Biochimica et Biophysica Acta 1850 (2015) 640–646

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Spontaneously hyperactive MEK-Erk pathway mediates paradoxical facilitation of cell proliferation in mild hypoxia

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ARTICLE INFO

Article history:
Received 29 July 2014
Received in revised form 12 November 2014
Accepted 4 December 2014
Available online 10 December 2014

Keywords:
Mild hypoxia
Cell proliferation
MAP kinase cascade
Erk
Caco2 cells
Cell reprogramming

ABSTRACT

Background: Oxygen is important for common eukaryotic cells to generate ATP. Pathophysiological conditions such as ischemic diseases cause tissue hypoxia. In addition, oxygen availability in deep tissues is supposed to be far lower than surrounding atmosphere even in healthy animals, and the oxygen partial pressures in most normal tissues are estimated to be around 40–50 mmHg, so-called mild hypoxia. Recent studies have demonstrated that mild hypoxia has distinct effects on living cells from severe hypoxia. For instance, mild hypoxia was reported to promote cell reprogramming. Although severe hypoxia is known to inhibit cell proliferation, mild hypoxia has been paradoxically demonstrated to increase cell proliferation. However, it has not been clarified by which molecular mechanisms mild hypoxia evokes the discontinuous increment of cell proliferation.
Methods: We established experimental conditions showing the opposite influences of mild and severe hypoxia on cell proliferation using undifferentiated Caco2 human colon carcinoma cells in order to clarify the underlying molecular mechanism.
Results: The basal activity of Erk, which is a typical mediator of mitogenic signals, is spontaneously increased specifically in cells exposed to mild hypoxia, and inhibition of MEK, an upstream kinase of the Erk, completely inhibited the mild hypoxia-induced enhancement of cell proliferation.
Conclusions: Spontaneous hyperactivation of the MEK-Erk pathway by mild hypoxia should be the plausible molecular mechanism of the paradoxical promotion of cell proliferation.
General Significance: Our findings will provide clues to the molecular basis of mild hypoxia-evoked phenomena such as cell reprogramming.

1. Introduction

Oxygen availability is a critical issue for most eukaryotic cells since they use oxygen as a fuel for oxidative phosphorylation to generate ATP, which is also known as a common energy transfer currency. Hypoxia, a state in which there are low oxygen levels, can be a consequence of pathophysiological conditions such as cardiovascular and cerebral ischemia, inflammation, pulmonary disorders and cancer. However, oxygen partial pressures in deep tissues are supposed to be far lower than the surrounding atmosphere even in healthy animals. Oxygen partial pressure in inspired air is about 160 mmHg corresponding to ~21% O_2 at sea level, and it decreases progressively within the body. The oxygen partial pressure in arterial circulation was reported as 90–110 mmHg (12–15% O_2) while that in venous circulation was 35–40 mmHg (~5% O_2) [1]. The oxygen partial pressures vary among the tissues depending on the type of organ in terms of such conditions as vascularization and location in the body. In rodents, tissue oxygen partial pressures were reported as 4–8 mmHg in spleen, 19–33 mmHg in thymus, 10–30 mmHg in liver, 11–16 mmHg in hypothalamus and 1–40 mmHg in cortex, and the oxygen partial pressures in most of the normal tissues are considered around 40–50 mmHg [1–4]. Furthermore, tumor tissues possess more extensive portions of hypoxia relative to the corresponding normal tissue [5]. However, most cell

Abbreviations: Erk, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; MEK, MAPK/Erk kinase; JNK, c-JUN N-terminal kinase; SOD2, manganese superoxide dismutase; HIF, hypoxia inducible factor; PI 3-kinase, phosphoinositide 3-kinase; BrdU, bromodeoxyuridine; LDH, lactose dehydrogenase

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http://dx.doi.org/10.1016/j.bbagen.2014.12.006
0304-4165/

culture experiments have been conducted under the surrounding ordinary atmosphere, while studies on hypoxia have been performed in severe hypoxia of around 1–3% O_2 in most cases. Therefore, more attention should be paid to the effects of mild hypoxia corresponding to the normal tissues. Recent studies have demonstrated that mild hypoxia has specific effects on living cells. Actually, mild (but not severe) hypoxia has been employed to facilitate cell reprogramming [6]. Low oxygen circumstances less than ~1% O_2 inhibit cell proliferation and possibly increase cell death. However, relatively mild hypoxic conditions around 5–7% O_2 have been contradictorily known to increase cell proliferation [7–9]. Although there are a number of reports mentioning either of these discontinuous effects of hypoxia on cell proliferation, there have been no reports clearly showing the discrepancy in the same experiment. In the present study, we established the assay conditions exhibiting the opposite influences of the mild and severe hypoxia on the cell proliferation using undifferentiated Caco2 human colon carcinoma cells in order to clarify the molecular mechanism underlying the distinct facilitation of cell proliferation by mild hypoxia, and here we propose spontaneous activation of Erks as the plausible mechanism.

