Molecular hydrogen induces metabolic reprogramming to promote differentiation of glioma stem cells

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Research

Abstract

Background: Our previous study showed that molecular hydrogen could effectively suppress glioblastoma multiforme (GBM) tumor growth via induction of glioblastoma stem cells (GSCs) differentiation. Metabolic reprogramming has been demonstrated to delicately regulate the stemness of cancer stem cells. In the present study, we explored whether metabolic reprogramming is involved in the hydrogen-induced GSCs differentiation.

Methods: Immunofluorescence staining was conducted to determine the expression of cell surface markers. Glucose uptake and lactate secretion was determined by spectrophotometric method. Untargeted metabolomics analyses were conducted to investigate the metabolic alteration in GSCs after hydrogen treatment. 13 C-labeled metabolic flux analysis was performed to explore the regulatory effects of molecular hydrogen on the glucose metabolism of GSCs.

Results: Immunofluorescence staining showed the up-regulated expression of oligodendroglial markers in hydrogen-treated GSCs. Both glucose uptake and lactate production in GSCs were significantly inhibited by hydrogen treatment. Untargeted metabolomics analyses showed hydrogen-induced promotion of de novo synthesis of nucleotides in GSCs. Metabolic flux analysis showed decreased glucose metabolism in GSCs induced by hydrogen. Conversely, the content of glycerol 3-phosphate, glutamate and glutamine were increased by hydrogen treatment.

Conclusions: Results from this study demonstrated that molecular hydrogen could inhibit glucose metabolism and promote de novo synthesis of nucleotides in GSCs, suggesting the involvement of metabolic reprogramming in the hydrogen-induced GSCs differentiation. Our study also provides important new clues to seek the target of molecular hydrogen.

Background

Glioblastoma multiforme (GBM), a grade IV malignant glioma according to WHO classification, is the most common and aggressive brain tumor with a median survival of 14.6 months despite multimodal therapy [1]. Recent evidence suggests that a subpopulation of GBM cells, namely glioblastoma stem cells (GSCs), may causally contribute to tumor initiation [2], recurrence of primary tumors [3] and therapeutic resistance [4, 5]. Therefore, targeting GSCs as the "root cells" initiating malignancy has been considered as a promising strategy for treating GBM. Recent study showed that differentiation of GSCs could induce therapy-sensitizing effects [6], suggesting that "differentiation therapy" may be a potent approach of anti-GBM therapy.

Molecular hydrogen (H_2) has been regarded as a novel therapeutic medical gas since the 2007-Nature Medicine publication [7]. Its potential benefits has been reported in various disease models, including cardiovascular and metabolic diseases [8], brain injury [9], inflammatory disease [10], ocular disease [11], radiation injury [12], cancer [13], and so on. We previously showed that hydrogen inhalation could effectively suppress GBM tumor growth via induction of GSCs differentiation [14]. The hydrogen-induced

inhibition of the stemness of GSCs could be observed both in vivo and in vitro. However, the mechanisms underlying the regulation of cancer stemness by hydrogen treatment remain unclear. Previous studies showed that metabolic reprogramming could delicately regulate the stemness properties of induced pluripotent stem cells (iPSCs) [15] or cancer stem cells [16]. We hypothesized that metabolic reprogramming may be involved in the hydrogen-induced GSCs differentiation.

In this study, we investigated the metabolic changes of GSCs induced by hydrogen treatment. Both untargeted metabolomics and stable isotope-labeling mass spectrometry analysis were performed to provide an unbiased view of the metabolic impact exerted by hydrogen on GSCs. Our data showed that hydrogen treatment significantly inhibits glucose metabolism and promote de novo synthesis of nucleotides in GSCs, indicating the involvement of metabolic reprogramming in hydrogen-induced GSCs differentiation.

Materials And Methods

Hydrogen-rich medium (HRM) preparation

High-purity hydrogen gas produced by a hydrogen gas generator was filtered through a sterile 0.22 μm syringe filter and dissolved in cell culture medium (final hydrogen concentration: 0.55 ~ 0.65 mM). The hydrogen concentration was monitored by using a needletype Hydrogen Sensor (Unisense A/S, Aarhus, Denmark).

Cell culture

Rat C6 glioma cells were obtained from the American Type Cell Collection (ATCC, Manassas, USA). Cells were routinely cultured at 5% CO_2 and 37 °C in DMEM medium (Gibco, NY, USA) supplemented with 10% fetal calf serum (Gibco, NY, USA) and 1% penicillin and streptomycin (Gibco, NY, USA).

C6 glioma cells derived glioma stem cells were obtained as the following method: C6 cells were collected and washed to remove serum, then suspended in serum-free DMEM medium at a density of 2.0×10^3/mL. Cells were cultured in normal serum-free medium or hydrogen-rich serum-free medium with B27 supplement (1:50, GIBCO, Grand Island, NY), 20 ng/mL of epidermal growth factor (EGF) (Pepro Tech Inc., Rocky Hill NJ), and 20 ng/mL of basic fibroblast growth factor (bFGF) (Pepro Tech). Medium was changed every 3 days. Over twenty passage GSCs were used in all the experiments.

