Resistance to hypoxia-induced necroptosis is conferred by glycolytic pyruvate scavenging of mitochondrial superoxide in colorectal cancer cells

C-Y Huang¹, W-T Kuo¹, Y-C Huang¹, T-C Lee¹,² and LCH Yu*¹

Cancer cells may survive under oxygen and nutrient deprivation by metabolic reprogramming for high levels of anaerobic glycolysis, which contributes to tumor growth and drug resistance. Abnormally expressed glucose transporters (GLUTs) are colocalized with hypoxia (Hx) inducible factor (HIF)1α in peri-necrotic regions in human colorectal carcinoma. However, the underlying mechanisms of anti-necrotic resistance conferred by glucose metabolism in hypoxic cancer cells remain poorly understood. Our aim was to investigate signaling pathways of Hx-induced necroptosis and explore the role of glucose pyruvate metabolism in mechanisms of death resistance. Human colorectal carcinoma cells were Hx exposed with or without glucose, and cell necroptosis was examined by receptor-interacting protein (RIP)1/3 kinase immunoprecipitation and 32P kinase assays. Our results showed increased RIP1/3 complex formation and phosphorylation in hypoxic, but not normoxic cells in glucose-free media. Blocking RIP1 signaling, by necrostatin-1 or gene silencing, decreased lactodehydrogenase (LDH) leakage and plasma membrane disintegration. Generation of mitochondrial superoxide was noted after hypoxic challenge; its reduction by antioxidants inhibited RIP signaling and cell necrosis. Supplementation of glucose diminished the RIP-dependent LDH leakage and morphological damage in hypoxic cells, whereas non-metabolizable sugar analogs did not. Hypoxic cells given glucose showed nuclear translocation of HIF1α associated with upregulation of GLUT-1 and GLUT-4 expression, as well as increase of intracellular ATP, pyruvate and lactate levels. The glucose-mediated death resistance was ablated by iodoacetate (an inhibitor to glyceraldehyde-3-phosphate dehydrogenase), but not by UK5099 (an inhibitor to mitochondrial pyruvate carrier), suggesting that glycolytic pathway was involved in anti-necrotic mechanism. Lastly, replacing glucose with cell-permeable pyruvate derivative also led to decrease of Hx-induced necroptosis by suppression of mitochondrial superoxide in an energy-independent manner. In conclusion, glycolytic metabolism confers resistance to RIP-dependent necroptosis in hypoxic cancer cells partly through pyruvate scavenging of mitochondrial free radicals.

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Malignant cells have developed adaptive mechanisms to survive under extreme conditions of tumor microenvironment, such as restricted oxygen supply and nutrients deprivation. The altered bioenergetic status may contribute to tumor growth and drug resistance against antiangiogenic and chemotherapeutic agents.¹,² One of the pro-survival mechanism of tumor cells is the high levels of anaerobic glycolysis, termed Warburg’s effect.³ A large body of evidence shows that upregulation of glycolytic enzymes and glucose transporters (GLUTs) are linked to transcription activity of hypoxia (Hx) inducible factor (HIF) 1 triggered by low oxygen condition.⁴,⁵ Moreover, presence of GLUT isoforms 1–4 and sodium/glucose transporter 1 (SGLT1), which are not normally expressed in colonocytes, has been widely documented in human colorectal carcinoma that colocalizes with HIF1α.⁶–¹² To date, glucose-mediated mechanisms involved in promoting tumor survival against hypoxic stress remain incompletely understood.

