

The protective role of 5-HMF against hypoxic injury

Ming-Ming Li · Li-Ying Wu · Tong Zhao · Lei Xiong · Xin Huang · Zhao-Hui Liu ·
Xue-Lai Fan · Cheng-Rong Xiao · Yue Gao · Yun-Bao Ma · Ji-Jun Chen ·
Ling-Ling Zhu · Ming Fan

Received: 25 July 2010 / Revised: 11 October 2010 / Accepted: 13 October 2010 / Published online: 6 November 2010
© Cell Stress Society International 2010

Abstract In an attempt to find new types of anti-hypoxic agents from herbs, we identified 5-hydroxymethyl-2-furfural (5-HMF) as a natural agent that fulfills the criterion. 5-HMF, the final product of carbohydrate metabolism, has favorable biological effects such as anti-oxidant activity and inhibiting sickling of red blood cells. The role of 5-HMF in hypoxia, however, is not yet. Our pilot results showed that pretreatment with 5-HMF markedly increased both the survival time and the survival rate of mice under hypoxic stress. The present study was aimed to further investigate the protective role

of 5-HMF and the underlying mechanisms in hypoxic injury using ECV304 cells as an in vitro model. ECV304 cells pretreated with or without 5-HMF for 1 h were exposed to hypoxic condition (0.3% O₂) for 24 h and then cell apoptosis, necrosis, the changes of mitochondrial membrane potential (MMP) and the expressions of phosphorylation- extracellular signal-regulated kinase (p-ERK) were investigated. Pretreatment with 5-HMF markedly attenuated hypoxia-induced cell necrosis and apoptosis at late stage ($p < 0.01$). Furthermore, pretreatment with 5-HMF rescued both the decline of the MMP and the increase of p-ERK protein under hypoxia. In a word, these results indicated that 5-HMF had protective effects against hypoxic injury in ECV304 cells, and its effects on MMP and p-ERK may be involved in the mechanism.

Ming-Ming Li and Li-Ying Wu are co-first author.

M.-M. Li · M. Fan
Beijing Institute for Neuro-Science,
Capital Medical University School of Basic Medical Sciences,
Beijing 100069, People's Republic of China

M.-M. Li · L.-Y. Wu · T. Zhao · L. Xiong · X. Huang · Z.-H. Liu ·
X.-L. Fan · L.-L. Zhu (✉) · M. Fan (✉)
Department of Brain protection and Plasticity,
Institute of Basic Medical Sciences,
No.27 Taiping Rd,
Beijing 100850, People's Republic of China
e-mail: linglingzhu@hotmail.com

M. Fan
e-mail: fanming@nic.bmi.ac.cn

C.-R. Xiao · Y. Gao
Beijing Institute of Radiation Medicine,
Beijing 100850, People's Republic of China

Y.-B. Ma · J.-J. Chen
State Key Laboratory of Phytochemistry and Plant Resources in
Western China, Kunming Institute of Botany,
Chinese Academy of Sciences,
Kunming 650204,
Yunnan, People's Republic of China

Keywords 5-HMF · Hypoxic injury · ECV304 cells · MMP

Abbreviations

5-HMF	5-hydroxymethyl-2-furfural
MTT	3-(4,5-dimethylthiazole-2-yl) 2,5-diphenyltetrazolium bromide
ECV304 cells	Human umbilical cord vein endothelial cells
MMP	Mitochondrial membrane potential
ERK	Extracellular signal-regulated kinase

Introduction

Oxygen is essential for the survival of mammals, and severe hypoxia can lead to highly detrimental events (Ngho et al. 2009). Environmental factors such as altitude and self-related factors such as atherosclerosis, cancer, and ischemia

may initiate hypoxic injury (Semenza 2004; Pattinson et al. 2005). Hypoxic injury is becoming a primary threat to the health of humans. There is an urgent need to develop effective therapies to prevent hypoxic injury.

It is well known that the hypoxic response in mammalian cells occurs through a number of mechanisms (Wotzlaw and Fandrey 2010). To our knowledge, extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinases (MAPKs) pathway plays a crucial role in almost all cell functions. Phosphorylation of ERK is activated in response to many kinds of stress and the activity of ERK can mediate two apparently opposing processes from cell proliferation to cell death (Cagnol and Chambard 2010; Khavari and Rinn 2007; Mebratu and Tesfaigzi 2009). There is now increasing evidence that ERK has the dual effects in hypoxia and compounds that influence ERK can function as protective agents (Montagut and Settleman 2009; Muslin 2008; Ohori 2008). Furthermore, mitochondria participate in the process of hypoxic response in different ways and the mitochondrial membrane potential (MMP) can reflect the function of mitochondria (Seol et al. 2009; Wang et al. 2009a, b; Snyder and Chandel 2009). It is reported that recovery of MMP can protect against ROS-mediated injuries such as hypoxia and ischemia (Iijima 2006; Zhou et al. 2009). Therefore, the maintenance of MMP is very important to prevent from hypoxic injury.