2. Materials and methods

2.1. Materials and reagents

All reagents were analytic grade and obtained from Wako Pure Chemical (Osaka, Japan) or Kanto Chemical (Tokyo, Japan) unless otherwise stated. U0126 was purchased from LC laboratories (Woburn, MA, US). Wortmannin, PD98059 and GW5074 were from Kyowa Medex (Tokyo, Japan), Cayman Chemical (Ann Arbor, MI, US) and Focus Biomolecules (Plymouth Meeting, PA, US), respectively. The concentrations of inhibitors used in this study were 100 nM for wortmannin, 10 μM for U0126, 25 μM for PD98059 and 5 μM for GW5074, respectively, unless otherwise stated. Human recombinant insulin was from Sigma Aldrich (St. Louis, MO, US). Caco2, human colorectal adenocarcinoma cells, were obtained from American Type Culture Collection (Manassas, VA, US).

2.2. Cell culture and hypoxic stimulations

Caco2 cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum with antibiotics. The cells were inoculated in the cell culture plate (Iwaki Glass, Tokyo, Japan) and incubated in humidified atmosphere with 5% CO_2 and 95% air for 24 h and then incubated in atmosphere with 5% CO_2 and 1% (~7.6 mmHg), 6% (~45.6 mmHg) or 20% (~152 mmHg) O_2 filled with inert N_2 for the indicated durations in an automatic multi-gas incubator (IncuSafe, MCO-5 M; Sanyo Electric, Osaka, Japan) as previously described [10]. As for short-term hypoxic stimulation within 30 min, culture media were equilibrated in the hypoxic atmosphere ahead of stimulation. The hypoxic stimulation was started with changing medium for the conditioned one, and then the cells were incubated under hypoxia.

2.3. Cell proliferation assay

Cell proliferation was assessed by MTT (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described with modification [11,12]. Briefly, 5×10^3 cells were inoculated in 96-well plates and incubated for 24 h, and then cultured under normoxic or hypoxic conditions as indicated, which was followed by reaction for 2 h with MTT at a concentration of 200 μg/ml. The formazan formed in the cells was dissolved in dimethyl sulfoxide, and the absorbance at 570/630 nm was measured using a microplate spectrophotometer (Varioskan Flash; Thermo Scientific, Wilmington, DE, US). The number of cells was expressed as a ratio to the control under normoxic conditions.

2.4. Expression levels of hypoxia-inducible factor (HIF)-1α and phosphorylation levels of Erk and Akt by Western blotting analysis

Cells were rapidly washed with ice-cold phosphate buffered saline (PBS) and lysed with Hepes/Triton-based lysis buffer as described previously [13,14]. For evaluation of the inhibitors, cells were treated with the compound for 10 min before being stimulated by 100 nM insulin for 60 min. Twenty micrograms of protein for each sample was subjected to SDS-PAGE using 7.5% polyacrylamide gel (Bio-Rad). Antibodies against HIF-1α were purchased from Cayman Chemical, and those against Akt, phospho-Akt, Erk and phospho-Erk were from Cell Signaling Technology. ECL reagents and Hyperfilm ECL (GE Healthcare) were used for detection. Perfection 2580 Photo Scanner (Seiko Epson, Suwa, Japan) and Image J software (NIH) were used for quantification. Two closely related isoforms, Erk 1 and 2, were detected and these isoforms were quantified altogether.

2.5. Bromodeoxyuridine (BrdU) incorporation assay

Cell proliferation activity was also confirmed by BrdU incorporation assay. The BrdU taken up into the cells was determined by a colorimetric ELISA kit using an anti-BrdU antibody (Millipore) in accordance with the manufacturer's instructions. Briefly, 2×10^4 cells were inoculated and incubated for 24 h, and then cultured under normoxic or hypoxic conditions as indicated, which was followed by incubation with the BrdU reagent for 4 h. The cells were fixed and the incorporated BrdU was immunologically detected by coupling with peroxidase and tetramethylbenzidine. Absorbance at 450/550 nm was quantified by the microplate spectrophotometer after acidification.

