Microtubular stability affects cardiomyocyte glycolysis by HIF-1α expression and endonuclear aggregation during early stages of hypoxia

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Teng M, Dang YM, Zhang JP, Zhang Q, Fang YD, Ren J, Huang YS. Microtubular stability affects cardiomyocyte glycolysis by HIF-1α expression and endonuclear aggregation during early stages of hypoxia. Am J Physiol Heart Circ Physiol 298: H1919–H1931, 2010. First published March 12, 2010; doi:10.1152/ajpheart.01039.2009.—Hypoxia-inducible factor (HIF)-1α is a key regulator of anaerobic energy metabolism. We asked the following question: Does the breakdown of microtubular structures influence glycolysis in hypoxic cardiomyocytes by regulating HIF-1α? Neonatal rat cardiomyocytes were cultured under hypoxic conditions, while microtubule-stabilizing (paclitaxel) and -depolymerizing (colchicine) agents were used to change microtubular structure. Models of high microtubule-associated protein 4 (MAP4) expression and RNA interference of microtubulin expression were established. Microtubular structural changes and intracellular HIF-1α protein distribution were observed with laser confocal scanning microscopy. Content of key glycolytic enzymes, viability, and energy content of cardiomyocytes were determined by colorimetry and high-performance liquid chromatography. HIF-1α protein content and mRNA expression were determined by Western blotting and real-time PCR, respectively. Low doses of microtubule-stabilizing agent (10 μmol/l paclitaxel) and enhanced expression of MAP4 stabilized the reticulostribular structures in hypoxic cardiomyocytes, increased the content of key glycolytic enzymes, ameliorated energy supply and enhanced cell viability, and upregulated HIF-1α protein expression and endonuclear aggregation. In contrast, the microtubule-depolymerizing agent (10 μmol/l colchicine) or reduced microtubulin expression had adverse effects on the same parameters, in particular, HIF-1α protein content and endonuclear aggregation. We conclude that microtubular structural changes influence glycolysis in the early stages of hypoxia in cardiomyocytes by regulating HIF-1α content. Stabilizing microtubular structures increases endonuclear and total HIF-1α expression, content of key glycolytic enzymes, and energy supply. These findings provide potential therapeutic targets for ameliorating cell energy metabolism during early myocardial hypoxia.

paclitaxel; colchicine; microtubule-associated protein 4; interference RNA; PCR; immunofluorescence; glycolytic enzymes; ATP; ADP; pyruvate kinase; hexokinase; phosphofructokinase; lactate; α-microtubulin

HYPOXIA IS a common pathophysiological phenomenon in all kinds of ischemic diseases, but it is also seen after a severe burn. Disturbances in energy metabolism are an important factor in hypoxia-induced cellular structural and functional damage. Previous studies on disturbances in energy metabolism of cardiomyocytes under hypoxic or ischemic/hypoxic conditions focused mainly on aerobic metabolism and glycolysis, in particular, changes in the content of key metabolic enzymes and regulation of glycolysis by certain factors [e.g., hypoxia-inducing factor (HIF)] (2, 12, 13, 44). However, the precise mechanism(s) leading to disturbances in energy metabolism under hypoxia remains unclear, and consequently, effective clinical measures are not available.

Microtubules and mitochondria change under hypoxic conditions, and there is a relationship between the changes in cardiomyocyte microtubules and cell signal pathways regulating energy metabolism. In hypoxia, mitochondrial function is rapidly downregulated and the energy from glycolysis is upregulated. Our previous studies suggested that a breakdown of microtubular structures in hypoxic cardiomyocytes might decrease energy content, and transfection of the physiological microtubule stabilizer microtubule-associated protein 4 (MAP4) could stabilize microtubules and ameliorate cell energy content during early hypoxia (4, 15a, 26, 37). Hypoxia may stimulate glycolysis in cardiomyocytes in less than a minute, thus increasing anaerobic ATP content and lactate synthesis by processes mainly regulated by HIF-1α (2). In addition, we demonstrated previously that upregulation of HIF-1α protein expression increases the content of key glycolytic enzymes in neonatal rat cardiomyocytes under hypoxic conditions.