Glucose uptake and lactate secretion assays

Glucose levels were determined using a Glucose Assay kit (BioVision, Inc.), and glucose consumption was calculated as difference in glucose concentration between the original medium and the medium from the cell cultures. The absorbance value of each well was read immediately at 563 nm using a microplate reader (Bio-Rad, Richmond, CA). Extracellular lactate levels were measured in the cell culture medium using a Lactate Assay kit (BioViosion, Inc.), according to the manufacturer's protocol. The results were normalized to the quantity of total protein in the control cells.

Immunofluorescent staining

Cells were seeded on coverslips, fixed with 4% paraformaldehyde (Sigma-Aldrich) for 10 min and permeabilized with 0.1% Triton X-100 in PBS and blocked with 1% BSA for 1 hr. Subsequently, cells were incubated overnight at 4 °C with primary antibodies including anti-SOX2 (1:100; Abcam), anti-Olig1 (1:500; merck), anti-MBP (1:500; Abcam) and then cells were incubated with FITC-conjugated secondary antibody (Abcam) for 1 h. The nuclei were stained with Hoechst and the fluorescence images obtained with Olympus IX71 inverted microscope.

Untargeted metabolomics analyses

For intracellular metabolite measurements, the medium was removed, cells were briefly washed with 1 mL ice cold saline solution (0.9% NaCl in water), followed by the addition of 1 mL methanol/acetonitrile/H2O (2:2:1, v/v, pre-chilled at -80 °C). Cells were then immediately transferred to 1.5 mL Eppendorf tube, followed by a rapid quenching in liquid nitrogen. Untargeted metabolomics analyses were conducted at the Metabolomics Facility at Tsinghua University Branch of China National Center for Protein Sciences (Beijing, China).

Metabolic flux analysis

DMEM lacking glucose was prepared from powder (Sigma), and then supplemented with uniform labeled ^{13}C-glucose, the resulting medium was used in cell culture. Cells were grown in 60-mm dishes until 80% confluent, then rinsed with PBS and cultured with isotopes-containing medium for 12 hr in the conditions as indicated in the experiments. After the incubation, the medium was replaced with 500 μL of 80% (v/v) HPLC-grade methanol (pre-chilled at -80 °C). Cultured cells were collected into a 1.5 mL eppendorf tube and vortexed for 1 min at 4 °C before overnight incubation at -80 °C. Cells were then centrifuged at 14,000 g for 20 min at 4 °C and transferred the same volume of supernatant to a new eppendorf tube. The supernatant was then dried in a vacuum centrifuge (Centrivap Concentrator, Labconco). The dried samples for LC/MS analysis were stored at -80 °C. LC/MS analyses were conducted on a TSQ Quantiva triple quadrupole mass spectrometer networked to a Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific) at the Metabolomics Facility at Tsinghua University Branch of China National Center for Protein Sciences (Beijing, China).

Statistical analysis

Groups from cell culture experiments were compared using two-tailed Student's t tests and results were presented as means ± SEM. All statistical analyses were performed using GraphPad Prism 8.0.2. A value of $p < 0.05$ was considered significant.

Results

Expression of oligodendroglial markers in GSCs were up-regulated by hydrogen treatment

Our previous finding showed that hydrogen treatment elevated the expression of astrocytes marker GFAP, to further investigate whether hydrogen could induce GSCs differentiate into oligodendroglia, the expression of two oligodendroglial markers, Olig1 and MBP, were determined by immunofluorescent staining. As shown in Fig. 1, both Oligo1 and MBP expression were up-regulated by hydrogen treatment, while the cancer stem-like cell marker SOX2 was down-regulated.

Hydrogen treatment inhibites glucose uptake and lactate secretion

Glucose uptake and lactate production in GSCs were determined after hydrogen treatment. As shown in Fig. 2A, compared with the controls, hydrogen induced significant derease in glucose uptake (11.55 ± 0.0093 mM vs. 12.27 ± 0.064 mM, $p = 0.0004$). The lactate production was also significantly reduced in hydrogen-treated GSCs (3.75 ± 0.27 mM vs. 6.04 ± 0.77 mM, $p = 0.0421$) (Fig. 2B).

Hydrogen treatment inhibites glucose metabolism in GSCs

To investigate the effects of hydrogen on the glucose metabolism, uniform labeled ^{13}C-glucose based mass isotopomer analysis was performed. As shown in Fig. 3, the uptake of ^{13}C-glucose was decreased (11.50%) by hydrogen treatment, although not significant ($p > 0.05$). The levels of ^{13}C-glucose derived glycolysis intermediates, including 2/3-phosphoglycerate (2/3-PG) (37.37%, $p = 0.0149$), phosphoenolpyruvate (PEP) (51.58%, $p = 0.0238$), and pyruvate (29.10%, $p = 0.0100$), were markedly decreased in hydrogen-treated GSCs, other metabolites including hexose-phosphate (Hexose-P) (52.96%, $p = 0.0600$), fructose 1,6-bisphosphate (FBP) (45.95%, $p > 0.05$), and dihydroxyacetone phosphate (DHAP) (27.11%, $p > 0.05$) were also reduced without statistical significance, suggesting that the glycolysis in GSCs was inhibited by hydrogen treatment. Conversely, the increased levels of ^{13}C labeled glycerol 3-phosphate (G-3-P) (42.01%, $p = 0.0289$) was observed in hydrogen-treating GSCs.

