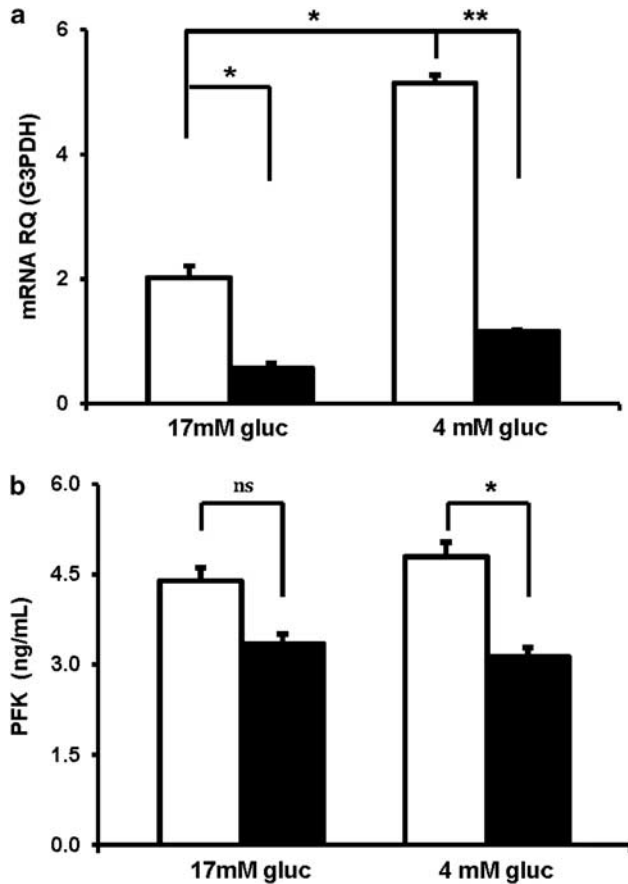
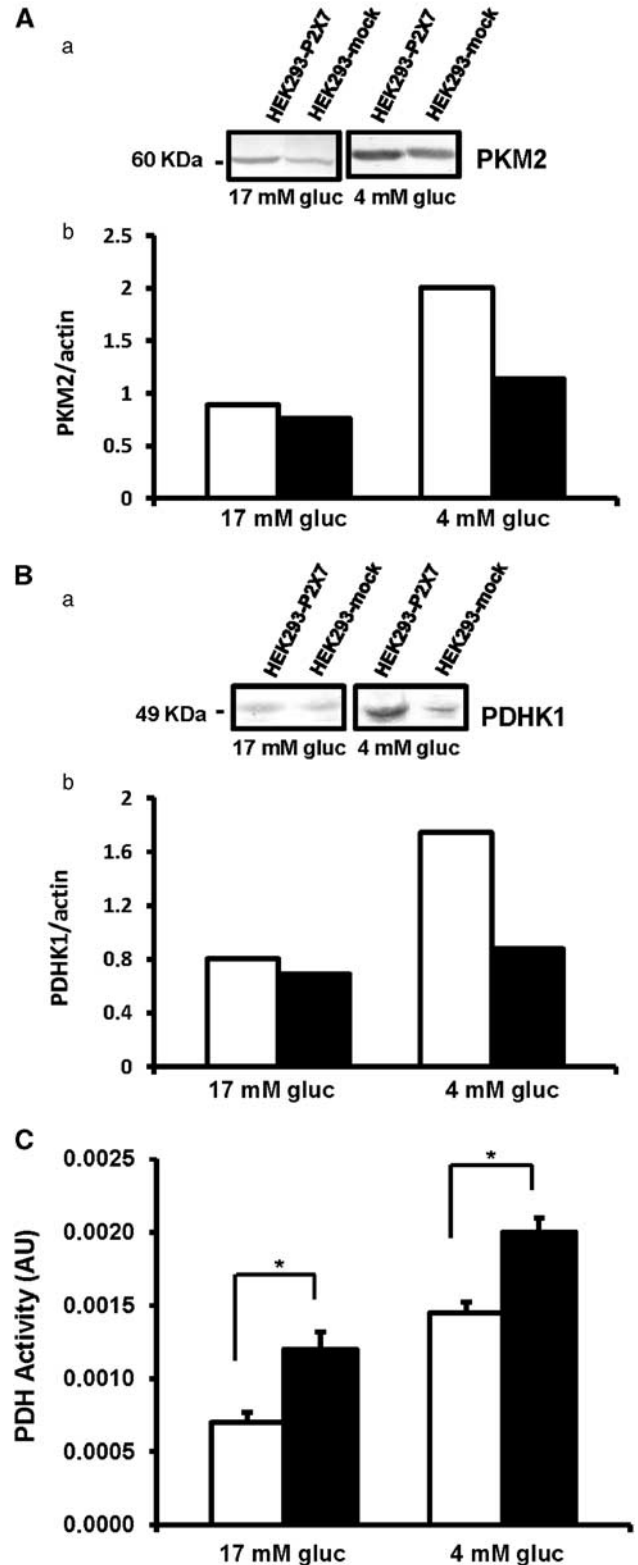