2.6. Cell death evaluation by MTT assay

We also determined the number of living Caco2 cells when the hypoxia and/or inhibitors treatment was applied to the cells already in full confluence. To put it concretely, we started by inoculating 5×10^4 cells and cultured them for 24 h under normal cultural circumstances. When the cells had grown confluently, the cells were incubated under normoxic or hypoxic conditions with or without inhibitors as indicated. The number of living cells was determined by MTT assay as aforementioned.

2.7. Cell death evaluation by lactose dehydrogenase (LDH) assay

Extracellular LDH activity is a typical marker of cell death. LDH activities in the culture media were determined by quantifying NADH production using LDH cytotoxicity detection kit (Takara Bio, Shiga, Japan). In brief, 50 μl of each culture medium was incubated with the premixed solution containing diaphorase, NAD and 2-[4-indophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride for 30 min at 30 °C, and the resultant formazan was estimated by absorbance at 490/600 nm. The number of dead cells was calculated by converting the values of controls under normoxic conditions as zero and those of 2% Triton X-100-treated samples as 100% according to the manufacturer's instruction.

2.8. Statistical analyses

Values are expressed as means ± S.E. Comparisons between groups were evaluated by Dunnett's or Tukey-Kramer's test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Mild hypoxia induces discontinuous facilitation of cell proliferation.

We determined the effects of mild (6% O_2) and severe (1% O_2) hypoxia on the proliferation of colon carcinoma Caco2 cells to clarify the cell

growth rate in hypoxic conditions similar to physiological tissue circumstances in vivo. We selected Caco2 cells because the relevance of hypoxia and/or HIFs in colorectal adenocarcinomas have been well studied. As expected, severe hypoxia reduced the number of living cells by ~20% in five days (Fig. 1A). However, mild hypoxia did not reduce but inconsistently increased the cells by ~15% in contrast (Fig. 1A). These effects of hypoxia on the cell proliferation were enlarged on the seventh experimental day (Fig. 1B). In contrast to the cell proliferation, the expression levels of HIF-1α increased in accordance with the intensity of the hypoxia (Fig. 1C).

3.2. Mild hypoxia augments the basal phosphorylation levels of Erk, while severe hypoxia suppresses it

We then searched signaling pathways related to cell proliferation for the plausible molecular mechanism leading to the discontinuous proliferative effect of mild hypoxia. Erk is a canonical molecule that belongs to mitogen-activated protein kinase (MAP kinase) family, and it is widely recognized to mediate mitogenic signals as its name designates. Therefore, we evaluated basal activities of Erk in cells cultured under mild and severe hypoxic states by determining phosphorylation ratio of Erk. After one day of incubation, severe hypoxia resulted in a decrease in the phosphorylation ratio of Erk while mild hypoxia did not cause significant effects on Erk (Fig. 2A). The diminishment of Erk phosphorylation continued for at least seven days (Fig. 2B, C). In contrast, the mild hypoxia oppositely led to an increase in the phosphorylation ratio of Erk after the fifth experimental day (Fig. 2B, C). The expression levels of Erk were not affected by the hypoxic stimulations. Two closely conserved isoforms, Erk 1 and 2, were detected, and all the changes in phosphorylation levels were in parallel between the isoforms. In addition, we also determined the effects of the mild and severe hypoxia within 30 min on the phosphorylation status of Erk. Such short durations of the hypoxic stimulations did not increase the phosphorylation levels but rather tended to decrease them when the cells were incubated for 30 min under mild or severe hypoxic conditions (Fig. 2D).

3.3. Mild hypoxia-induced facilitation of proliferation is dependent on MEK-Erk pathway

To elucidate the significance of the increments of the basal Erk phosphorylation evoked in mild hypoxia, we investigated the effects of U0126, a specific inhibitor of the upstream kinase, MEK [15,16]. Without U0126, the differences in the cell number were observed after the fifth experimental day, consistent with the data shown in Fig. 1 (Fig. 3A). Extended duration of severe hypoxia reduced the number of cells alive in seven days (Fig. 3B). In the presence of U0126, the facilitation of cell proliferation induced in mild hypoxia was completely inhibited to the same extent as that in normoxic conditions (Fig. 3).