Cells depleted of oxygen and nutrients undergo necrotic death, which is commonly seen in hypoxic cores of colorectal tumors,¹³,¹⁴ and also in cases of mesenteric ischemia/reperfusion and necrotizing enterocolitis.¹⁵,¹⁶ Although necrosis has been traditionally regarded as an uncontrolled form of cell death, recent data indicated that programmed necrosis or necroptosis is regulated by receptor-interacting protein (RIP) kinase through RIP1 and RIP3 complex formation and phosphorylation.¹⁷–²¹ Mitochondrial bioenergetic alterations and reactive oxygen species (ROS) derived from respiratory chain have also been implicated as necrotic effectors.¹⁸,²¹ However, the molecular mechanisms of Hx-induced necrotic

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Abbreviations: RIP, receptor-interacting protein kinase; GPD, glyceraldehyde-3-phosphate dehydrogenase; MPC, mitochondrial pyruvate carrier; LDH, lactate dehydrogenase; TER, transepithelial electrical resistance; ROS, reactive oxygen species; GLUT, glucose transporter; SGLT1, sodium/glucose transporter 1; BHA, butylated hydroxyanisole; ZO, zonula occludens; IA, iodoacetate

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death and signaling pathways of anti-necrotic resistance in cancer cells are still unclear. Recent reports documented that HIF1α and GLUT-1 colocalize at peri-necrotic regions in human colorectal tumors,\textsuperscript{13,22} suggesting that glucose metabolism may confer anti-necrotic resistance to hypoxic stress. Glucose is catalyzed to ATP and pyruvate by a cascade of glycolytic enzymes, such as glucokinase and glyceraldehyde-3-phosphate dehydrogenase (GPD).\textsuperscript{23} The final glycolytic product pyruvate is also the starting substrate for tricarboxylic acid cycle after being transported across inner mitochondrial membrane by mitochondrial pyruvate carrier (MPC).\textsuperscript{24,25} Aside from its critical role as the link between glycolysis and mitochondrial respiration, pyruvate also scavenges ROS through a non-enzymatic reaction.\textsuperscript{26} Numerous studies have suggested that chemoresistance may be due in part to glycolytic ATP as a preferential energy source for promoting cancer cell survival.\textsuperscript{27,28} However, whether glycolytic pyruvate metabolite has a role in circumventing Hx-induced necrotic death has yet to be explored.

Results

Hypoxic challenge triggers RIP-dependent necroptosis in human colorectal carcinoma cells. Human colorectal carcinoma Caco-2 cells were exposed to normoxia (Nx) or Hx in glucose-free media (Φ) for various time points, and a time-dependent increase of lactodehydrogenase (LDH) leakage was observed in Hx + Φ but not Nx + Φ cells (Figure 1a). Live images revealed cytosolic vacuolation, widening intercellular space and cell detachment in a timely order following hypoxic challenge, whereas no morphological change was observed in normoxic counterparts (Figure 1b). No sign of apoptosis was found after hypoxic challenge as evidenced by the lack of oligonucleosome formation and caspase-3 activation (Supplementary Figure S1). Similar results of Hx-induced cell necrosis were seen in another human colorectal tumor cell line HT29 (Supplementary Figures S2-A and B).

The mitochondrial transmembrane potential was determined by using a cationic JC-1 dye. Exposure to Hx resulted in a transient increase and then decline in red fluorescence intensity (the aggregated form of JC-1) followed by a display of green fluorescence (the monomer form of JC-1) in the cytoplasm at later time points (Figure 1c). Quantification results indicated that the ratios of J-aggregate/monomer in cells after 8- and 24-h Hx were 221.1 ± 49.0% and 20.5 ± 2.8%, respectively, of that of the normoxic controls (Figure 1d), suggesting that Hx caused a transient hyperpolarization and a final collapse of mitochondrial transmembrane potential. Furthermore, plasma membrane disintegration paralleled with loss of tight junctions in hypoxic cells, evidenced by reduction of transepithelial electrical resistance (TER), increase of apical-to-basolateral dextran flux and structural disruption of zona occluden-1 (ZO-1) (Figure 1e–g).