5-hydroxymethyl-2-furfural (5-HMF) is the final product of carbohydrate metabolism. It exists abundantly in a lot of plants and food which have carbohydrate (Nguyen et al. 2005; Wu 2009). Its molecular formula is $C_3H_6O_3$. It has been reported that 5-HMF has favorable biological effects such as anti-oxidant activity, inhibiting sickling of red blood cells and ameliorate hemorheology (Abdulmalik et al. 2005; Okpala 2006; Lin et al. 2008; Li et al. 2009; Villela et al. 2009).

As we know, the mechanism of hypoxic injury contains the process of oxidative stress (Kehrer and Park 1991; Zhu et al. 2009). Since 5-HMF has anti-oxidant activity, we investigated whether it may protect against hypoxic injury and further explored the possible mechanisms involved.

Materials and methods

Materials

The materials used for this study include the following: RPMI1640 (GIBCO, USA), 5-HMF and Rho123 (Sigma, USA), LDH kits (Zhongsheng, China); Annexin V&PI apoptosis test kit (Baosai, China), and p-ERK and ERK antibody (Santa Cruz, USA); β -actin antibody (Sigma, USA).

Cell culture and hypoxia

ECV304 cells were maintained in RPMI 1640 medium (GIBCO, USA) supplemented with 10% fetal calf serum (Hyclone, USA), 100 U/ml penicillin and 100 U/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. After the cells were pretreated with or without 5-HMF for 1 h, they were exposed to 0.3% O_2 at 37°C for 24 h in a hypoxic chamber (Oligo, USA). The normoxic cells were simultaneously cultured in a humidified atmosphere containing 5% CO_2 at 37°C.

Flow cytometric detection of apoptotic cells

Cells were collected by centrifugation and washed with phosphate buffered saline (PBS). According to the manufacturer's instructions of the kit (Baosai, China), the pellet was suspended in 200 μ l of binding buffer, and then 10 μ l of Annexin V-FITC (20 μ g/ml) and 5 μ l of PI (50 μ g/ml) were added. After 15 min, another 300 μ l of binding buffer was added to the mixture, and it was then analyzed by flow cytometry. Fluorescence was detected through a filter by using a FACS Calibur flow cytometer (Becton Dickinson, USA). At least 10,000 cells were analyzed in each sample. Annexin V-positive and PI-negative cells were defined as apoptosis at early stage, while both Annexin V- and PI-positive cells were defined as apoptosis at late stage.

Trypan blue exclusion assay

Cells were collected by trypsinization and centrifugation. The pellet was suspended in culture medium, and then 100 μ l of suspension was added to 400 μ l of 0.4% trypan blue solution. The number of blue-stained dead cells and non-stained living cells were then counted with a hemacytometer within 3 min. The cell mortality rate (%) was calculated as the number of dead cells divided by the number of total cells, multiplied by 100 (Fang et al. 2010; Valadares et al. 2009; Wang et al. 2009a, b).

LDH assay

At the end of incubation, the supernatant was collected from the plates and the LDH content was determined using the LDH assay kit according to the manufacturer's instructions.

Determination of mitochondrial membrane potential

To determine the change of MMP in cells, flow cytometry was applied using Rhodamine 123 (Rh-123) staining (Wu et al. 2008). The cells were harvested and washed with ice-cold PBS twice by centrifugation at 1,000 rpm for 10 min,

and then 0.5 ml PBS contained 10 $\mu\text{g/ml}$ Rh-123 was added to the cells. The tubes were vortexed gently and incubated at 37°C in the dark for 30 min, then were washed with ice-cold PBS twice by centrifugation at 1,000 rpm for 10 min. 0.5 ml PBS was added to the cells. Flow cytometric analysis was carried out by FACScan.

Western blot analysis

After treatment, cells were washed with PBS, scraped, and centrifuged for 30 s at 4,500 rpm. Total protein extract buffer (RIPA buffer—50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), which contained protease inhibitors and phosphatase inhibitors was added to each sample on ice for 30 min, and then samples were centrifuged for 20 min at 14,000 rpm. Protein concentration was determined by the Bradford protein assay, and the samples were stored at -80°C . For western blot analysis, 100 μg of protein was subjected to SDS-PAGE under reducing conditions, and proteins were then transferred to nitrocellulose membranes. The membrane was blocked for 2 h at room temperature with 5% *w/v* nonfat dried milk in PBST. The blots were incubated with p-ERK, ERK, and β -actin antibodies (1:1,000, 1:1,000, and 1:10,000 dilutions) overnight at 4°C, followed by incubation for 2 h with the secondary antibody (1:1,000 dilution). The presence of the target proteins was revealed by a chemiluminescence assay.

Statistical analysis

Data were expressed as mean \pm SD and assessed with one-way ANOVA followed by SPSS software. Comparisons between normoxic group and hypoxic group or hypoxic group and group pretreated with 5-HMF under hypoxia were made with unpaired Student's *t* test. Differences between groups were considered to be significant at $P<0.05$.