3.4. Validation of the MEK inhibitor, U0126 in Caco2 cells

We estimated the effects of U0126 to confirm the efficacy and specificity of U0126 in Caco2 cells. Pretreatment of U0126 dose-dependently suppressed basal and insulin-stimulated phosphorylation of Erk (Fig. 4A). As much as 1–10 μM of U0126 completely inhibited Erk phosphorylation. At a concentration of 10 μM, U0126 did not affect basal and insulin-evoked phosphorylation of Akt, while a typical phosphoinositide (PI) 3-kinase inhibitor, wortmannin, completely inhibited Akt phosphorylation but did not affect Erk, although we missed the apparent insulin-evoked increase in Erk phosphorylation at that time point (Fig. 4B).

3.5. The mild hypoxia-induced facilitation of proliferation is sensitive to other Raf-MEK-Erk pathway inhibitors

We also evaluated the effects of other inhibitors against Raf-MEK-Erk pathway on mild hypoxia-induced cell proliferation. In the presence of another popular MEK inhibitor, PD98059, the facilitation of cell proliferation induced in mild hypoxia was completely suppressed to the same extent as that in normoxic conditions (Fig. 4C). In addition, GW5074, a Raf inhibitor, also completely inhibited mild hypoxia-induced cell proliferation (Fig. 4C). While the MEK inhibitors, U0126 and PD98059, reduced the number of living cells even in the normoxic and severe hypoxic conditions, GW5074 did not affect them (Figs. 3 and 4C).

3.6. Mild hypoxia induces discontinuous facilitation of DNA synthesis

We tried confirming that the mild hypoxia-induced increase in cell number actually reflects the facilitation of cell proliferation using BrdU incorporation assay. In accordance with the results of MTT assay, severe hypoxia reduced DNA synthesis by ~60% in five days, and mild hypoxia augmented DNA synthesis by ~40% in contrast (Fig. 4D). The MEK inhibitors, U0126 and PD98059, completely inhibited the mild hypoxia-induced facilitation of DNA synthesis to the same extent as those in normoxia (Fig. 4D).

3.7. Mild hypoxia did not suppress cell death

To eliminate the possibility that the number of the living cells we observed does not reflect cell proliferation but is dominantly affected by cell death rate, we determined the effects of hypoxia and inhibitors on the number of dead cells. At first, MTT assay was conducted using cells already fully confluent in culture plates (Fig. 5A). In theory, the number of formazan-positive cells should be decreased only when the rate of cell death exceeds that of proliferation in this experimental condition. The number of living cells was not decreased by the mild hypoxic stimulation, while the severe hypoxia reduced it (Fig. 5A). Under normoxic or mild hypoxic conditions, neither U0126 nor

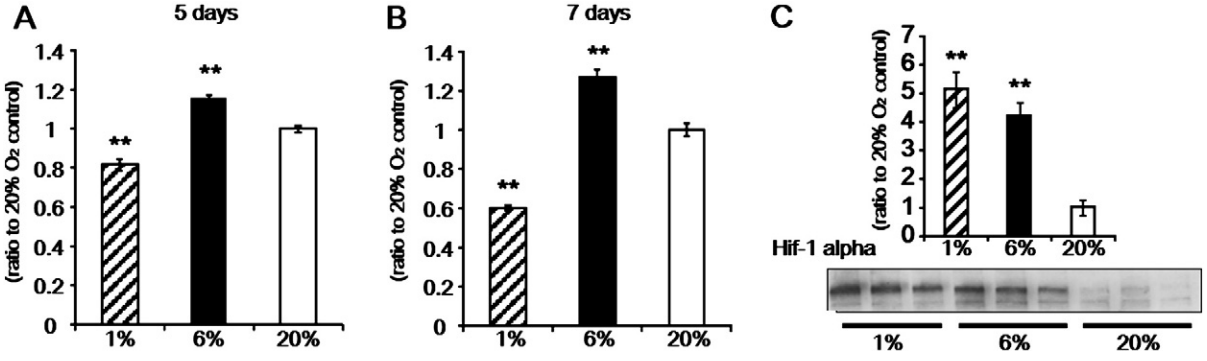

Fig. 1. Cell proliferation and HIF-1α expression levels under mild and severe hypoxic conditions. The number of living Caco2 cells cultured in the 1% (hatched bar), 6% (filled bar) or 20% (open bar) O_2 conditions for five (A) or seven days (B). Absolute values of the cell numbers for 1.0 were 1.5×10^4 cells (A) and 4.5×10^4 cells (B). $n = 5$. HIF-1α expression levels after 24 h culture at 1% (hatched bar), 6% (filled bar) or 20% (open bar) O_2 (C). $n = 3$; **, $p < 0.01$ vs. 20% O_2 control.