Figure 4 P2X7R expression causes overexpression of the key glycolytic enzymes G3PDH and PFK. HEK293-P2X7 (open bars) and HEK293-mock cells (closed bars) were incubated in 17 or 4 mM glucose-supplemented medium, and G3PDH (a) and PFK (b) expression was measured by quantitative PCR and ELISA, respectively. G3PDH mRNA content is expressed as a RQ ratio normalized onto P2Y1 mRNA. G3PDH and PFK determinations are average \pm S.D. of three separate experiments, each performed in duplicate. Statistical significance, * $P < 0.05$; ** $P < 0.03$; and n.s., not significant

Figure 5 P2X7R expression causes overexpression of PKM2 and PDHK1 and inhibition of PDH activity. HEK293-P2X7 (open bars) and HEK293-mock cells (closed bars) were incubated in 17 or 4 mM glucose-supplemented medium, and PKM2 (A) and PDHK1 (B) expression was measured by western blot. Protein bands (a) in panels (A and B) were quantified by densitometry (b) and normalized onto endogenous actin band. PKM2 and PDHK1 determinations are from one experiment representative of four similar. PDH (C) activity was measured as described in Materials and methods and expressed in arbitrary units. Data are averages \pm S.D. of multiple determinations ($n=3$). Statistical significance, * $P < 0.05$



The net effect of PKM2 and PDHK upregulation will thus be an inhibition of mitochondrial functions and a preferential diversion of pyruvate to lactate. To verify this hypothesis, we performed a semiquantitative *in vitro* assay of mitochondrial function using two selective fluorescent mitochondrial probes: Mito Tracker green and tetramethyl rhodamine methyl ester (TMRM). Mito Tracker green accumulates into the mitochondria in a potential-insensitive fashion, thus allowing a precise morphological identification of these organelles independently of their energy charge. On the contrary, TMRM is a potential-sensitive dye that localizes to the mitochondria in a

mitochondrial potential-sensitive fashion, and thus allows to measure the energy state of mitochondria.

Figures 6A and B, shows that, in agreement with our previous findings,¹² HEK293-P2X7 cells show a larger TMRM uptake, to witness higher mitochondrial potential, compared with HEK293-mock. In low glucose, mitochondrial potential of HEK293-P2X7 dropped to about the same level as that of HEK293-mock, indicating that glucose deprivation inhibited mitochondrial function. Mito Tracker green staining shows that mitochondria of HEK293-P2X7 cells were thicker and swollen in physiological glucose, while those of HEK293-mock