The ^{13}C labeled metabolites in the mitochondrial tricarboxylic acid (TCA) cycle, including aconitate, α-Ketoglutarate (α-KG), succinate, fumarate, and malate, were also significantly diminished in GSCs after hydrogen treatment (Fig. 4). The decrease in succinate, fumarate, and malate was less evident than aconitate and α-KG. On the contrary, hydrogen treatment induced up-regulation of both glutamate (Glu) and glutamine (Gln) levels in GSCs.

Hydrogen treatment promotes de novo synthesis of purine and pyrimidine nucleotides in GSCs

To achieve a global view of the metabolic alteration induced by hydrogen treatment, untargeted metabolomics analyses were performed. As shown in Fig. 5, hydrogen treatment significantly increased the intracellular pools of both purine and pyrimidine metabolites, indicating the promotion of nucleotides biosynthesis by hydrogen.

The results of metabolic flux analysis also showed increased de novo synthesis of nucleotides induced by hydrogen, evidenced by elevated levels of glucose derived nucleotide monophosphates, including IMP (23.27%, $p = 0.0069$), AMP (92.81%, $p = 0.0003$), GMP (100.05%, $p < 0.0001$), and UMP (43.12%, $p = 0.0002$) (Fig. 6). On the contrary, the content of nucleotide triphosphates including ATP (56.00%, $p = 0.0842$) and UTP (39.80%, $p > 0.05$) was increased, although not significant. The levels of nucleotide diphosphates were not significantly changed.

Discussion

The effects of molecular hydrogen on GBM have been investigated in our previous study [14]. Inhalation of 67% hydrogen gas has been shown to have an inhibitory effect on GBM. Both in vivo and in vitro experiments showed the hydrogen induced differentiation of GSCs as evidenced by a reduction of CD133 expression and an upregulation of GFAP expression. The present study provides further evidence that hydrogen treatment could also increase Olig1 and MBP expression, indicating the oligodendroglial-oriented differentiation of GSCs. It is noteworthy that GSCs were treated with HRM for only one time, and were then incubated in humidified air containing 5% CO_2. In this condition, the hydrogen concentration in HRM reduced by more than 90% within 6 hours [17], which indicated that the effect of molecular hydrogen on GSCs is a short-course action. The rapid effect of molecular hydrogen has also been observed in previous study, which showed that inhalation of 2% hydrogen gas for only 30 min could significantly attenuated brain injury in neonatal hypoxia-ischemia rat model [18].

Different types of cells may use different metabolic pathways. Nearly all types of cancer cells carry out glycolysis at a much higher rate than normal cells, even when oxygen is available, which have been known as "Warburg effect" [19]. This heavier reliance on glycolysis has also been found in GSCs. Previous study reported that compared with the GBM cells, GSCs showed low mitochondrial respiration and elevated glycolysis [20]. However, this observation has been challenged by Vlashi et al., who suggested that GSCs relied mainly on oxidative phosphorylation for energy supply [21]. In the present study, hydrogen treatment showed inhibitory effect on glucose uptake and lactate secretion, indicating that the glycolysis metabolism was signifincantly down-regulated by hydrogen, which was further confirmed by metabolic flux analysis. These observations suggested that GSCs exhibit a higher rate of glycolysis than differentiated glioma cells. The results of metabolic flux analysis also showed reduced rate of oxidative phosphorylation, however, the changes in the content of TCA intermediates was less dramatic than glycolytic intermediates, indicating that the decreased oxidative phosphorylation rate may be mainly attributed to the decline in glycolysis.

In view of the whole process of glucose metabolism, the decrease in the content of intermediates began at the step of glucose uptake, indicating that glucose transporters (GLUT) may be the potential targets of hydrogen. The decreased glucose uptake could be resulted from the reduced GLUT expression through PPAR α activation [22]. Previous study has reported that molecular hydrogen could also induce PPAR α activation [23]. Thus molecular hydrogen may decrease glucose uptake by down-regulation of GLUT via PPAR α activation. Considering the changes in glucose uptake was much smaller than Hexcose-P, it is

also possible that the effect on glucose uptake may be the feedback inhibition of reduced glycolysis rate. Thus the most likely targets of hydrogen during the process of glucose metabolism may be the enzymes involved in the convertion of Glucose to Hexcose-P. Because our metabolic flux analysis can not distinguish glucose-6-phosphate and fructose 6-phosphate, either hexokinase (HK) or phosphoglucose isomerase (PGI) may be the potential target of hydrogen. HK2 has been considered as a key mediator of glycolysis in GBM [24], which may be down-regulated by hydrogen treatment. It is also possible that molecular hydrogen could decrease HK2 activity directly, as the direct modulation of enzyme activity by hydrogen has also been found in our previous study [25]. In addition, the inhibition of glycolysis flux could be induced by decreased PGI activity via mTOR activation [26]. The regulation of mTOR pathway by molecular hydrogen has also been found in previous study [27], indicating that the inhibitory effect on glycolysis may be caused by hydrogen induced decrease in PGI activity via mTOR pathway.