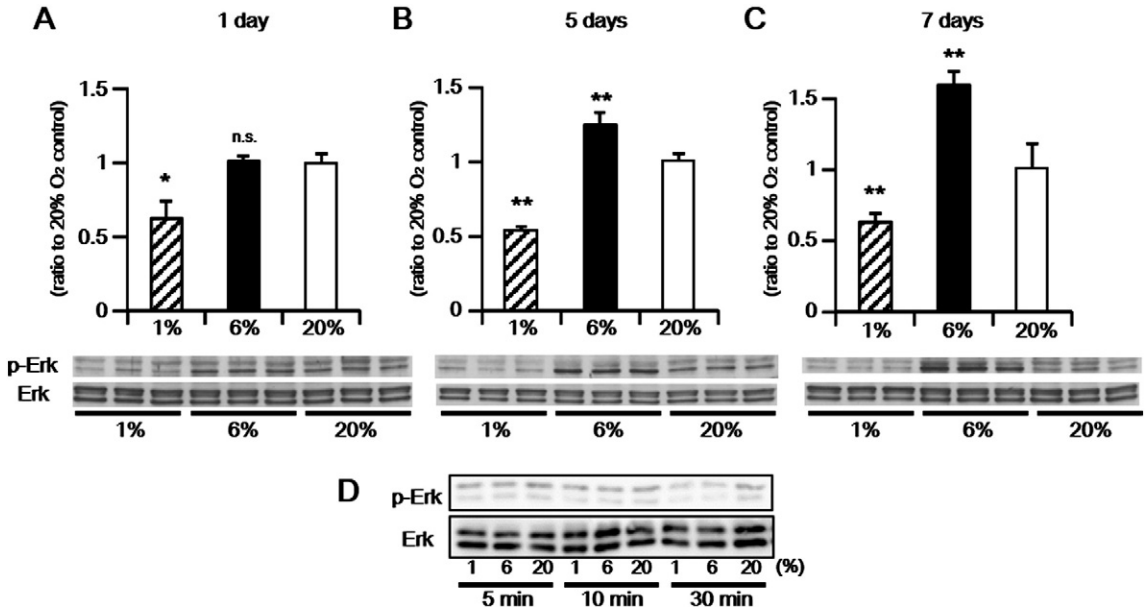

Fig. 2. Change in the basal phosphorylation ratio of Erk in mild and severe hypoxic conditions. The ratios of phosphorylation of Erk in the quiescent Caco2 cells cultured in the 1% (hatched bar), 6% (filled bar) or 20% (open bar) O_2 conditions for one (A), five (B) or seven days (C). $n = 3$. Phosphorylation status of Erk under hypoxic conditions for 5, 10 and 30 min (D). n.s., not significant; *, $p < 0.05$; **, $p < 0.01$ vs. 20% O_2 control.

PD98059 decreased the number of living cells, but GW5074 did slightly (Fig. 5A).

Activities of the extracellular LDH, a typical marker of cell mortality, were also determined. The lower the oxygen partial pressure was, the higher the extracellular LDH activity was in the absence of the inhibitors (Fig. 5B). None of the inhibitors used here increased mortality in normoxia (Fig. 5B). U0126 reduced cell death under the severe and mild hypoxic conditions, and PD98059 suppressed it under severe hypoxia. GW5074 did not affect the cell mortality even under hypoxic conditions (Fig. 5B).

4. Discussion

In the present study, we successfully demonstrated the opposite influences of mild and severe hypoxia on cell proliferation in the presence of serum within the same assay conditions (Fig. 1A, B). In contrast, we confirmed the gradual increase in expression levels of HIF-1α, which is widely accepted as a dominant player in the hypoxic response, as O_2 levels decreased consistent with previous reports (Fig. 1C) [17–20].

It has been unclear by which molecular mechanisms the mild hypoxia evokes the discontinuous increment of cell growth inconsistently with the severe hypoxia, although this mild hypoxia-caused proliferative effect is well known. Erk, which is a representative molecule of the MAP kinase family, is a typical mediator of mitogenic signals. Many kinds of extracellular stimuli including growth factors achieve mitogenic actions through the highly conserved Ras-Raf-MEK-Erk signaling cascade. Aberrant regulation of the Ras-Raf-MEK-Erk pathway often leads to disorders in cell proliferation. Actually, a series of activating mutations of the molecules in this pathway have been found in various kinds of human cancers [21–24]. We therefore paid attention to Erk in search of plausible molecular mechanisms leading to the paradoxical facilitation of cell proliferation by mild hypoxia. The activities of each isoform of Erk 1 and 2 were evaluated by phosphorylation ratios as the phosphorylation of threonine 202 and tyrosine 204 residues of Erk 1, and threonine 185 and tyrosine 187 residues of Erk 2, which lie in the activation loop and are specifically phosphorylated by upstream kinases MEK 1 and 2, correlating well with their activities [25,26].