Pretreatment with necrostatin-1 (Nec-1; a specific RIP1 inhibitor) and gene silencing of RIP1 reduced the level of LDH leakage caused by hypoxic challenge (Figures 2a and b). A ~50% knockdown of RIP1 protein by siRNA was confirmed by western blots (Figure 2b). Using immunoprecipitation and \textsuperscript{32}P kinase assays, formation of RIP1/3 complex and phosphorylation of RIP1 were found in Hx + Φ but not Nx + Φ cells (Figure 3a), indicating the activation of RIP1/3 signaling. The Hx-induced morphological damage and cell detachment were also inhibited by Nec-1 (Figure 2c). However, the mitochondrial transmembrane potential change was not reverted by Nec-1 (Figure 2d), suggesting that RIP1 activation may not be upstream of mitochondrial dysfunction. In hypoxic cells treated with necrostatin-1, a transient increase in red fluorescence was seen after 8 h followed by a switch to green fluorescence after 24 h (Figure 2d), of which the quantification results of JC-1 staining were 277.2 ± 25.2% and 40.2 ± 13.6%, compared with normoxic controls with necrostatin-1 at respective time points.

Glucose uptake abolishes Hx-induced RIP signaling and necroptosis. Administration of glucose (25 mM) reduced the RIP1/3 complex formation and phosphorylation (Figure 3a) and decreased LDH leakage in hypoxic cells in a dose-dependent manner (Figure 3b). Non-metabolizable sugar analogs (i.e., 3-O-methyl-glucopyranoside and mannitol) or glutamate did not reduce the LDH activity (Supplementary Figure S3). Moreover, glucose addition also ablated Hx-induced morphological damages (data not shown), mitochondrial transmembrane potential damage (Figure 3c) and tight junctional disruption (Figure 3d–f). Addition of glucose did not modify the apoptotic levels in hypoxic cells (data not shown).

To confirm that cells still perceive hypoxic stress after glucose addition, activation of HIF1α and Hx-targeted genes were examined. Nuclear translocation of HIF1α (Figure 4a) correlated with increased expression of GLUT-1 and GLUT-4.

Figure 1 Necrotic death was triggered by hypoxic challenge in human colonic carcinoma cells. (a) Caco-2 cells were exposed to normoxia (Nx) or hypoxia (Hx) in glucose-free media (Φ) for various time points. Increased LDH activity was found in the cell media of Hx + Φ, but not Nx + Φ cells, in a time-dependent manner. *P < 0.05 versus Nx + Φ (n = 6/group). (b) Representative time-lapse images showing morphological changes in Hx + Φ cells for 24 h. Cytosolic vacuolation (→) and cell detachment (↑) were noted in Hx + Φ cells. (c) Representative time-lapse images showing temporal alterations of mitochondrial transmembrane potential in Hx + Φ, but not Nx + Φ cells. The aggregated form of JC-1-J-aggregate; red fluorescence) accumulated in functional mitochondria in normoxic cells throughout each time point. In hypoxic cells, a transient increase in red fluorescence intensity was seen after 4–8 h followed by a decline at later time points (16–24 h) associated with an increase in green fluorescence (the monomer form of JC-1-J-monomer) in the cytoplasm. (d) The ratio of J-aggregate to monomer was quantified in Nx + Φ and Hx + Φ cells at various time points. In contrast to normoxic cells, hypoxic challenge induces transient hyperpolarization and a final collapse of the mitochondrial transmembrane potential. *P < 0.05 versus Nx + Φ at individual time points (n = 8/group). (e) Hypoxic challenge decreased the transepithelial resistance (TER) of cells compared with normoxic conditions. Data are presented as the absolute TER value at various time points. *P < 0.05 versus Nx + Φ (n = 6/group). (f) Hypoxic cells displayed heightened apical-to-basolateral flux of dextran probe in a time-dependent manner. *P < 0.05 versus Nx + Φ (n = 6/group). (g) Representative images of tight junction ZO-1 staining in cells exposed to Nx and Hx for 16 h. Tight junction disruption and cell detachment (asterisks) were observed in hypoxic cells (n = 6/group).
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a

LDH activity (unit/L)

0  4  8  16  24

Time (hr)

b

Nx+Φ

Hx+Φ

0 hr 4 hr 8 hr 12 hr 16 hr 24 hr

c

Nx+Φ

Hx+Φ

0 hr 4 hr 8 hr 12 hr 16 hr 24 hr

d

J-aggregate/monomer ratio (% of Nx+Φ)