Results

5-HMF protected cells against hypoxia-induced necrosis and apoptosis at late stage

Our pilot data suggest that 5-HMF protects against hypoxic injury *in vivo*. We next investigated the role of 5-HMF in anti-hypoxic injury *in vitro* using ECV304 cells. Cells were exposed to hypoxia for 24 h after treatment with different concentrations of 5-HMF. The MTT assay revealed decreases in ECV304 cell survival after exposure to hypoxia for 24 h compared with the normoxic group, and 200 $\mu\text{g/ml}$ 5-HMF treatment significantly increased the

viability compared with the hypoxic control (data not shown). We subsequently tested the effects of 5-HMF on apoptosis and necrosis after cells were exposed to hypoxia for 24 h. Cells were stained with annexin V and PI, and the percentage of apoptotic cells was determined by flow cytometry. Apoptotic cells at early stage were defined as Annexin V positive and PI negative, while apoptotic cells at late stage and necrotic ones were defined as Annexin V and PI positive. As shown in Fig. 1a, under normoxia, there were few apoptotic cells ($7.1\pm 2.0\%$) and 200 $\mu\text{g/ml}$ 5-HMF did not affect the apoptotic rate ($6.8\pm 0.5\%$). After hypoxia, there was obvious increase in the number of apoptotic cells as compared with those under normoxia (Fig. 1b, the apoptotic rate of $34.0\pm 5.7\%$ vs. $7.1\pm 2.0\%$; $p<0.01$), and 200 $\mu\text{g/ml}$ 5-HMF decreased the apoptotic rate about 7% compared with the hypoxic control. Furthermore, interestingly, 5-HMF protected cells against hypoxia-induced necrosis and apoptosis at late stage more significantly than the apoptotic cells at early stage (Fig. 1c, d).

To confirm this, we therefore investigated whether 5-HMF could rescue cells from necrosis and apoptosis at late stage induced by hypoxia. Necrosis which is characterized by cell membrane disruption and cell content release was evaluated by the trypan blue exclusion assay and the LDH leakage assay. The leakage rate of LDH and the trypan blue exclusion were measured as indexes of cell necrosis and apoptosis at late stage.

As shown in Fig. 2, using the trypan blue exclusion assay, the cell mortality rate increased significantly from $0.6\pm 1.3\%$ under normoxia to $37.3\pm 10.1\%$ under hypoxia while 200 $\mu\text{g/ml}$ 5-HMF reduced the cell mortality rate by 20% compared with hypoxia control ($17.7\pm 5.5\%$ vs. $37.3\pm 10.1\%$; $p<0.01$).

After hypoxia, the LDH leakage rate significantly increased compared with the normoxia control (Fig. 3, $11.7\pm 1.1\%$ vs. $2.3\pm 0.4\%$; $p<0.01$), while 200 $\mu\text{g/ml}$ of 5-HMF decreased the LDH leakage rate compared with the hypoxic control (Fig. 3, $8.0\pm 1.3\%$ vs. $11.7\pm 1.1\%$; $p<0.01$). All these results suggested that 5-HMF could protect cells against hypoxia-induced necrosis and apoptosis at late stage.

5-HMF up-regulated MMP after hypoxia

Mitochondria participates in the process of apoptosis and necrosis in different ways and the MMP reflects the function of mitochondria (Chen et al. 2010). To investigate the underlying protective mechanisms of 5-HMF against hypoxia, we assessed the changes of MMP using rhodamine123. Flow cytometry showed that MMP declined after ECV304 cells were exposed to hypoxia for 24 h compared with the normoxia group (Fig. 4; 225.8 ± 20.6 vs. 526.1 ± 33.3 ; $p<0.01$), and 200 $\mu\text{g/ml}$ of 5-HMF rescued the