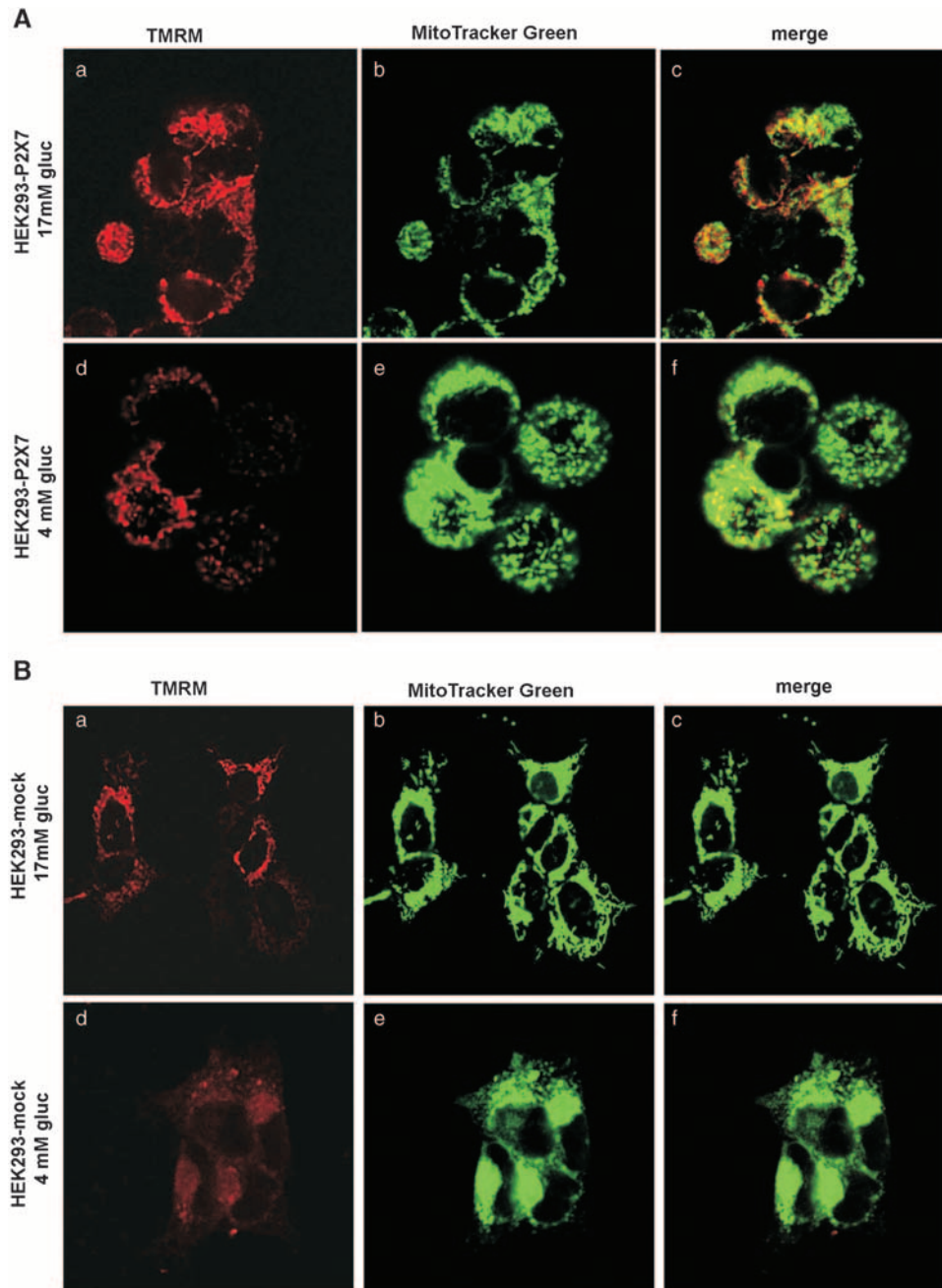


Figure 6 Glucose depletion causes a decrease in mitochondrial potential. HEK293-P2X7 (A) and HEK293-mock (B) cells were incubated under the different conditions for 24 h, and at the end of this incubation were labeled with TMRM (panels a and d) and Mito Tracker Green (panels b and e). Image acquisition and analysis were performed as described in Materials and Methods. Panels (c and f) show merge of the red and green channels

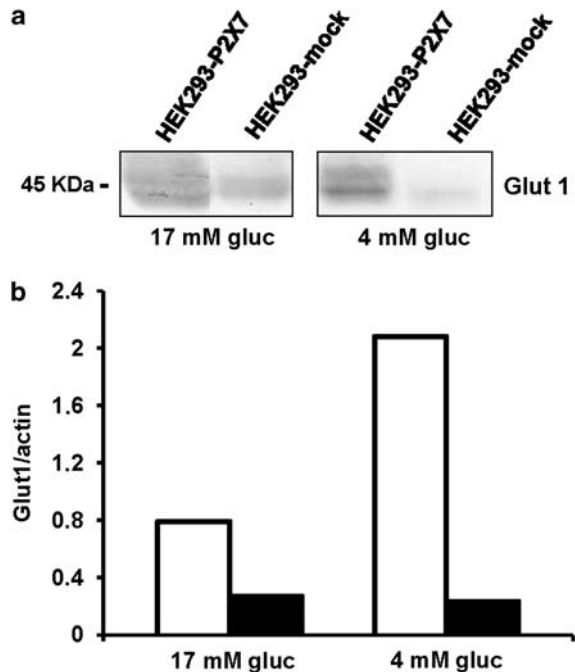


Figure 7 Glucose depletion causes upregulation of Glut1 in HEK293-P2X7, but not in HEK293-mock, cells. Samples were processed as described in Materials and Methods and protein bands (a) were quantified by densitometry (b) and normalized onto endogenous actin band. HEK293-P2X7, open bars and HEK293-mock, closed bars. One experiment representative of four similar is shown

were organized in the typical ramified network. In low glucose, alongside with the collapse of mitochondrial potential, we also observed fragmentation of the filamentous mitochondrial network in both cell types, an indication of mitochondrial injury. These data indicating an inhibition of mitochondrial energy metabolism in low glucose suggest that HEK293 cells should increase their ability to use glucose under nutrient deprivation, and thus improve glucose uptake.

The main membrane carrier responsible for non-insulin-dependent glucose uptake is Glut1. Figure 7 shows that HEK293-P2X7 cells had a higher level of expression of Glut1 both in physiological and low glucose. This suggests that HEK293-P2X7 cells may adapt to low nutrient conditions by upregulating expression of glucose carrier, thus maximizing nutrient uptake. However, it has been recently reported that glucose deprivation also increases use of intracellular glycogen stores in tumor cells.²⁷ Therefore, we checked whether HEK293 cells might also compensate reduced glucose supply by degrading intracellular glycogen. As shown in Figure 8, glycogen stores were higher in HEK293-P2X7 than in HEK293-mock cells. Incubation in low-glucose medium caused a fast depletion of glycogen stores, thus presumably providing glucose for glycolysis and lactate generation. Therefore, P2X7R expression allows cells to exploit multiple sources of glucose, exogenous and endogenous, to support glycolytic metabolism.