0  50  100  150  200  250

Time (hrs)

N  X  +Φ  Hx+Φ

0  4  8  16  24

e

TER (Ohm·cm²)

150  200  250  300  350

Time (hr)

N  X  +Φ  Hx+Φ

0  4  8  12  16

f

% Apical dextran flux/1cm²

0.00  0.05  0.10  0.15  0.20  0.25  0.30  0.35

Time (hr)

N  X  +Φ  Hx+Φ

0  8  12  16  24

g

Nx+Φ

Hx+Φ

*
at the transcriptional and translational levels in hypoxic cells given glucose (Figure 4b–d). The experiment was not carried out on hypoxic cells in glucose-free media due to cell necrosis (i.e., plasma membrane disintegration and release of cellular contents). Moreover, similar results of glucose-mediated death resistance, HIF1α activation and GLUT upregulation were also seen in HT29 cells under hypoxic stress (Supplementary Figure S4-A, C, D).

Anaerobic glycolytic metabolism is involved in anti-necrotic resistance to Hx stress. To verify the metabolic process that is involved in death resistance, cells were pretreated with iodoacetate (IA, a glycolytic inhibitor to GPD) and UK5099 (UK, a MPC inhibitor) prior to hypoxic challenge in the presence of glucose. Blockade of glucose-mediated resistance was noted in cells pretreated with IA, whereas no inhibitory effect was seen with UK (Figure 5a), suggesting that glycolytic products unrelated to tricarboxylic acid cycles were involved in anti-necrotic mechanisms. For hypoxic cells in glucose-free media, IA and UK had no effect on LDH activity (data not shown).

The intracellular ATP, pyruvate, lactate contents were next quantified to examine the bioenergetic status of cells. A significant reduction of intracellular ATP and pyruvate levels were seen in hypoxic cells compared with their normoxic controls in glucose-free media (Figures 5b and c), whereas comparable lactate production was noted between the two groups (Figure 5d). Addition of glucose partially prevented the drop of ATP and pyruvate caused by Hx, and significantly increased the lactate contents (Figure 5b–d). The effect of IA was confirmed by lower levels of ATP, pyruvate and lactate production compared with those given glucose without inhibitors in both normoxic and hypoxic conditions (Figure 5b–d). In contrast, UK had no effect on these parameters (Figure 5b–d).

Pyruvate is involved in death resistance through mitochondrial superoxide scavenging without restoration of cellular energy. The specific role of pyruvate in the mechanisms of death resistance was examined by replacing glucose with cell-permeable ethyl pyruvate derivative in hypoxic cells. Addition of pyruvate derivative significantly

![Figure 2](image-url)

**Figure 2**  Hypoxia (Hx)-induced necrotic cell death is dependent on RIP signaling pathways. (a) Pretreatment with necrostatin-1 (Nec-1; a specific RIP1 inhibitor) decreased the Hx-induced LDH leakage in a dose-dependent manner. *P < 0.05 versus respective normoxia (Nx) + F groups; #P < 0.05 versus ‘0 mM’ in Hx + F cells (n = 6/group). (b) Knockdown of RIP1 by siRNA reduced LDH leakage in hypoxic cells. No effect was seen by negative control (CON) siRNA. Knockdown efficiency of transfected cells was confirmed by Western blots. *P < 0.05 versus respective Nx + F groups. †P < 0.05 versus CON (n = 3/group). (c) Representative images showing that Nec-1 inhibited morphological damage and cell detachment caused by 8-h and 24-h Hx. (d) Representative images showing transient mitochondrial hyperpolarization (an increase in red fluorescence intensity after 8 h) and a final collapse of transmembrane potential (an increase in green fluorescence intensity after 24 h) in hypoxic cells treated with Nec-1. The results suggest that RIP1 may not be upstream of mitochondrial dysfunctions (n = 4/group).
reduced the LDH leakage, RIP1/3 complex formation and morphological damage in hypoxic cells (Figure 6a–c). However, the ATP levels in cells given pyruvate were comparable to those without supplementation (Figure 6d). Presence of pyruvate neither altered cellular ATP contents nor suppressed dextran flux in hypoxic cells (Figure 6e), indicating that death resistance by pyruvate was uncoupled with ATP production and energy-dependent processes (e.g., tight junctional restoration). Unlike glucose, pyruvate did not suppress Hx-induced mitochondrial transmembrane potential changes (Figure 6f). These results suggest that pyruvate confers resistance to necroptosis through an alternative, energy-independent mechanism.