This is the first report demonstrating the facilitation of basal Erk activity caused by mild hypoxia to the best of our knowledge. On the other hand, severe hypoxia was not suggested to activate but rather inactivate Erk, which may be consistent with previous studies denying the involvement of Erk in hypoxic signals [27]. In contrast, hypoxic stimulation has been reported to activate other MAP kinase family proteins, JNK and p38. However, JNK and p38 are mainly believed to

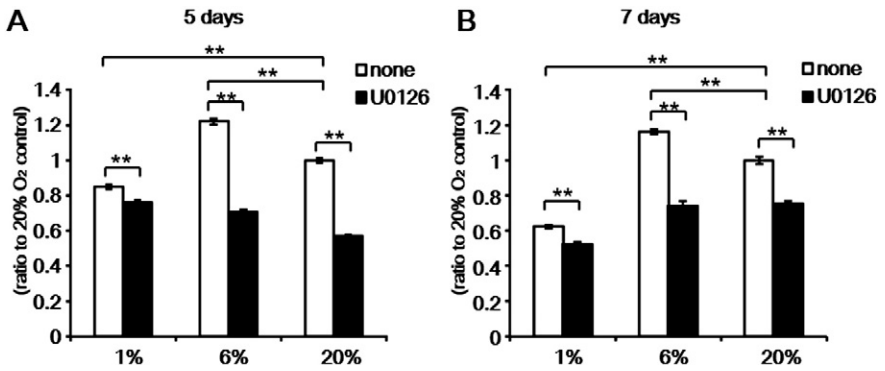

Fig. 3. Effects of MEK inhibitor on cell proliferation under mild and severe hypoxic conditions. The number of living Caco2 cells cultured in the 1%, 6% or 20% O_2 conditions with (filled bar) or without (open bar) a MEK inhibitor, U0126, for five (A) or seven days (B). $n = 12$; **, $p < 0.01$.