Over the last few years, it has become clear that serine/threonine kinase Akt/PKB and the transcription factor HIF-1 α

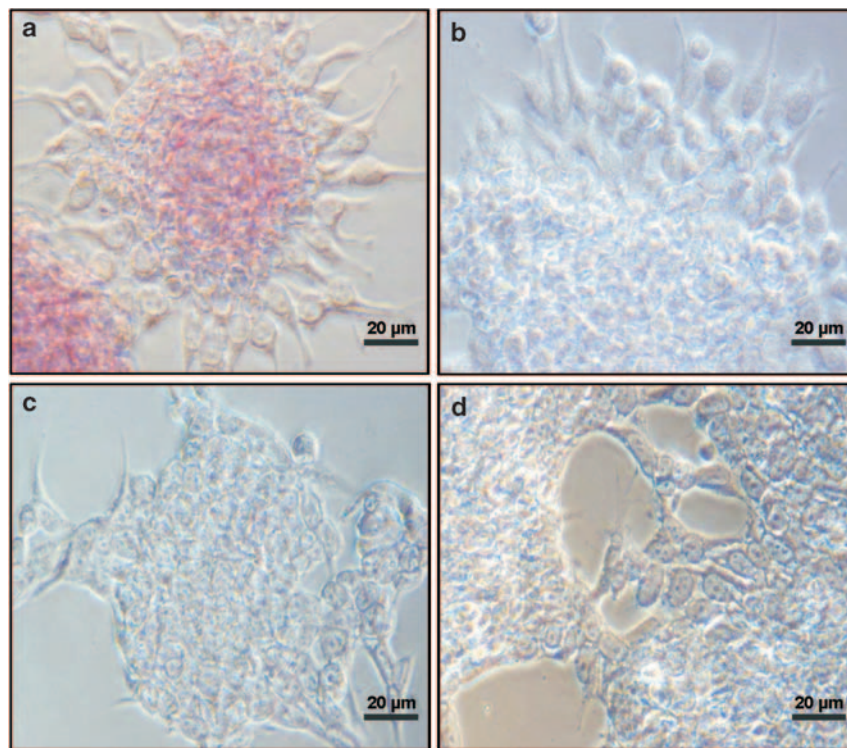


Figure 8 HEK293-P2X7 cells possess larger glycogen stores than HEK293-mock cells. HEK293-P2X7 (a and b) and HEK293-mock (c and d) were plated in 24 well per plates and incubated under the different experimental conditions as described in Materials and Methods. After 24 h, they were PAS-stained and analyzed by phase contrast microscopy with a $\times 40$ objective. Glycogen deposits are revealed by the pink stain. For each experimental condition, three wells per plate were analyzed