Generation of mitochondria-derived oxidative free radicals has been implicated in the cell necrotic pathways triggered by cytotoxic agents, and we sought to examine its role in hypoxic necrosis. Increased mean fluorescence of MitoSox (an indicator of mitochondrial superoxide production) was observed in hypoxic cells compared with normoxic controls in glucose-free media (Figure 7a). Decreasing the mitochondrial superoxide levels with butylated hydroxyanisole (BHA, a free radical scavenger) and apocynin (an inhibitor to nicotinamide adenine dinucleotide phosphate oxidase) (Figure 7a) led to partial inhibition of LDH leakage in hypoxic cells (Figure 7b). Moreover, pretreatment with BHA also diminished the RIP1/3 complex formation (Figure 7c). These results indicate that ROS production, which is upstream of RIP signaling, is involved in Hx-induced necroptotic pathways.

The modulatory effect of glucose and pyruvate on free radical levels was next addressed. Addition of glucose decreased Hx-induced mitochondrial ROS levels (Figure 7d), but did not alter the redox activities of catalase, superoxide dismutase, glutathione reductase or glutathione-S-transferase (Supplementary Figure S5), suggesting a non-enzymatic scavenging mechanism. The glucose-mediated reduction of mitochondrial ROS may be inhibited by pretreatment with IA but not with UK (Figure 7d). Lastly, replacing glucose with cell-permeable pyruvate derivative also significantly suppressed the mitochondrial ROS levels in hypoxic cells (Figure 7d). Similar results of pyruvate-mediated resistance were seen in HT29 cells under hypoxic stress (Supplementary Figure S4-B, E).
Discussion

Malignant cells develop adaptive mechanisms to evade necrotic death caused by depletion of oxygen and nutrients. Our study demonstrates a novel mechanism through which glycolytic pyruvate confers resistance to RIP-dependent necroptosis in hypoxic colorectal carcinoma via mitochondrial superoxide scavenging. To the best of our knowledge, this study is the first to provide evidence of anti-necroptotic pathways against hypoxic stress in colorectal cancer cells.

Despite long-standing observation of cell necrosis in the hypoxic core of colorectal tumors, there is an apparent lack of knowledge on its molecular mechanisms. In our study, we demonstrated that oxygen and glucose deprivation induced RIP1/3 signaling and morphological necrotic features, that is, rupture of plasma membrane, 8–24 h after the onset of challenge in colorectal carcinoma cells. Recent studies from other laboratories have also showed necroptosis in intestinal epithelial cells in models of chronic intestinal inflammation in gene-deficient mice. Although there is no data on the timing of necrotic death caused by hypoxic stress in normal human epithelial cells, rapid villous necrosis in jejunum and colon was found in rats after 60 min of hemorrhagic shock or 40 min of mesenteric ischemia, showing that normal cells are more sensitive to oxygen and nutrient deprivation compared with cancer cells. It is noteworthy that normoxic controls in glucose-free media displayed no sign of necroptosis, indicating that glucose deprivation alone did not trigger necrotic death.