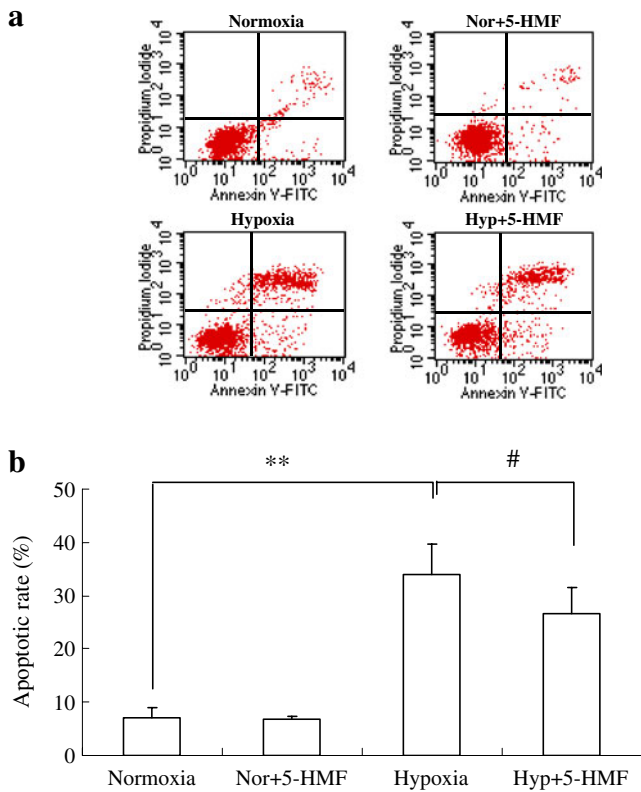
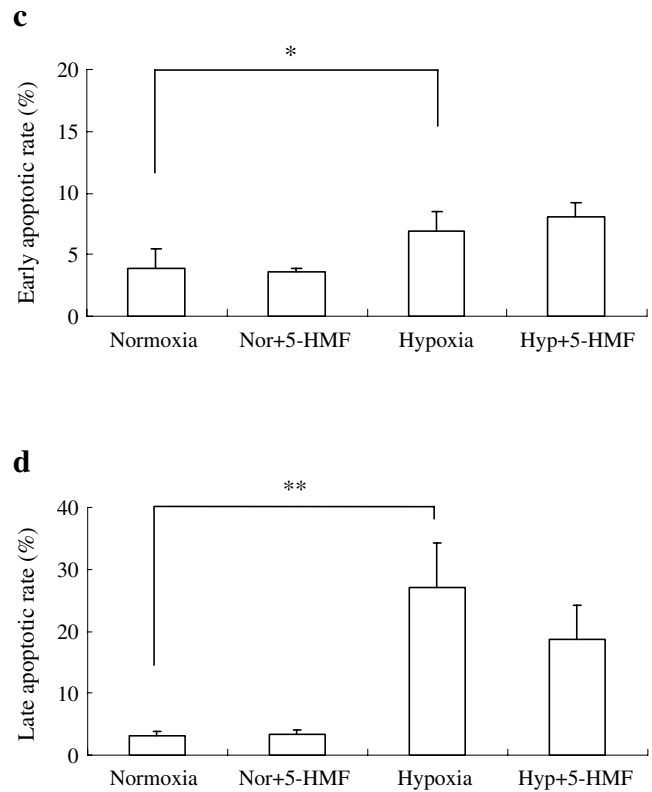


Fig. 1 Effect of 5-HMF on hypoxia-induced ECV304 cell apoptosis and necrosis. **a** Effect of 5-HMF on hypoxia-induced ECV304 cell apoptosis and necrosis determined with flow cytometry; **b** percentage of apoptotic rate in ECV304 cells from control groups or pretreated groups under normoxia and hypoxia. Values are expressed as a percentage (%), which was the number of apoptotic cells divided by the total cells, multiplied by 100; **c** percentage of apoptotic rate in ECV304 cells from control groups or pretreated groups at early stage. Values are expressed as a percentage (%), which was the number of



apoptotic cells at early stage divided by the total cells, multiplied by 100; **d** percentage of apoptotic rate in ECV304 cells from control groups or pretreated groups at late stage and necrosis. Values are expressed as a percentage (%), which was the number of apoptotic cells at late stage and necrosis divided by the total cells, multiplied by 100. All data were expressed as means \pm SD, $n=4$; ** $p<0.01$; * $p<0.05$ versus normoxia control; # $p<0.05$ versus hypoxia control. *Nor* normoxia, *Hyp* hypoxia, *5-HMF* 200 μ g/ml 5-HMF

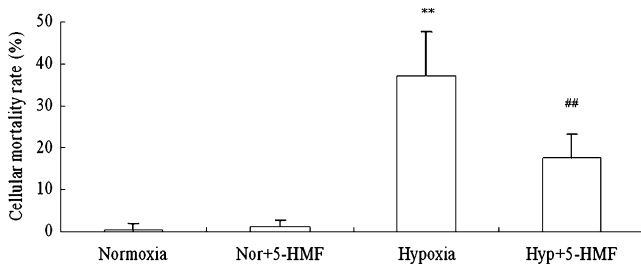


Fig. 2 Effect of 5-HMF on hypoxia-induced cell mortality. Cells were pretreated with 200 μ g/ml 5-HMF for 1 h followed by treatment with hypoxia for 24 h. Cell mortality was measured as described in the materials and methods section. 5-HMF treatment decreased cell mortality in hypoxia-stressed ECV304 cells. Values are expressed as a percentage (%), which was the number of dead cells divided by the total cells, multiplied by 100. All data were expressed as means \pm SD, $n=5$; ** $p<0.01$ versus normoxia control; ## $p<0.01$ versus hypoxia control. *Nor* normoxia, *Hyp* hypoxia, *5-HMF* 200 μ g/ml 5-HMF