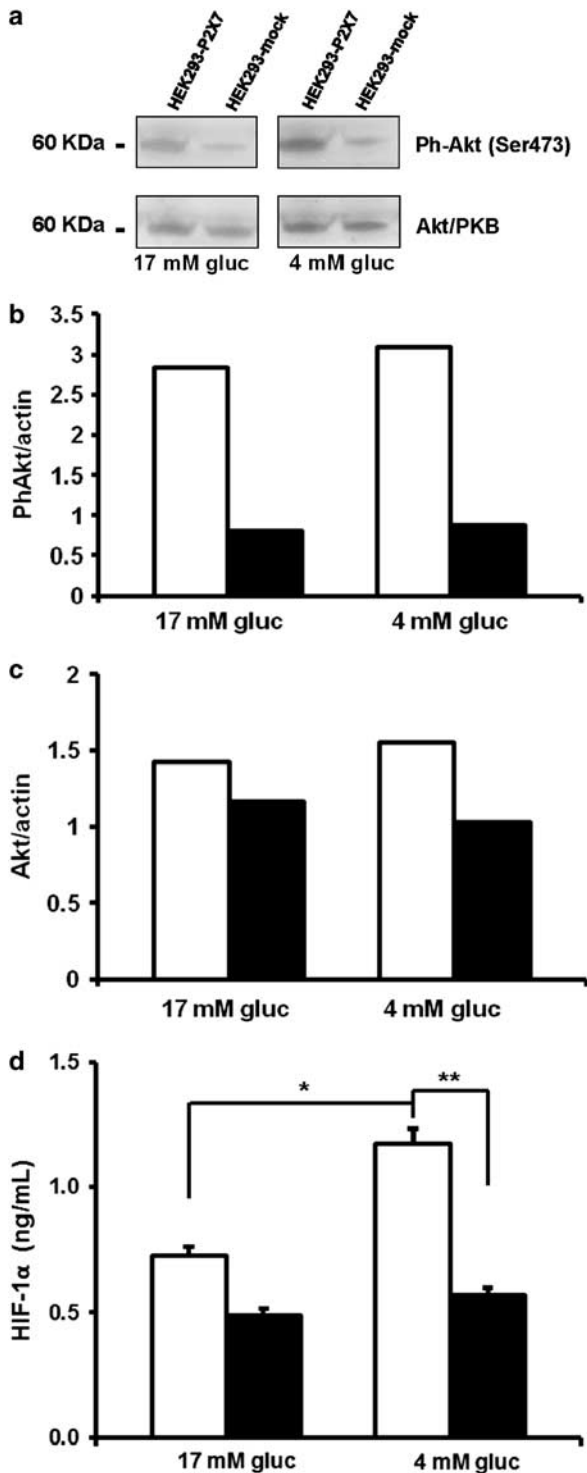


Figure 9 P2X7R expression increases Akt/PKB phosphorylation and HIF-1 α expression. HEK293-P2X7 (open bars) and HEK293-mock cells (closed bars) were incubated for 24 h under the different experimental conditions and processed as detailed in Materials and Methods. Protein bands (a) were quantified by densitometry (b and c) and normalized onto endogenous actin band. One experiment representative of six similar is shown. HIF-1 α expression (d) was determined by ELISA as described in Materials and Methods. Data are average \pm S.D. of multiple determinations ($n = 3$). Statistical significance, * $P < 0.05$ and ** $P < 0.03$

are crucial factors controlling glucose uptake, glycolysis, lactate production and cell proliferation.^{28–30} Akt/PKB is potentially relevant in the context of purinergic modulation of energy metabolism because recent reports show that P2X7R modulates its activity,^{31,32} whereas no information is available on possible P2X7R-HIF-1 α interactions. Figures 9a–c show that Akt/PKB is expressed to about the same level in both HEK293-P2X7 and HEK293-mock cells, and that, more importantly, while in HEK293-mock cells Akt/PKB is dephosphorylated in the key Ser473 residue, and thus inactive, in HEK293-P2X7 cells Akt/PKB is phosphorylated. Interestingly, HIF-1 α was expressed to a higher level in HEK293-P2X7 than in HEK293-mock cells, and reduced glucose enhanced its expression in the former but not in the latter (panel d).

Discussion

Extracellular environment is a key factor determining cell growth and differentiation. It is a fact that extracellular matrix and tissue biochemical milieu profoundly affect proliferation, motility, cell differentiation and death.³³ Cells that are better equipped to exploit the extracellular nutrient supply gain an undisputable growth advantage over other cells. Cancer cells grow in the presence of much lower serum concentrations, as well as in the presence of lower glucose levels, than normal cells.¹ These adaptations are probably the consequence of the selective pressure imposed by the fluctuating levels of nutrients within solid tumors and it is clear that they will provide a strong selective advantage over normal cells. The ability of cancer (but in general of all fast-growing) cells to conserve a strong glycolytic activity during normoxia and in the presence of often fully functional mitochondria (the ‘Warburg effect’) is probably the result of the pressure placed on cancer cells to maximize the use of all possible sources for energy metabolism and structural synthetic processes.²⁴

P2X7R is mainly expressed by inflammatory and immune cells.³⁴ However, more recent data show that it is also upregulated in several cancers, such as acute and chronic lymphocytic leukemia, acute myeloid leukemia, prostate carcinoma, breast cancer, neuroblastoma and thyroid papillary carcinoma.^{15,25,35–37} The biological meaning of P2X7R upregulation in tumors is unknown, but it has led to the operational proposal to use pharmacological P2X7R agonists to kill cancer cells, as it is well known that this receptor is a potent inducer of cell death when overstimulated. A few years ago, we showed that in human leukemic lymphocytes and human neuroblastoma cells the P2X7R is uncoupled from apoptosis, and rather sustains growth.^{15,25} This observation was in line with the results of experiments in which P2X7R was heterologously expressed in lymphoblastoid or HEK293 cells. To our surprise, these experiments showed that basal expression of this receptor, far from being detrimental, produced a strong trophic activity.^{12,38} We investigated the biochemical basis of this trophic effect and discovered that it was at least in part due to an enhanced mitochondrial energy state, a better handling of ER Ca²⁺, and a sustained activation of the transcription factor NFATc1.^{12,21} Better mitochondrial performance was due to a tightly controlled increase in matrix mitochondrial Ca²⁺, which in

turn increased NADH dehydrogenase activity and the overall efficiency of the respiratory chain.