Mitochondrial dysfunctions and free radical generation have also been implicated in the necrotic process caused by cytotoxic agents. The transient mitochondrial
The hyperpolarization observed in our study may reflect a temporal reversal of electron transport chain ATP synthase activity because of a decline in intracellular oxygen, leading to adverse proton pumping against the electrochemical gradient to intermembranous spaces. Moreover, the final mitochondrial potential collapse correlated well with organelle swelling at later time points (16–24 h) of Hx. Interestingly, we identified high levels of mitochondrial superoxide production prior to plasma membrane disintegration in hypoxic colonic carcinoma cells. This seemingly paradoxical situation of ROS emission in Hx has also been previously documented in cardiomyocytes after infarction. The generation of mitochondrial ROS has been suggested to be caused by electron leak to oxygen in the respiratory chain complexes, whereby...
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Figure 7  Pyruvate-mediated mitochondrial superoxide scavenging has a critical role in resistance to hypoxia (Hx)-induced necroptosis. (a) Increased MitoSOX fluorescence units were seen in hypoxic cells at the 8-h time point, suggesting mitochondrial superoxide production upon hypoxic stress. The generation of mitochondrial ROS may be partially prevented by antioxidants (200 μM BHA or 1 mM apocynin), whereas vehicle control (veh) had no effect. *P < 0.05 versus respective normoxia (Nx) groups; #P < 0.05 versus respective Hx groups. The Hx-induced LDH leakage was abolished by pretreatment with antioxidants, suggesting that cell necrosis was dependent on mitochondrial ROS production. *P < 0.05 versus respective Nx groups; #P < 0.05 versus veh (n = 8/group). (b) Immunoprecipitation blots showing the formation of RIP1/3 complex in hypoxic cells, which was inhibited by BHA. The experiments were repeated twice and similar results were obtained. (n = 3/group). (d) Supplementation of glucose decreased mitochondrial ROS levels in hypoxic cells, which was reversed by pretreatment with iodoacetate (IA, 1 mM), but not UK5099 (UK, 10 μM) or vehicle (veh). Replacing glucose with a pyruvate derivative (pyr) also reduced mitochondrial ROS in hypoxic cells. *P < 0.05 versus respectiveNx groups; #P < 0.05 versus veh. (n = 8/group)
degradation. Hx sensing downregulates PHD activity and thus, stabilizes HIF1α levels, leading to its nuclear translocation and downstream transcription of target genes.\textsuperscript{44,45} Paradoxically, PHDs are themselves target genes of HIF1 transcription, indicating a negative feedback mechanism.\textsuperscript{46,47}

In addition, HIF1α degrades within 8–12 h without de novo synthesis.\textsuperscript{48} Therefore, we speculate that the fluctuation of GLUTs expression after the onset of Hx may reflect the HIF1α/PHD loop. Experiments to address the temporal relationship, cellular distribution and pathophysiological significance of various isoforms of GLUTs by HIF1 in colonic carcinomas are currently under progress.

Angiogenic pathways and mediators have been extensively studied as potential anti-cancer drugs for several decades. In some cases, patients fail to respond to antiangiogenic agents or simply develop drug resistance. Tumors may display some cases, patients fail to respond to antiangiogenic agents studied as potential anti-cancer drugs for several decades. In

Materials and Methods

Cell culture models. Human colonic carcinoma Caco-2 and HT29 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen, Grand Island, NY, USA) containing 5 mM glucose and without pyruvate.\textsuperscript{5,51} The media was supplemented with 10% fetal bovine serum, 15 mM HEPES, 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma, St. Louis, MO, USA). Cells were seeded in 96-well (10^5 cells/well) or 24-well (10^6 cells/well) tissue culture plates supplemented with 10% fetal bovine serum, 15 mM HEPES, 100 U/ml penicillin, 1 mM pyruvate (25 mM), was also examined in hypoxic cells. All reagents were purchased from Dharmacon, Lafayette, CA, USA. Cells were transfected with RNAi-mediated knockdown of RIP1.