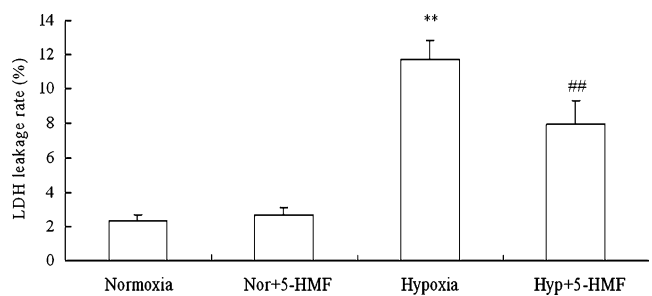


Fig. 3 Effect of 5-HMF on hypoxia-induced LDH leakage rate of ECV304 cells. Cells were pretreated with 200 μ g/ml 5-HMF for 1 h followed by treatment with hypoxia for 24 h. The LDH leakage rate was measured as described in the materials and methods section. 5-HMF treatment decreased LDH leakage in hypoxia-stressed ECV304 cells. The LDH leakage rate (%) is the activity of the cell supernatant divided by the activity of cells in total. All data were expressed as means \pm SD, $n=10$; ** $p<0.01$ versus normoxia control; ## $p<0.01$ versus hypoxia control. *Nor* normoxia, *Hyp* hypoxia, *5-HMF* 200 μ g/ml 5-HMF

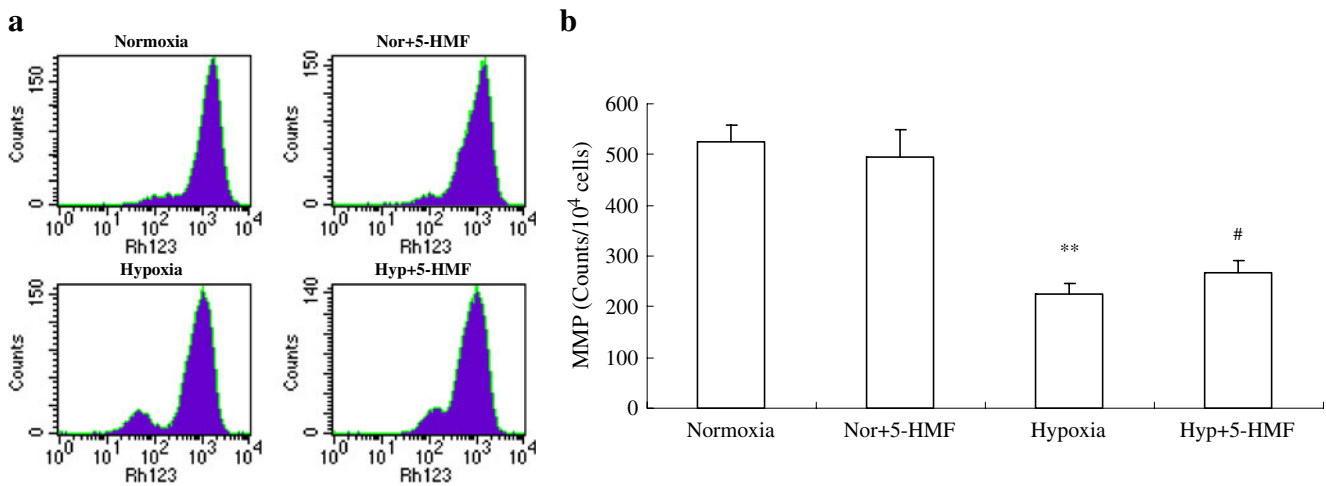


Fig. 4 Effect of 5-HMF on hypoxia-induced MMP. Cells were pretreated with 200 $\mu\text{g/ml}$ 5-HMF for 1 h followed by treatment with hypoxia for 24 h. The MMP-related fluorescence intensity of ECV304 cells was measured by flow cytometry. **a** Changes of the MMP-related

fluorescence intensity of ECV304 cells. **b** Quantitative analysis of the effect of 5-HMF on MMP. All data were expressed as means \pm SD, $n=4$; $**p<0.01$ versus normoxia control; $\#p<0.05$ versus hypoxia control. *Nor* normoxia, *Hyp* hypoxia, *5-HMF* 200 $\mu\text{g/ml}$ 5-HMF

decline of MMP partly compared with the hypoxia group (Fig. 4; 268.1 ± 23.6 vs. 225.8 ± 20.6 ; $p<0.05$). These results suggested that the protective role of 5-HMF in anti-hypoxia might be involved in regulation of mitochondrial function.

Effect of 200 $\mu\text{g/ml}$ 5-HMF on p-ERK expression

Meanwhile, ERK has been shown previously to mediate cellular adaptive processes to a hypoxic stress as a pivotal factor. But it is unclear whether ERK was also associated with the process of 5-HMF protection against hypoxia-induced cell necrosis and apoptosis at late stage. We measured the expression of ERK by western blotting. As reported, hypoxia led to increased p-ERK protein. Interestingly, compared with the hypoxic cells, the immunoblots revealed a decrease in the levels of p-ERK protein of the 5-HMF pretreated cells under hypoxia (Fig. 5). The above data indicated that 5-HMF inhibited the expression of p-ERK, which might be involved in the protective role of 5-HMF against hypoxic injury.