In addition to a more efficient oxidative phosphorylation, HEK293-P2X7 cells also have a more active glycolytic metabolism. The surprising finding is that lactate output is substantially increased in low glucose, both in HEK293-mock and HEK293-P2X7 cells, but to a higher extent in these latter cells. Furthermore, in low glucose, HEK293-P2X7 have a better growth performance. This suggests that under conditions of glucose deprivation, cells that express P2X7R preferentially activate aerobic glycolysis and succeed to obtain from this metabolic pathway all the necessary energy intermediates and building blocks to sustain proliferation. This switch from oxidative phosphorylation to glycolysis is likely due to the upregulation in low glucose of two key enzymes: PKM2 and PDHK1. PKM2 is one of the four PK isoforms present in mammalian cells, which catalyze the dephosphorylation of phosphoenol pyruvate, with the net production of one molecule of ATP. PKM2 has become a hot focus of investigation in oncology when it was discovered not only that tumors overexpress this isoform but also that this enzyme is the substrate of several oncogenic kinases, such as BCR-ABL, FGFR-1 and pp60v-src.³⁹

As it is well known, cancer cells convert large amounts of glucose into lactate even in the presence of oxygen. This phenomenon bears the name of its discoverer, Otto Warburg, and is thus referred to as the Warburg effect.⁶ Over the years, details of energy production by tumors have been extensively investigated and clarified, unveiling that 'aerobic glycolysis' by no means implies that oxidative phosphorylation is shutoff in cancer cells, but rather that these cells may have a very active aerobic conversion of pyruvate to lactate, alongside with a normal oxidative phosphorylation. However, dissection of the metabolic pathways involved in the 'Warburg effect' has not been paralleled by an equally advanced understanding of the biological significance of this phenomenon. To this aim, some hints may be offered by the typical upregulation of PKM2 occurring in cancer cells. In fact, this isoform in tumors is preferentially expressed as a dimer rather than a tetramer. Dimeric PKM2 has lower affinity for enolpyruvate than the tetramer. This might appear paradoxical, considering that cancer cells have a higher lactate output, but it is not if we think that by slowing down glucose degradation to lactate PKM2 allows the upstream building-up of metabolic intermediates (for example, glucose 6-P, glyceraldehyde 3-P and glycerate 3-P) to be used for synthetic purposes, a strong requirement of actively proliferating cells.⁸ The mechanism of the preferential degradation of pyruvate to lactate is unknown, but it might have to do with the ability of PKM2 to associate with other glycolytic enzymes, such as hexokinase, G3PDH, phosphoglyceromutase or enolase.³⁹

The other relevant metabolic feature of P2X7R-expressing cells is PFK and PDHK1 overexpression. PFK is a key regulatory enzyme in the glycolytic pathway, often overexpressed in cancer, that catalyzes phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate. PDHK1 catalyzes phosphorylation, and thus inactivation of PDH, the key enzyme for the entry of pyruvate into the citric acid cycle. PDHK1 is also overexpressed in cancer cells, and it is thought that this is another important factor that allows the preferential

degradation of pyruvate to lactate rather than its conversion to acetyl-CoA. In keeping with PDHK1 overexpression, we found that PDH activity is depressed in HEK293-P2X7 cells, although, contrary to our anticipation, it is decreased rather than increased by glucose deprivation. HEK293-P2X7 cells also have high expression of the ubiquitous glucose transporter Glut1 and G3PDH. Both Glut1 and G3PDH are overexpressed in highly glycolytic cells. Alongside with overexpression of Glut1, larger glycogen stores provide HEK293-P2X7 cells a large fuel supply for glycolysis.

It is well known that the two mastermind factors that controls expression of genes involved in cellular energy metabolism and growth are Akt/PKB and HIF-1 α .^{40,41} Akt/PKB kinase is overexpressed in tumors, is activated by plasma-membrane receptors for growth factors, supports aerobic glycolysis, modulates mitochondrial metabolism and is also known to be switched on by P2X7R. We found that the mere expression of P2X7R is sufficient to drive Akt/PKB activation, which is higher in low glucose. The mechanistic details of P2X7R–Akt/PKB communication have yet to be unveiled, but it is clear that this is an additional feature that corroborates the strong growth-promoting effect of P2X7R. Recently, it has been shown that HIF-1 α silencing downmodulates P2X7R expression in cancer cells,⁴² but it is not clear whether P2X7R feeds back on HIF-1 α . Our data show that cells overexpressing P2X7R upmodulate HIF-1 α and that glucose depletion enhances this effect.