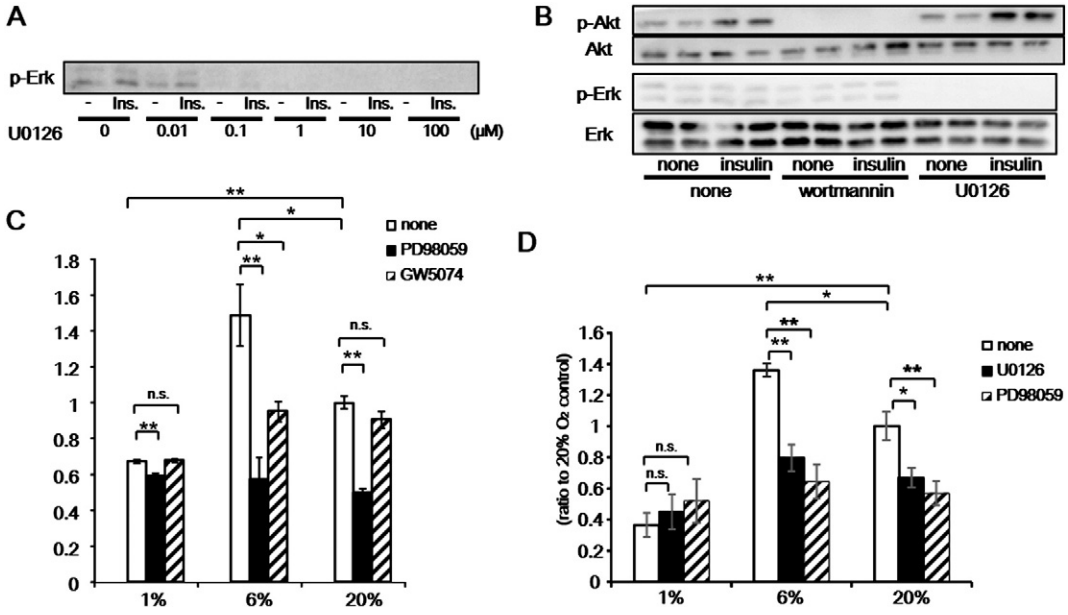

Fig. 4. Evaluation of the effects of U0126 and other Raf-MEK-Erk pathway inhibitors. Dose-responsive inhibitory effects of U0126 on the Erk phosphorylation in Caco2 cells (A). The effects of wortmannin and U0126 on the phosphorylation status of Akt (Thr308) and Erk (B). The indicated samples were from the cells stimulated by 100 nM insulin for 60 min following inhibitor pretreatments. The number of living cells cultured under the 1%, 6% or 20% O_2 conditions in the absence (open bar) or presence of another MEK inhibitor, PD98059 (filled bar) or a Raf inhibitor, GW5074 (hatched bar) for five days (C). Bromodeoxyuridine (BrdU) incorporation in the cells cultured under the 1%, 6% or 20% O_2 conditions in the absence (open bar) or presence of U0126 (filled bar) or PD98059 (hatched bar) for five days (D). $n = 4$–6. n.s., not significant; *, $p < 0.05$; **, $p < 0.01$.

regulate stress and immune responses rather than mitogenic actions and are differentially regulated [28]. Therefore, JNK or p38 are not suggested to play dominant roles in mild hypoxia-evoked cell proliferation, although we do not have enough data to exclude the possibility. By contrast, U0126, a specific inhibitor of MEK, almost completely inhibited the mild hypoxia-enhanced cell proliferation to the same extent as the normoxia (Fig. 3). We confirmed that U0126 completely inhibits basal and enhanced phosphorylation of Erk but does not affect that of Akt, which could mediate PI 3-kinase-dependent signals to promote cell proliferation at the 10 μM concentration used here (Fig. 4A, B). Furthermore, another popular specific MEK inhibitor, PD98059, also exhibited similar inhibitory effects (Fig. 4C). These results were replicated by BrdU incorporation assays evaluating DNA synthesis, which should be correlated with proliferation rate (Fig. 4D). Thus, our observations clearly demonstrate the significance of the MEK-Erk pathway in cell proliferation evoked by mild hypoxia. In addition, GW5074, an inhibitor of Raf, an upstream kinase of MEK, also completely inhibited the effects of mild hypoxia on cell proliferation (Fig. 4C). Therefore, Raf is supposed to be involved in mild hypoxic signaling, although it remains to be determined what makes the spontaneous hyperactivation of the Raf-MEK-Erk pathway in mild hypoxia.

Differently from our observations, it was reported that not mild (10% O_2) but severe (1–3% O_2) hypoxia promotes the proliferation of primary fibroblasts from bovine neonatal aortic adventitia even without serum [29]. The article also showed that the severe hypoxia evokes transient facilitation of phosphorylation of MAP kinases including Erk1 and 2 within one hour, while we did not find any increase in Erk phosphorylation in Caco2 cells by mild or severe hypoxia within such a short duration (Fig. 2D). The sensitivity to oxygen availability may be different among the cell types.

In fact, the cell proliferation assays adopted in the current study, MTT assay (Figs. 1A, 1B, 3, and 4C) and BrdU incorporation assay (Fig. 4D) cannot identify which causes changes in the number of living cells, proliferation or cell death. Thus, we evaluated the cytotoxic effects of the hypoxia and inhibitors. Extracellular LDH activities increased in response to the severity of hypoxia, suggesting that cell mortality should be dependent on oxygen availability (Fig. 5B). The facilitation of cell proliferation should exceed the increase in cell death under mild hypoxia because the number of living cells determined by MTT assay did not change when cells in full confluence were used in the assay (Fig. 5A). In contrast, the number of living cells in the MTT assay using confluent cells decreased by a similar extent to the increase in

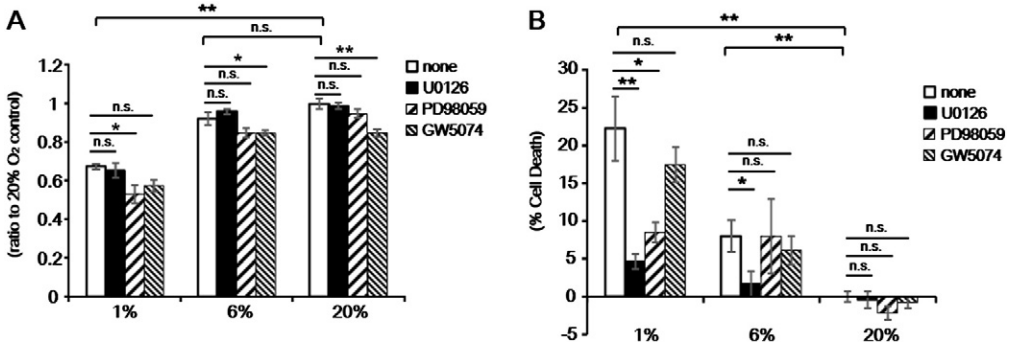

Fig. 5. Evaluation of cell death induced by the hypoxic conditions and/or inhibitors. The number of living Caco2 cells cultured under 1%, 6% or 20% O_2 conditions for five days with or without inhibitors as indicated when fully confluent cells were used for the assay (A). The number of dead cells were evaluated by lactose dehydrogenase (LDH) activities released into the culture medium when cells were cultured under the indicated conditions for five days (B). $n = 4$–6. n.s., not significant; *, $p < 0.05$; **, $p < 0.01$.