It is noteworthy that the levels of G-3-P in GSCs were significantly increased after hydrogen treatment. G-3-P formed either from glycerol released by triacylglycerol breakdown or by the reduction of dihydroxyacetone phosphate (DHAP) from glycolysis. The DHAP derived G-3-P is oxidized by glycerol 3-phosphate dehydrogenase (GPDH). The increase in G-3-P levels may be attributed either to elevated GPDH expression or to enhanced GPDH activity. The expression of GPDH has been demonstrated to be regulated by PPAR α or γ activation [28]. Molecular hydrogen has also been shown to induce PPAR α or γ activation in previous study [23]. Therefore, we supposed that the increase in G-3-P levels in GSCs may be caused by hydrogen induced up-regulation of GPDH via PPAR α or γ activation. Another important finding is that an increased production of both Glu and Gln in GSCs was induced by hydrogen treatment. As the changes in content of Glu was much more evident than Gln, the elevated levels of Gln may be arised from increased Glu levels. The conversion of Glu from α-KG is catalyzed by glutamate dehydrogenase (GDH). The increased production of Glu may caused by the up-regulation of GDH expression or GDH activity.

In our study, both untargeted metabolomics and metabolic flux analysis showed consistent results that the nucleotides biosynthesis was significantly promoted by hydrogen treatment. Hydrogen-induced increase in the content of purine mononucleotides seems to be much more evident than pyrimidine mononucleotides. In addition, the content of nucleotide triphosphates, especially the ATP levels, were decreased by hydrogen treatment, which is consistent with the lower rate of glucose metabolism in hydrogen-treated GSCs. The effects of hydrogen on the rate-limiting pyrimidine and pyrimidine synthetic enzymes need to be further investigated.

Conclusion

The present study provides a first detailed glucose metabolic analysis of the hydrogen-treated GSCs, which gave important new clues to seek the target of molecular hydrogen. The results demonstrated that molecular hydrogen could inhibit glucose metabolism and promote de novo nucleotides synthesis in GSCs, suggesting the involvement of metabolic reprogramming in the hydrogen-induced GSCs differentiation.

Abbreviations

GBM: Glioblastoma; GSCs: Glioma stem cells; iPSCs: induced pluripotent stem cells; HRM: hydrogen-rich medium; CTRL: Control; EGF: epidermal growth factor; bFGF: basic fibroblast growth factor; 2/3-PG: 2/3-phosphoglycerate; Hexose-P: hexose-phosphate; FBP: fructose 1,6-bisphosphate; DHAP: dihydroxyacetone phosphate; G-3-P: glycerol 3-phosphate; α-KG: α-Ketoglutarate; Glu: glutamate; Gln: glutamine; GLUT: glucose transporters; HK: hexokinase; PGI: phosphoglucose isomerase; DHAP: dihydroxyacetone phosphate; GPDH: glycerol 3-phosphate dehydrogenase; GDH: glutamate dehydrogenase; R-5-P: ribose-5-phosphate; PRPP: 5-phosphoribosyl-1-pyrophosphate; ASP: aspartate; DHO: dihydroorotate; ADS: Adenylosuccinate; XMP: xanthosine-5-monophosphate; IMP: inosine-5-monophosphate.

Declarations

Ethics approval and consent to participate

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

MXM and XF designed the overall project. XF and MSN analyzed the data and wrote the manuscript. MSN, ZY, YTT and LMY performed the research. ZXK and ZPX revised the manuscript.

Consent for publication

Not applicable.

Competing interests

The authors report no conflicts of interest in this work.