Hypoxic challenge and glucose deprivation. Cells were deprived of oxygen and glucose as previously described.\textsuperscript{61–63} Hypoxic (Hx) challenge was conducted using a modular incubator chamber (Billups-Rothenberg, Del Mar, CA, USA) by incubation of 5% CO\textsubscript{2} and 95% N\textsubscript{2} at 10 h for 5 min; normoxic (Nx) controls were kept at 5% CO\textsubscript{2} and 95% N\textsubscript{2}. In some groups, cells were pretreated with Nec-1 (a specific RIP1 inhibitor), BHA (200 μM; a free radical scavenger), apocynin (1 mM; an inhibitor to nicotinamide adenine dinucleotide phosphate oxidase) or vehicle controls prior to hypoxic challenge, and then subjected to fluoremetric readings. Alternatively, cells were analyzed by time-lapse microscopy using Application Solution Multi-Dimensional Workstation (Leica Microsystems, Manheim, Germany). Cells were loaded with JC-1 (10 μg/ml) for 30 min before infusion of 5% CO\textsubscript{2} and 95% N\textsubscript{2} into the temperature-controlled moisture chamber of the Application Solution Multi-Dimensional Workstation for live cell imaging.

LDH leakage assay. The leakage of intracellular enzyme LDH into the surrounding environment indicates rupture of plasma membrane, which is a hallmark of cell necrosis. The cell culture supernatant was collected after hypoxic challenge for the measurement of LDH activity. Briefly, a reaction mixture of 0.2 mM NADH and 0.36 mM sodium pyruvate was dissolved in Krebs–Henseleit (K–H) buffer containing 2% bovine serum albumin. The K–H buffer is composed of 116 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO\textsubscript{4}, 2.5 mM CaCl\textsubscript{2}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4} and 24 mM NaHCO\textsubscript{3} (pH 7.4). Ten microliters of cell supernatant and 190 μl of reaction mixture were mixed well in 96-well plates prior to spectrophotometric kinetic readings. Owing to the differences in the absorption spectra of NADH and NAD\textsuperscript{+}, changes in the NADH concentration can be detected at 340 nm. The decrease in absorbance measured every minute over a 10-min period represents the activity of LDH. One unit of LDH activity is defined as the quantity for oxidation of 1 μmol NADH per minute; the LDH activity of cell supernatant was expressed in Units per liter (Unit/l).

Analysis of mitochondrial functions by time-lapse microscopy. Mitochondrial transmembrane potential changes and ROS production were measured by using cell-permeant cationic fluorescent dyes, including 5,5’6,6’-tetrachloro-1,1’3,3’-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) reagents and MitoSOX (Invitrogen). The JC-1 reagent emits red fluorescence in its aggregated form when it accumulates in the negatively charged mitochondrial matrix of viable cells. The monomeric form of JC-1 emits green fluorescence when the dye is dispersed in the cytoplasm because of the loss of mitochondrial transmembrane potential. MitoSOX Red, which selectively targets functional mitochondria, exhibits red fluorescence after oxidation by superoxide. Cells grown on 96-well culture plates or 8-well chamber slides (2 × 10^5 cells/well, Costar) were incubated with JC-1 (10 μg/ml) or MitoSOX (5 μM) for 20 min, and washed twice prior to hypoxic challenge, and then subjected to fluorometric readings. Alternatively, cells were analyzed by time-lapse microscopy using Application Solution Multi-Dimensional Workstation (Leica Microsystems, Manheim, Germany). Cells were loaded with JC-1 (10 μg/ml) for 30 min before infusion of 5% CO\textsubscript{2} and 95% N\textsubscript{2} into the temperature-controlled moisture chamber of the Application Solution Multi-Dimensional Workstation for live cell imaging.

Immuno precipitation of RIP1–RIP3 complex and in vitro kinase assay. Cells lysates were immunoprecipitated with anti-human RIP1 (BD Biosciences, Franklin Lakes, NJ, USA) overnight and then incubated with protein G agarose beads for 1 h at 4°C followed by centrifugation. The pellet was dissolved in electrophoresis sample buffer for heat denaturation. The immune complexes were subjected to reducing SDS-PAGE, and the membranes were incubated with anti-RIP1 (1:1000, BD Biosciences) or polyclonal rabbit anti-RIP3 (1:1000, Abcam, Cambridge, UK) for immunoblotting. For in vitro kinase assays, the bead pellets were incubated in kinase reaction buffer supplemented with 10 μM cold ATP and γ-32P-ATP for 30 min at 30°C. The samples were resolved by SDS-PAGE and exposed to film for autoradiography as previously described.\textsuperscript{19}

RNAi-mediated knockdown of RIP1. RIP1 siRNA and negative control were purchased from Dharmacon, Lafayette, CA, USA. Cells were transfected with 100 nM siRNA oligonucleotides using DharmaFECT siRNA transfection reagents as per manufacturer’s protocol. Knockdown efficiency of transfected cells was confirmed by western blotting 96 h post transfection.