Altogether, these data show that expression of the P2X7R induces the expression of several metabolic features, typical of cells adapted to grow under limited nutrient supply, which are also typical of cancer cells. These metabolic characteristics are in line with another property typical of cancer cells conferred by P2X7R expression, that is, the ability to grow in the absence of serum. Furthermore, P2X7R-expressing cells also show an enhanced ER Ca²⁺ handling, an activated NFATc1 and a more efficient oxidative phosphorylation. This latter feature might seem at odd with a cancer phenotype; however, it is now well known that mitochondrial function is not necessarily hampered in cancer, but, on the contrary, cancer cells have often a very active and efficient oxidative phosphorylation. On the basis of these findings, it is tempting to equate P2X7R to an oncogene, as this receptor confers a substantial growth advantage by turning on all the most important intracellular pathways responsible for generation of energy and for the production of the building blocks needed for amino-acid, lipid and nucleic-acid synthesis. Thus, it might not be a chance finding that P2X7R is overexpressed by many cancers. In conclusion, we have highlighted a novel metabolic activity of the P2X7R that sheds new light on the functions of this atypical ATP receptor and on the regulation of cellular energy metabolism.

Materials and Methods

Cell lines and proliferation assay. HEK293 cells have been previously extensively investigated in our laboratory for P2X-receptor expression and function and found to lack endogenous expression of all P2X-receptor subtypes (see also Supplementary Figure 1).^{43–45} For the purposes of the present study, HEK293 cells were transfected to express the plasmid encoding the wild-type human P2X7R sequence or with an empty vector as previously described.⁴⁶ Briefly, cells (2×10^6) were transfected with plasmids (20–40 μ g) encoding human P2X7R, or with an empty vector, by calcium phosphate precipitation, cloned and

kept under selection in the presence of 0.2 mg/ml G418 sulfate (geneticin). P2X7R expression by the transfectants was checked by RT-PCR, western blot and stimulation with ATP or benzoyl-ATP (Supplementary Figure 1). HEK293 cells were cultured in DMEM-F12 (Sigma-Aldrich, Milan, Italy) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μ g/ml streptomycin (all from Celbio EuroClone, Milan, Italy). ACN neuroblastoma cells were maintained in RPMI (Celbio EuroClone) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μ g/ml streptomycin (all from Celbio EuroClone) and 1% non-essential amino acids (GIBCO, Life Technologies, Paisley, Scotland, UK). Cells were detached with the Cell dissociation solution (Sigma-Aldrich), plated in 75 cm² Falcon flasks (Microtec, Naples, Italy) and incubated at 37 °C in a humidified incubator in the presence of 5% CO₂. For cell-proliferation assay, 5 × 10⁴ cells per well were plated in Falcon multiwell plates and counted at 24 and 48 h.

Treatments and drugs. All experiments were performed in the absence of serum (e.g., serum-free DMEM or RPMI media). Low glucose experiments were performed in the presence of 4 mM glucose (low-glucose DMEM or RPMI, from Sigma-Aldrich or Cellbio Euroclone, respectively). Cells were plated overnight in complete medium, then this medium was replaced with serum- or glucose-free medium for the next 4 h. ATP (Roche, Milan, Italy) was used at a concentration of 1 mM, FCCP (Sigma-Aldrich) was used at a concentration of 250 nM, the P2X7R inhibitor A740003 (Tocris Bioscience, Ellisville, MS, USA) was used at a concentration of 200 μ M. Mitochondria were stained with the potential-insensitive green stain Mito Tracker Green (Invitrogen Italia, Milan, Italy), and the potential-sensitive red stain TMRM (Invitrogen, Paisley, Scotland). TMRM was stored as a 10-mM stock solution in ethanol, whereas Mito Tracker Green was stored as a 1-mM stock solution in DMSO (Sigma-Aldrich).

Measurement of intracellular ATP. Intracellular ATP levels were measured by luminometric assay using the Enliten ATP assay system (Promega, Italia, Milan, Italy). A total of 15 × 10³ cells per sample were lysed with 10 μ l lysis buffer (FireZyme, San Diego, CA, USA), and supplemented with 90 μ l of diluent buffer (FireZyme) to stabilize ATP. Samples were then placed in a Victor 3 multilabel counter (Perkin Elmer) equipped with a Wallac liquid injector (Perkin Elmer, Wellesley, MA, USA) that allowed rapid injection of the luciferin-luciferase solution (100 μ l).

Lactate measurement. A total of 25 × 10³ cells per well were plated in the different media as described above. Supernatants were withdrawn at the various time points and lactate release was measured with a Lactate Colorimetric Assay Kit (Abcam, Cambridge, UK), according to the manufacturer's indication.

Real-time PCR. Real-time PCR was performed in a Step One Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). A total of 10⁶ cells per well were plated under the different experimental conditions. Total RNA was extracted with TRIZOL reagent (Invitrogen). Reverse transcription was performed starting from 1 μ g of total RNA per well, with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) as described by the manufacturer. A solution of 2 μ l cDNA was used as template. Amplification was performed with custom-made primers and Taqman probes (Applied Biosystems) as detailed below, and with an internal P2Y1 reference (Pre-developed TaqMan assay reagents, Applied Biosystems). A comparative C_T analysis ($\Delta\Delta C_T$) was done to allow determination of the change of expression (fold increase) of the target cDNA in the experimental samples relative to the reference sample.