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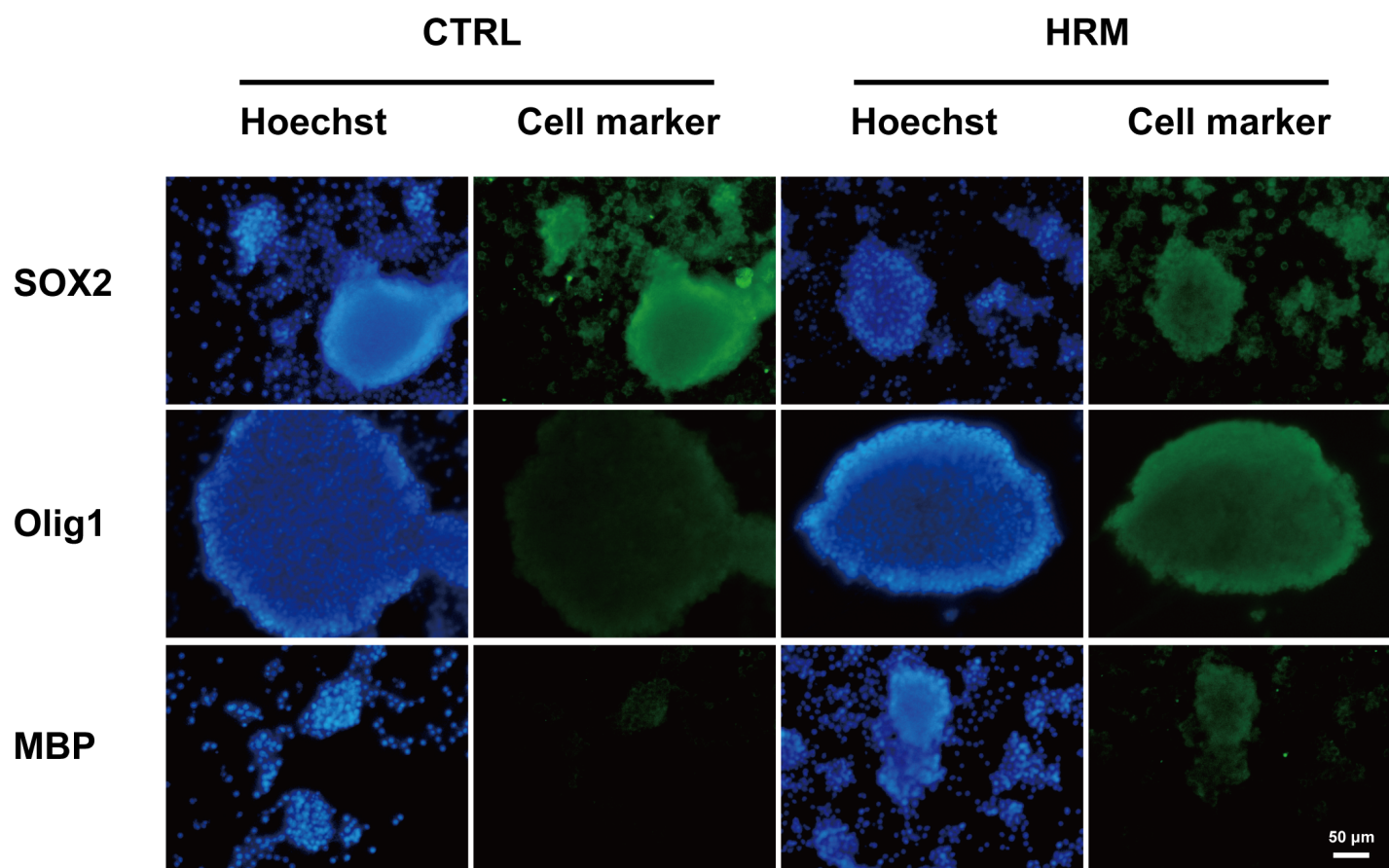
Figures