It has been reported that structural breakdown of microtubules and other cytoskeleton components downregulates HIF-1α protein expression, thus altering downstream proteins or signal pathways (3, 7, 8, 18, 40, 43). We hypothesized that the breakdown of microtubular structures in hypoxic cardiomyocytes would decrease the content of key glycolytic enzymes by regulating HIF-1α content and endonuclear aggregation and, in addition, influence energy supply in cardiomyocytes during early hypoxia. This study represents part of a wider study on the effects of severe burns on cardiac tissue, and we examined glycolysis because cardiac energy metabolism in severe burns will have a direct effect on heart function and patient survival rate. Elucidation of the actions of microtubular structure will help to identify potential targets for ameliorating disturbances in the energy metabolism of hypoxic cells.

MATERIALS AND METHODS

Primary cardiomyocyte culture. Neonatal Sprague-Dawley rats (1–2 days old) were provided by the Animal Center of the Third Military Medical University. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Pub. No. 85-23, revised 1996), and the project was reviewed and approved by the Animal Experiment Ethics Committee of the Third Military Medical University. Rat ventricular muscles were harvested and digested with trypsin and then cultured according to protocols published previously (37, 44). Neo-
natal rat ventricular cardiomyocytes were cultured in DMEM-F-12 (Hyclone) with 5-bromodeoxyuridine (BrdU; 31 mg/l), 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), penicillin G (100 U/ml), and streptomycin (100 mg/ml) before hypoxia treatment (20, 21).

**Experimental model and groups.** A hypoxia model was established as described previously (2, 7). All experimental groups were subjected to protocols at 37°C and treated under the same conditions. After 2 days of primary culture, cardiomyocytes were divided into five groups: 1) **H** group: hypoxia alone; 2) **HT** group: hypoxia plus pretreatment for 8 h (7) with 5, 10, or 15 μmol/l paclitaxel (Sigma; final concentration; HT5, HT10, and HT15 groups, respectively); 3) **HC** group: hypoxia plus pretreatment for 8 h with 10 μmol/l colchicine (Sigma) (7, 8); 4) **hypo** plus transfection for MAP4 high expression; and 5) **hypo** plus transfection for α-microtubulin low expression. Paclitaxel was used to polymerize microtubules, whereas colchicine was used to depolymerize microtubules. Although colchicine is known to disrupt microtubule assembly, it also causes cell cycle arrest, a possible confounding effect. However, only a very few cultured neonatal rat cardiomyocytes normally go through cell division and proliferate; moreover, most of the proliferation was found in the first 2 days of primary culture. In our model, we add colchicine on the third day of primary culture, thus eliminating the effect of colchicine on cardiomyocyte cell cycles. Moreover, the addition of BrdU inhibits fibroblast cell division but scarcely inhibits cardiomyocyte division.

Cardiomyocytes were transfected for 36 h with a recombinant adenovirus containing the MAP4 gene or the α-microtubulin interference plasmid. Hypoxic conditions were produced by placing culture plates in sealed vacuum bags containing 94% N2, 5% CO2, and 1% O2 at 37°C. We used the hypoxia group at 0 h as the nonhypoxia control value. The O2 content in the anaerobic jar was <1.5 vol% and was verified with an oxygen content radiometer.

**Construction of recombinant adenovirus vector carrying MAP4 and establishment of cardiomyocyte model highly expressing MAP4.** After cloning and amplification of the rat MAP4 cDNA, the samples were subcloned into pShuttle2 plasmids to construct the recombinant shuttle plasmid pShuttle2-MAP4. The pShuttle2-EGFP (enhanced green fluorescent protein) was used as a negative control.

**In vitro cardiomyocyte model with low microtubulin expression by RNA interference of α-microtubule mRNA.** Four RNA interference fragments were designed based on rat α1-microtubulin cDNA sequence and a rat α-microtubulin interference plasmid, Tuball-4, constructed with pGensil-4 vector. A hexokinase (HK) plasmid (negative control) was constructed with pGensil-1 vector. The two plasmids were used to construct recombinant adenovirus vectors for the small interfering RNA and HK groups.

**Immunofluorescence assay of cardiomyotube microtubules and HIF-1α.** Immunocytochemical staining was performed as described previously (37, 40). Primary antibodies used were anti-rat α-microtubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-rat HIF-1α antibody (Chemicon, Temecula, CA). Secondary antibodies used were FITC- and tetramethylrhodamine isothiocyanate (TRITC)-conjugated antibodies (Jackson ImmunoResearch, West Grove, PA). Finally, counterstaining of nuclei was performed with 4',6-diamidino-2-phenylindole (DAPI; Biotium, Hayward, CA).