Discussion

It is well known that oxygen homeostasis represents a fundamental physiological process and hypoxia is a serious stress that can cause a lot of detriments. Necrosis and apoptosis play a great role in the process (Bacon and Harris 2004; Kulkarni et al. 2007; Bursch et al. 2008). Blocking the necrosis and apoptosis process induced by hypoxia may help to slow down or even prevent the occurrence and progression of the injuries. Therefore, it is very important to find a critical molecule involved in the mechanisms of

protecting against hypoxia-induced necrosis and apoptosis to prevent hypoxic injury. 5-HMF, the final product of carbohydrate metabolism, has been reported to have favorable biological effects such as anti-oxidant activity, inhibiting sickling of red blood cells and ameliorate hemorheology. However, the role of 5-HMF in anti-hypoxic injury is still unclear and the possible mechanism remains to be clarified, especially at cellular level. In the present study, we have focused on demonstrating the protective effects of 5-HMF against hypoxia and the possible molecular mechanisms underlying the effects.

Our pilot study showed that 5-HMF could increase both the survival time and the survival rate of mice (data not shown). In this study, we further approached the protective effects of 5-HMF against hypoxia in vitro. The results indicated that 5-HMF could improve the viability of cells

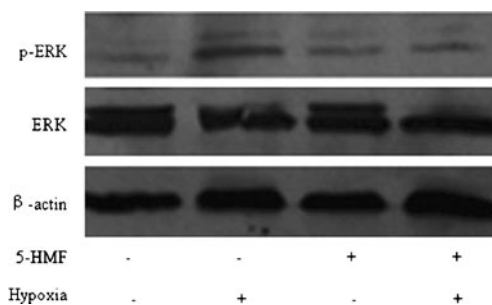


Fig. 5 Effect of 5-HMF pretreatment on the expression of p-ERK protein. Cells were pretreated with 200 $\mu\text{g/ml}$ 5-HMF for 1 h followed by treatment with hypoxia for 24 h. Total cell extracts were analyzed with Western blotting using a mouse polyclonal antibody to p-ERK, β -actin levels were performed to assess the total amount of proteins loaded on the gel. *Nor* normoxia, *Hyp* hypoxia, *5-HMF* 200 $\mu\text{g/ml}$ 5-HMF

under hypoxic conditions, thus it confirmed protective effects of 5-HMF against hypoxia.

The degree of injury to capillary endothelial cells under hypoxia and ischemia and the integrity of their tight junctions are important for the integrity of the BBB (Torii et al. 2007; Culot et al. 2009). Human umbilical cord vein endothelial cells (ECV304 cells) are morphologically and biochemically similar to primary endothelial cells. They are therefore favorable for the study of endothelial cells in vitro and have been used extensively in this respect (Luo et al. 2002; Zhao et al. 2008). Thus, we further verified the protective effects of 5-HMF primarily in ECV304 cells. Our present study confirmed that hypoxia for 24 h significantly increased cell necrosis and apoptosis at late stage as evidenced by elevated apoptotic rate, LDH leakage rate, and mortality rate. However, pretreatment with 200 µg/ml of 5-HMF greatly reduced apoptotic rate, LDH leakage rate, and mortality rate. These findings strongly suggested that 5-HMF might exert a protective effect against the hypoxia-induced cytotoxicity and inhibit cell necrosis and apoptosis at late stage in response to hypoxia.

On the basis of the obtained results that 5-HMF protected against necrosis and apoptosis at late stage induced by hypoxia, further investigation was performed with focuses on the possible mechanisms involved in the protective effects of 5-HMF. It is well known that mitochondria participates in the process of anti-apoptosis and necrosis in different ways and the MMP can reflect the function of mitochondria (Seol et al. 2009; Wang et al. 2009a, b). It is reported that recovery of MMP can protect against injuries such as diabetes, hypoxia, and ischemia (Iijima 2006; Manna et al. 2009; Seol et al. 2009). Interestingly, we found that hypoxia for 24 h declined MMP and 200 µg/ml of 5-HMF rescued the decline of MMP partly. These findings suggested that 5-HMF protected against necrosis and apoptosis at late stage induced by hypoxia via a mitochondria-mediated pathway.

Recent studies show that ERK activation functions upstream of mitochondrial dysfunction and further initiates apoptosis or necrosis (Dagda et al. 2009; Zhuang et al. 2008). ERK/MAPKs pathway is responsible for activation of cell survival/damaging mechanisms (Seger and Krebs 1995). As noted above, phosphorylation of ERK is activated in response to many kinds of stress, and its effect changes with different conditions, depending on the stimulus and its persistence time, and inhibition of p-ERK expression by U0126-reduced endothelial cell death by OGD (Narasimhan et al. 2009). In this study, we hypothesized that the mechanism by which 5-HMF inhibited hypoxic stress-induced cell necrosis and apoptosis at late stage lies in the inhibition of p-ERK protein. In agreement with the previous study, phosphorylation of ERK was significantly activated in hypoxia as shown in Fig. 5.