Figure 1

Hydrogen treatment up-regulated the expression of oligodendroglial markers and down-regulated the cancer stem-like cell marker in GSCs.
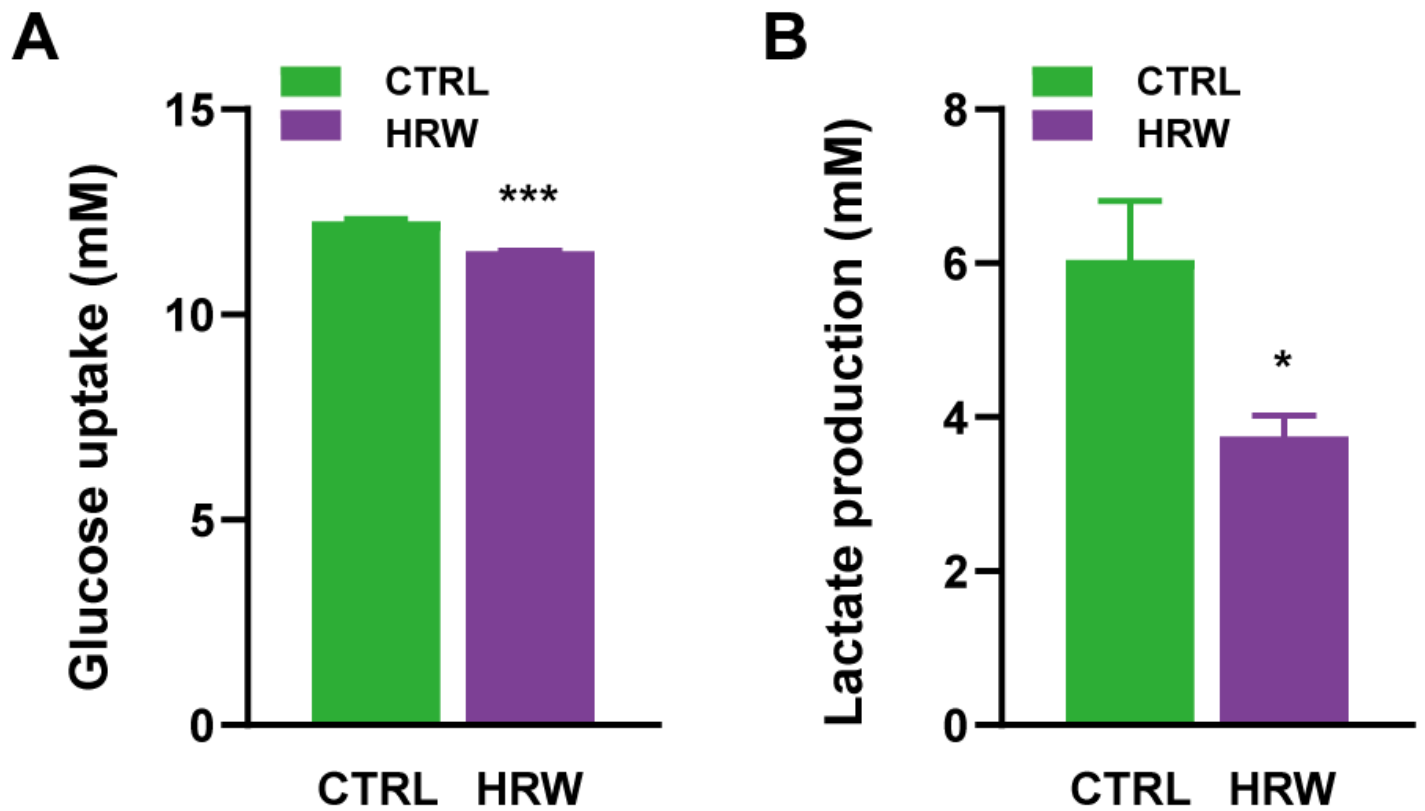
Figure 2

Hydrogen treatment induced significant derease in glucose uptake and lactate production in GSCs. (A) Glucose uptake and (B) lactate production were determined by spectrophotometric method. *p < 0.05, ***p < 0.001.
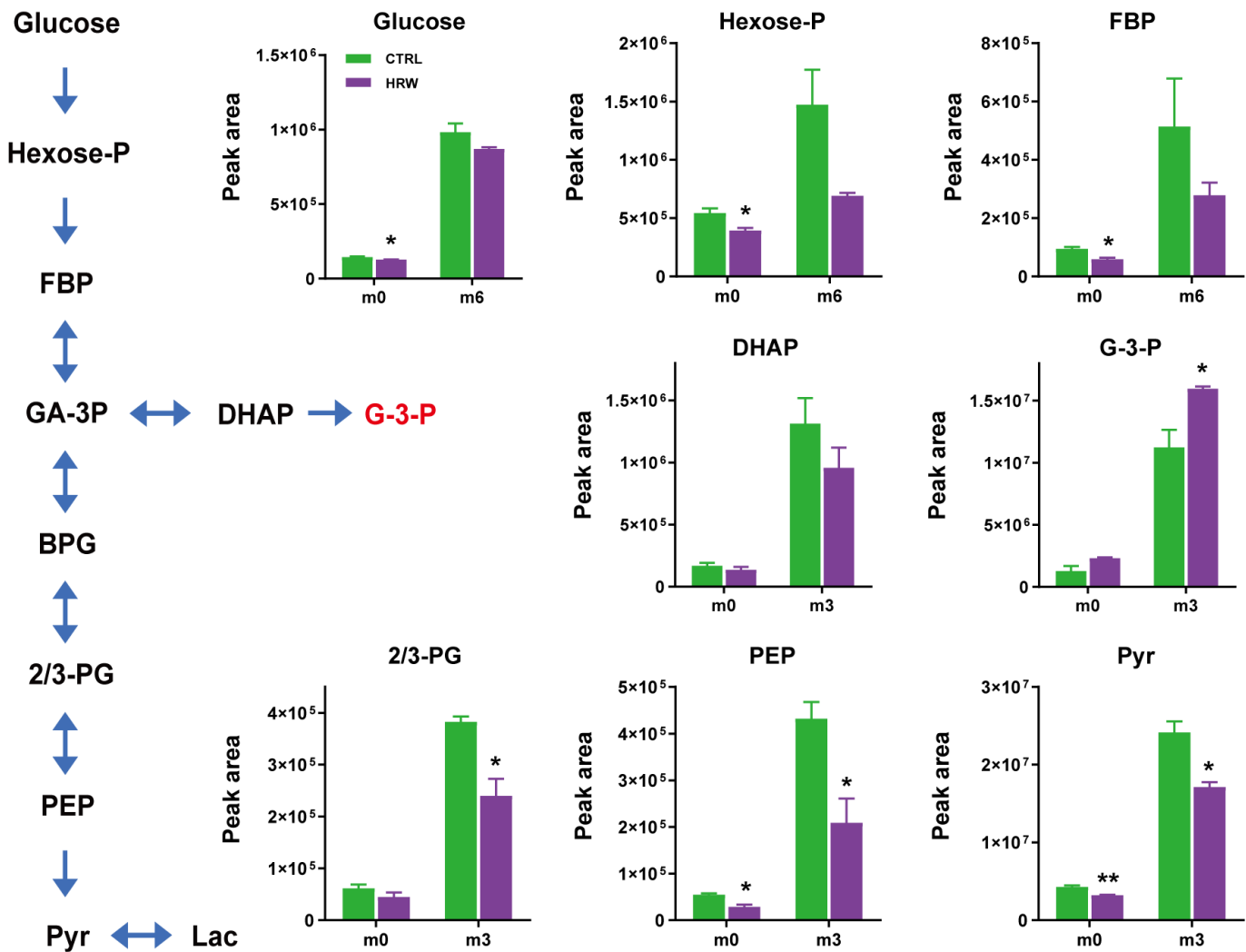
Figure 3