Western blot analysis. Cells were plated at the concentration of 5 × 10⁵ per well. Proteins were extracted in ice-cold, Ca²⁺-free, lysis buffer containing phenylmethylsulfonyl fluoride (1 mM), benzamide (1 mM) and 0.1% Triton (all by Sigma-Aldrich), processed and blotted as described previously.¹⁵ Membranes were incubated with the primary mAbs overnight at 4 °C. The following mAbs were used: anti-PKM2, anti-PDHK1, anti-Glut1, anti-ph-Akt/PKB (Ser473) and anti-Akt/PKB (all from Cell Signaling, Milan, Italy), at a dilution of 1 : 1000 in TBS-t buffer (TBS with 0.1% Tween 20) supplemented with 1% BSA. An anti-actin mAb, diluted 1 : 500 (Sigma-Aldrich), was used as loading control. Then, membranes were incubated with a secondary goat anti-rabbit, HRP-conjugated antibody (Bio-Rad, Copenhagen, Denmark) and diluted 1 : 3000 in TBS-t buffer. Bands were visualized by enhanced chemiluminescence detection kit (Amersham, GE Healthcare, Milan, Italy). Densitometric analysis of the protein bands was carried out with the Alpha

VIEW program (Alpha Innotech Corporation, San Leandro, CA, USA). Band densitometry was normalized on the actin ratio.

HIF-1- α measurement. HIF-1- α content was evaluated with the HIF-1- α Human Elisa Kit (Abcam), according to manufacturer's indication. Briefly, 4 mg of total homogenates were loaded. Samples were then placed in a Victor 3 multilabel counter (Perkin Elmer). Conversion of absorbance at 450 nm into ng/ml of protein was performed with a calibration standard curve by reference to the manufacturer's indication. Total homogenates of HEK293-P2X7 and HEK293-mock cells were obtained as suggested by the manufacturer, whereas total protein contents were measured with Bradford method.

PFK measurement. PFK content was evaluated with the *in vitro* quantitative Enzyme-linked Immunosorbent Assay Kit for human PFK (Uscn Life Science Inc., Wuhan, China), according to the manufacturer's indication. Briefly, 15 ng of total homogenates were loaded. Samples were then placed in a Victor 3 multilabel counter (Perkin Elmer). Conversion of Absorbance at 450nm into ng/ml of protein was performed with a calibration standard curve by reference to the manufacturer's indication. Total homogenates of HEK293-P2X7 and HEK293-mock cells were obtained as suggested by the manufacturer, whereas total protein contents were measured with Bradford method.

PDH activity assay. PDH activity was evaluated in HEK293-P2X7 and HEK293-mock with the PDH Activity Assay Kit (Novagen, Merck for Biosciences, Frankfurt, Germany), according to the manufacturer's indication. Briefly, 900 μ g of total homogenates were loaded. Samples were then placed in a Victor 3 multilabel counter (Perkin Elmer). Absorbance at 450 nm was measured every 20 s for a total of 15 min. PDH activity is expressed as slopes of the curves generated by kinetic analysis.

Visualization of mitochondria and semiquantitative determination of mitochondrial potential. Cells, plated onto 24-mm glass coverslips, were loaded with 1 μ M Mito Tracker green and 20 nM TMRM in the different culture media for 30 min at 37 °C. At the concentration used, TMRM is mainly localized in the mitochondria, emitting a level of fluorescence proportional to the absolute value of mitochondrial potential.¹² Confocal images were acquired with a Zeiss LSM 510 confocal microscope equipped with a plan-apochromat × 63 oil immersion objective (Carl Zeiss, Oberkochen, Germany) as previously described.¹²

Glycogen-store detection. Cells, 25 × 10³, that were plated overnight in Polylin 1X-coated, UV-sterilized 24-well plates. Glycogen was revealed with periodic acid-Schiff (PAS) Kit (Sigma-Aldrich). Cells were fixed with methanol 96% (Sigma-Aldrich), processed as suggested by the manufacturer and analyzed with an Olympus IMT-2 phase contrast microscope equipped with a × 40 objective (Olympus Life Science Europe, Munich, Germany) geared with a DS-2Mv high-speed color camera (Nikon, Tokyo, Japan).

Data analysis. All data are shown as mean ± S.D. Tests of significance were performed by Student's *t*-test using GraphPad InStat software (GraphPad, San Diego, CA, USA).

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

FA performed most of the experiments, analyzed the data and contributed to writing. SF performed real-time PCR and western blot in the experiments. EA performed

some of the lactate-release experiments and helped with cell transfections. DF participated in the design of the experiments and in data analysis. FDV designed research, participated in data analysis and wrote the paper.

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