Interestingly, compared with hypoxia control, the expression level of p-ERK protein in cells pretreated with 5-HMF was decreased under hypoxia. These results indicated that 5-HMF inhibited the activation of ERK protein, which might be involved in the protective role of 5-HMF against hypoxia.

Taken together, our findings suggest directly that 5-HMF can prevent damage from severe hypoxia. Although the mechanisms are not fully elucidated, the protective role of 5-HMF is closely associated with the function of mitochondria and the expression of p-ERK protein. The detailed mechanisms need to be further investigated.

Acknowledgements This work was supported by the National Basic Research Program of China (2006CB504100) and a key grant of the Natural Sciences Foundation of China (30393130 and 30730112).

References

- Abdulmalik O, Safo MK, Chen Q, Yang J, Brugnara C, Ohene-Frempong K, Abraham DJ, Asakura T (2005) 5-hydroxymethyl-2-furfural modifies intracellular sickle haemoglobin and inhibits sickling of red blood cells. *Br J Haematol* 128:552–561
- Bacon AL, Harris AL (2004) Hypoxia-inducible factors and hypoxic cell death in tumour physiology. *Ann Med* 36:530–539
- Bursch W, Karwan A, Mayer M, Dornetshuber J, Frohwein U, Schulte-Hermann R, Fazi B, Di Sano F, Piredda L, Piacentini M, Petrovski G, Fesus L, Gerner C (2008) Cell death and autophagy: cytokines, drugs, and nutritional factors. *Toxicology* 254:147–157
- Cagnol S, Chambard JC (2010) ERK and cell death: mechanisms of ERK-induced cell death—apoptosis, autophagy and senescence. *FEBS J* 277:2–21
- Chen Y, Lewis W, Diwan A, Cheng EH, Matkovich SJ, Dorn GW (2010) Dual autonomous mitochondrial cell death pathways are activated by Nix/BNip3L and induce cardiomyopathy. *Proc Natl Acad Sci* 107:9035–9042
- Culot M, Mysiorek C, Renftel M, Roussel BD, Hommet Y, Vivien D, Cecchelli R, Fenart L, Berezowski V, Dehouck MP, Lundquist S (2009) Cerebrovascular protection as a possible mechanism for the protective effects of NXY-059 in preclinical models: an in vitro study. *Brain Res* 1294:144–152
- Dagda RK, Zhu J, Chu CT (2009) Mitochondrial kinases in Parkinson's disease: converging insights from neurotoxin and genetic models. *Mitochondrion* 9:289–298
- Fang WT, Li HJ, Zhou LS (2010) Protective effects of prostaglandin E1 on human umbilical vein endothelial cell injury induced by hydrogen peroxide. *Acta Pharmacol Sin* 31:485–492
- Iijima T (2006) Mitochondrial membrane potential and ischemic neuronal death. *Neurosci Res* 55:234–243
- Kehrer JP, Park Y (1991) Oxidative stress during hypoxia in isolated-perfused rat heart. *Adv Exp Med Biol* 283:299–304
- Khavari TA, Rinn J (2007) Ras/Erk MAPK signaling in epidermal homeostasis and neoplasia. *Cell Cycle* 6:2928–2931
- Kulkarni AC, Kuppusamy P, Parinandi N (2007) Oxygen, the lead actor in the pathophysiologic drama: enactment of the trinity of normoxia, hypoxia, and hyperoxia in disease and therapy. *Antioxid Redox Signal* 9:1717–1730
- Li YX, Li Y, Qian ZJ, Kim MM, Kim SK (2009) In vitro antioxidant activity of 5-HMF isolated from marine red alga *Laurencia*