Hydrogen treatment significantly dereased the rate of glycolysis in GSCs. *p < 0.05, **p < 0.01.

Figure 4

Hydrogen treatment significantly reduced rate of oxidative phosphorylation in GSCs. *p < 0.05, **p < 0.01, ***p < 0.001.

R-5-P → PRPP → IMP

IMP → XMP, IMP → ADS

XMP → GMP

ADS → AMP

ASP → DHO → Orotate → UMP → CMP

De novo Purine Synthesis

De novo Pyrimidine Synthesis

CTRL   HRM

R-5-P
Hypoxanthine
Inosine
Guanosine
Xanthine
IMP
ADS
AMP
GMP

Uridine
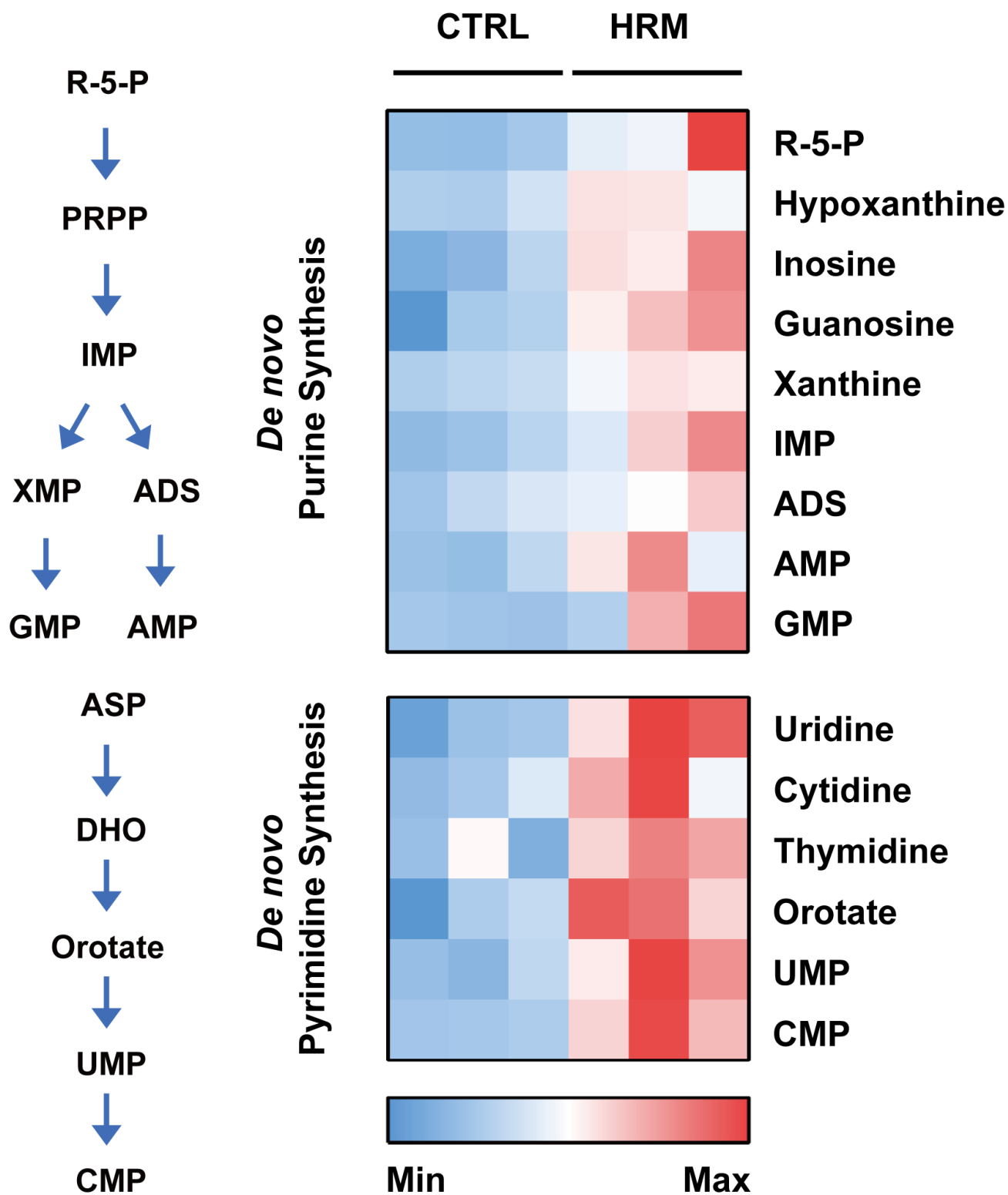Cytidine
Thymidine
Orotate
UMP
CMP