dead cells determined by the LDH assay in severe hypoxic conditions (Fig. 5). Thus, it is not suggested that the cell proliferation rate itself is promoted under severe hypoxia.

Furthermore, neither U0126 nor PD98059 increased cell death under mild hypoxia (Fig. 5). Therefore, these MEK inhibitors should indeed block the mild hypoxia-induced cell proliferation. It is interesting that MEK inhibitors suppressed the severe hypoxia-induced LDH leakage even though Erk phosphorylation did not increase under the 1% oxygen conditions (Figs. 2 and 5B). These inhibitors should also work on cell proliferation because the total number of living cells did not increase eventually (Figs. 3 and 4D). GW5074 also exhibited no or quite small cytotoxicity, supporting the significance of Raf in the mild hypoxia-evoked cell proliferation (Fig. 5).

Mild hypoxia has been attracting considerable attention recently in terms of cell reprogramming. The rate of reprogramming of mouse embryonic fibroblasts induced by the retroviral introduction of four factors, Oct3/4, Sox2, Klf4 and c-Myc was reported to be augmented not under 1% but under 5% O_2 circumstances, which is similar to the distinct cell proliferation by mild hypoxia we observed in the present study [6]. They also showed that exposure to mild hypoxia increased the efficiency of induced pluripotent (iPS) cells from human dermal fibroblasts. Although the molecular mechanism of the efficient cell reprogramming into iPS cells has not been clarified, it may be related to c-Myc signals since the mild hypoxia-induced increase in reprogramming rate achieved by introducing only three factors (excluding c-Myc) was as high as that by the full four factors [6].

c-Myc is a proto-oncogenic transcription factor that regulates cell growth, cell cycle progression, DNA repair and metabolism [30,31]. Activation of c-Myc has been suggested to facilitate cell proliferation. Several reports have suggested the involvement of c-Myc in hypoxic responses. Dr. Chan and his colleagues demonstrated that mild hypoxia at 5% O_2 circumstances promotes survival and proliferation instead of cell cycle arrest of primary cultured astrocytes from manganese superoxide dismutase (SOD2) homozygous knockout mice [7,8]. They observed an increase in c-Myc expression induced by 5% O_2 hypoxia even though it occurred only in SOD2-null astrocytes [7]. HIFs are believed to play dominant roles in hypoxic responses as mentioned above. c-Myc was shown to lie downstream of HIF-2α and was proposed to facilitate neoplastic progression in hypoxic tumor cells, and deacetylation of histone H4 by hypoxic stimulation was diminished in c-Myc null Rat-1 cells, although they adopted severe hypoxic stimulations around 0.5% or 1% O_2 instead of mild hypoxia in their studies [32,33]. It is possible that c-Myc mediates mitogenic signals evoked by the mild hypoxia because c-Myc can be subject to the regulation of the MEK-Erk pathway. The spontaneous hyperactive MEK-Erk pathway should function more upstream in mild hypoxic response as Erk was reported to phosphorylate and stabilize c-Myc [34,35]. The facilitation of the MEK-Erk pathway we found here may also play a role in the promotion of cell reprogramming caused by mild hypoxia.

In conclusion, we found that mild hypoxia facilitates spontaneous activation of MEK-Erk signals, which is suggested to be a plausible molecular mechanism of the increase in cell proliferation caused by mild hypoxia, and the MEK-Erk pathway should be an important mediator of mild hypoxia-evoked phenomena such as the promotion of cell reprogramming. An upstream kinase, Raf, should be involved in the mechanism of MEK-Erk signal activation. In addition, our findings reinforce the significance of total MAP kinase signaling cascades in tumor expansion in deep tissues under circumstances that are found in mild hypoxia.

Acknowledgments

We thank Kayoko Miyoshi for secretarial assistance and Drs. Toshiaki Tamaki, Kazuhiko Tsutsumi and Satoko Nakanishi for helpful advice. This work was partly supported by a grant from the Regional Cluster Program, Japan.

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