Measurement of TER and paracellular permeability. Cells grown to confluency underwent Nx or Hx for the indicated times. The monolayer TER was measured using an electrovolumeter (World Precision Instruments, Sarasota, FL, USA). Paracellular permeability was assessed by apical-to-basal transport of a dextran probe (MW3000) conjugated to fluorescein (Invitrogen) as described previously.\textsuperscript{20,24}

Immunofluorescent staining of tight junction structures. Cells were exposed to Nx or Hx for 16 h, fixed with 4% paraformaldehyde for 1 h on ice and quenched with 50 mM NH\textsubscript{4}Cl in PBS for 10 min at room temperature. After blocking with 0.1% bovine serum albumin in PBS for 1 h, monolayers were incubated with a polyclonal rabbit anti-human ZO-1 antibody (1:100, Invitrogen) in a permeabilizing buffer (0.05% saponin and 0.1% bovine serum albumin in PBS) for 1 h. Cells were then incubated with secondary antibodies of goat anti-rabbit IgG conjugated to Alexa 488 (1:1000, Invitrogen) for 1 h in the dark and then stained with...
with a Hoechst dye to visualize cell nuclei. The slides were mounted with aqueous mounting media and viewed under a Zeiss fluorescence microscope.

**Measurement of cell apoptosis.** DNA fragmentation, which is a final stage of apoptosis, was measured using a cell detection ELISA kit (Roche Applied Science, Indianapolis, IN, USA) for oligonucleosome amount as previously described. The caspase-3 activity assay (Anaspec, Fremont, CA, USA) was based on spectrophotometric detection of chromophore rhodamine 110 after cleavage from the labeled substrate DEVD–rhodamine 110 according to the manufacturer’s instructions.

RNA extraction and polymerase chain reaction for GLUT transcripts. Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. For semiquantitative polymerase chain reaction (PCR) analysis, the RNA (2 μg) was reverse transcribed with oligo(dT) using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, West Palm Beach, FL, USA) in 20 μl reaction volume. The reverse transcribed product containing 0.1 μg of initial RNA was subjected to PCR amplification in a thermal cycler. The specific primer pairs of human GLUTs 1–4 and β-actin and the thermal cycling procedures were described previously. The amplification conditions were as followed: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 50 s in a total of 30 cycles. The PCR products were separated onto 1% agarose gel containing ethidium bromide and visualized by ultraviolet transillumination.

**Measurement of intracellular pyruvate, ATP and lactate contents.** Cell lysates were used for the assessment of intracellular pyruvate (Biovision, Milpitas, CA, USA) following the manufacturer's instructions. Measurement of intracellular redox enzyme activities. Signaling, Danvers, MA, USA). Band density was determined using the software Gel-pro Analyzer 4.0 (Media Cybernetics, Rockville, MD, USA).

**Measurement of intracellular redox enzyme activities.** The activities of catalase, superoxide dismutase, glutathione reductase or glutathione-S-transferase were measured in cell lysates using commercial assay kits (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer’s instructions.

**Statistical analysis.** All values were expressed as mean ± S.E.M. and the means were compared by one-way analysis of variance followed by a Student–Newman–Keul test. Significance was established at P < 0.05.

**Conflict of Interest**

The authors declare no conflict of interest.

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**Author contributions**

LC-HY and C-YH designed the research; C-YH, W-TK and Y-CH conducted the research; C-YH, T-CL and LC-HY analyzed the data; LC-HY, C-YH and T-CL wrote the paper (and made a major contribution).

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