Min   Max

Figure 5

Untargeted metabolomics analyses showed the hydrogen-induced promotion of de novo nucleotides synthesis in GSCs.
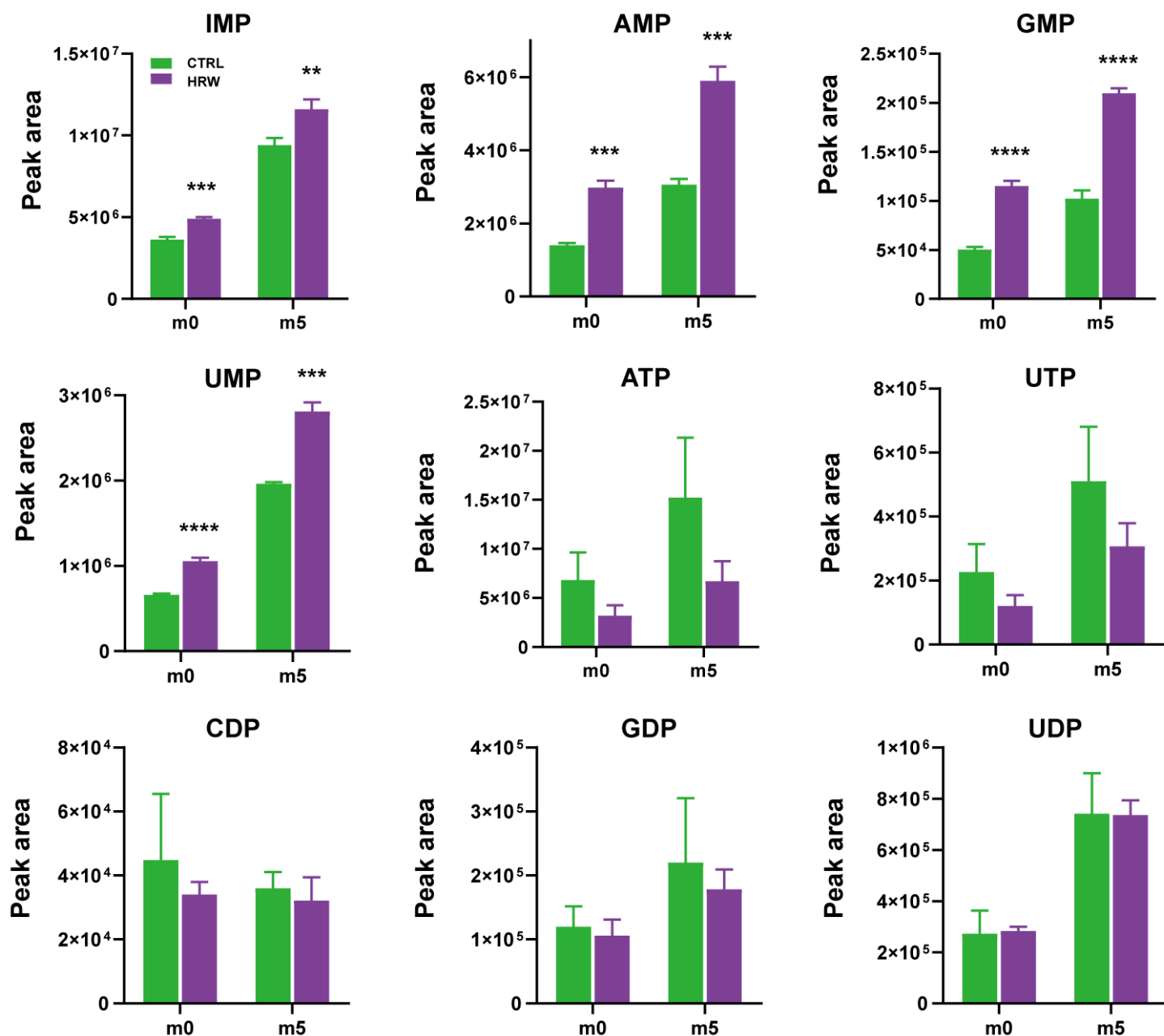
Figure 6

Metabolic flux analysis showed the effects of hydrogen on the content of nucleotides in GSCs. **p < 0.01, ***p < 0.001, ****p < 0.0001.