- undulata* in free-radical-mediated oxidative systems. *J Microbiol Biotechnol* 19:1319–1327
- Lin AS, Qian K, Usami Y, Lin L, Itokawa H, Hsu C, Morris-Natschke SL, Lee KH (2008) 5-Hydroxymethyl-2-furfural, a clinical trials agent for sickle cell anemia, and its mono/di-glucosides from classically processed steamed *Rehmanniae Radix*. *J Nat Med* 62:164–167
- Luo WB, Dong L, Wang YP (2002) Effect of magnesium lithospermate B on calcium and nitric oxide in endothelial cells upon hypoxia/reoxygenation. *Acta Pharmacol Sin* 23:930–936
- Manna P, Sinha M, Sil PC (2009) Protective role of arjunolic acid in response to streptozotocin-induced type-I diabetes via the mitochondrial dependent and independent pathways. *Toxicology* 257:53–63
- Mebratu Y, Tesfaigzi Y (2009) How ERK1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? *Cell Cycle* 8:1168–1175
- Montagut C, Settleman J (2009) Targeting the RAF-MEK-ERK pathway in cancer therapy. *Cancer Lett* 283:125–134
- Muslin AJ (2008) MAPK signalling in cardiovascular health and disease: molecular mechanisms and therapeutic targets. *Clin Sci (Lond)* 115:203–218
- Narasimhan P, Liu J, Song YS, Massengale JL, Chan PH (2009) VEGF Stimulates the ERK 1/2 signaling pathway and apoptosis in cerebral endothelial cells after ischemic conditions. *Stroke* 40:1467–1473
- Ngoh GA, Facundo HT, Hamid T, Dillmann W, Zachara NE, Jones SP (2009) Unique hexosaminidase reduces metabolic survival signal and sensitizes cardiac myocytes to hypoxia/reoxygenation injury. *Circ Res* 104:41–49
- Nguyen AT, Fontaine J, Malonne H, Claeys M, Luhmer M, Duez P (2005) A sugar ester and an iridoid glycoside from *Scrophularia ningpoensis*. *Phytochemistry* 66:1186–1191
- Ohuri M (2008) ERK inhibitors as a potential new therapy for rheumatoid arthritis. *Drug News Perspect* 21:245–250
- Okpala I (2006) Investigational agents for sickle cell disease. *Expert Opin Investig Drugs* 15:833–842
- Pattinson KT, Sutherland AI, Smith TG, Dorrington KL, Wright AD (2005) Acute mountain sickness, vitamin c, free radicals, and hif-1alpha. *Wilderness Environ Med* 16:172–173
- Seeger R, Krebs EG (1995) The MAPK signaling cascade. *FASEB J* 9:726–735
- Semenza GL (2004) Hydroxylation of hif-1: oxygen sensing at the molecular level. *Physiol Bethesda* 19:176–182
- Seol JW, Lee HB, Lee YJ, Lee YH, Kang HS, Kim IS, Kim NS, Park SY (2009) Hypoxic resistance to articular chondrocyte apoptosis—a possible mechanism of maintaining homeostasis of normal articular cartilage. *FEBS J* 276:7375–7385
- Snyder CM, Chandel NS (2009) Mitochondrial regulation of cell survival and death during low-oxygen conditions. *Antioxid Redox Signal* 11:2673–2683
- Torii H, Kubota H, Ishihara H, Suzuki M (2007) Cilostazol inhibits the redistribution of the actin cytoskeleton and junctional proteins on the blood-brain barrier under hypoxia/reoxygenation. *Pharmacol Res* 55:104–110
- Valadares MC, de Carvalho IC, de Oliveira JL, Vieira Mde S, Benfica PL, de Carvalho FS, Andrade LV, Lima EM, Kato MJ (2009) Cytotoxicity and antiangiogenic activity of grandisin. *J Pharm Pharmacol* 61:1709–1714
- Villela NR, Cabrales P, Tsai AG, Intaglietta M (2009) Microcirculatory effects of changing blood hemoglobin oxygen affinity during hemorrhagic shock resuscitation in an experimental model. *Shock* 31:645–652
- Wang L, Luo HS, Xia H (2009a) Sodium butyrate induces human colon carcinoma HT-29 cell apoptosis through a mitochondrial pathway. *J Int Med Res* 37:803–811
- Wang WD, Xu XM, Chen Y, Jiang P, Dong CZ, Wang Q (2009b) Apoptosis of human Burkitt's lymphoma cells induced by 2-N, N-diethylaminocarbonyloxymethyl-1-diphenylmethyl-4-(3, 4, 5-trimethoxybenzoyl) piperazine hydrochloride (PMS-1077). *Arch Pharm Res* 32:1727–1736
- Wotzlav C, Fandrey J (2010) Monitoring of cellular responses to hypoxia. *Methods Mol Biol* 591:243–255
- Wu CD (2009) Grape products and oral health. *J Nutr* 139:1818S–1823S
- Wu JN, Huang J, Yang J, Tashiro S, Onodera S, Ikejima T (2008) Caspase inhibition augmented oridonin-induced cell death in murine fibrosarcoma 1929 by enhancing reactive oxygen species generation. *J Pharmacol Sci* 108:32–39
- Zhao Y, Lu N, Li H, Zhang Y, Gao Z, Gong Y (2008) High glucose induced human umbilical vein endothelial cell injury: involvement of protein tyrosine nitration. *Mol Cell Biochem* 311:19–29
- Zhou YJ, Zhang SP, Liu CW, Cai YQ (2009) The protection of selenium on ROS mediated-apoptosis by mitochondria dysfunction in cadmium-induced LLC-PK(1) cells. *Toxicol In Vitro* 23:288–294
- Zhu D, Wu L, Li CR, Wang XW, Ma YJ, Zhong ZY, Zhao HB, Cui J, Xun SF, Huang XL, Zhou Z, Wang SQ (2009) Ginsenoside Rg1 protects rat cardiomyocyte from hypoxia/reoxygenation oxidative injury via antioxidant and intracellular calcium homeostasis. *J Cell Biochem* 108:117–124
- Zhuang S, Kinsey GR, Yan Y, Han J, Schnellmann RG (2008) Extracellular signal-regulated kinase activation mediates mitochondrial dysfunction and necrosis induced by hydrogen peroxide in renal proximal tubular cells. *J Pharmacol Exp Ther* 325:732–740