**Determination of content of lactate dehydrogenase in culture supernatants, key glycolytic enzymes, and lactic acid content.** Phosphofructokinase (PFN) content was detected according to the methods.
of Lilling and Beitner (17); the content of HK, pyruvate kinase (PK), and lactate dehydrogenase (LDH) and the lactic acid content were assessed with conventional colorimetric methods according to kit instructions (Nanjing Jiancheng Biotech, Nanjing, China), and conventional colorimetry was performed with a Beckman DC-7 ultraviolet-visible light spectrophotometer. Each experiment was repeated twice, and each group was repeated five times (n = 10).

**Determination of ATP and ADP contents.** ATP and ADP were extracted with perchloric acid and measured by HPLC (Gilson) as described previously (25). Each experiment repeated twice, and each group was repeated five times (n = 10).

**Determination of HIF-1α protein expression.** Western blotting was performed as described previously (32). The results were analyzed with the DOC Gel2000 gel imaging system (Bio-Rad). β-Actin (Santa Cruz Biotechnology) was used as an internal standard (7, 18). Each experiment was repeated twice, and each group was repeated five times (n = 10).

**Real-time PCR assay of HIF-1α mRNA.** Total RNA was extracted from cardiomyocytes with TRizol reagent according to the manufacturer’s instructions (Invitrogen). HIF-1α mRNA was subjected to RT-PCR with a Realtime 7500 PCR apparatus (Applied Biosystems) according to the instruction manual (SYBRII Green Realtime PCR; Toyobo) and published methodology (32). The results were analyzed with Applied Biosystems 7500 system v1.4.0 software. Each experiment repeated twice, and each group was repeated five times (n = 10).

**Statistical analysis.** Normal distribution and homoscedasticity of tests of the data were confirmed, and two-way ANOVA was performed with SPSS 12.0 software. Results are given as means ± SD, and statistical significance was set at P ≤ 0.05.

**RESULTS**

**Effect of paclitaxel and colchicine on microtubular structures in cardiomyocytes.** The effects of paclitaxel and colchicine on nonhypoxic cardiomyocytes were studied with respect to microtubular structural organization. Three different concentrations (5, 10, and 15 μmol/l) of paclitaxel and colchicine were chosen according to previous reports, and the cultures were examined after 12 h. All three concentrations of paclitaxel increased the measured fluorescence activity and polymerized microtubules (Fig. 1). On the other hand, all three concentrations of colchicine depolymerized microtubules, and there was a breakdown of reticular microtubular structures (RMS) in a dose-dependent manner in the cultures, although the 5 μmol/l colchicine dosage had only a marginal effect on microtubule structure. However, 15 μmol/l colchicine aggravated damage to cardiomyocytes and significantly decreased key glycolytic enzymes, LDH release, and ATP content. The 10 μmol/l colchicine dosage was chosen because of its significant depolymerizing effect on microtubules while at the same time minimizing effects on other processes.

Under normoxic conditions, reticular microtubules were distributed evenly and densely in the cytoplasm of cardiomyocytes. The reticular distribution of microtubules was distinct near the cytomembrane (Fig. 2, A–C). RMS broke down after hypoxia and fluorescence intensity was weaker, and these changes were aggravated by increasing the time of hypoxia (Fig. 2, D–F, H). In the colchicine-treated groups after 1 h of hypoxia, a breakdown of the RMS was evident as well as an indistinct RMS near the cytomembrane; microtubules were unevenly distributed throughout the cell (Fig. 2, D–F, HC). Such changes were aggravated by prolonging the hypoxia. Paclitaxel showed a dose-related response in the cultures, in which higher dosages increased the measured fluorescent activity compared with hypoxia alone (Fig. 2G). There appeared to be no significant differences between the hypoxia group at 1 h and 3 h and the 5 μmol/l dosage group, whereas at 6 h, and at all other dosages and times, the fluorescence intensity was significantly higher with paclitaxel treatment compared with the hypoxia group. While the hypoxia-induced breakdown of RMS appeared slightly less with the low paclitaxel dose, higher dosages appeared to have an increasing effect on the RMS such that treatment with 15 μmol/l paclitaxel caused a significant increase in microtubule fluorescence associated with RMS that was extremely uneven in its distribution, with patchy aggregations of microtubulin observed in the cytoplasm (Fig. 2, D–F, HT15). These effects were similar to those observed in normothermic cultures treated with 5, 10, and 15 μmol/l paclitaxel for 12 h. However, RMS in these normothermic cultures was clearer than in the hypoxia group with paclitaxel, and fluorescence intensity was higher.

**Effect of paclitaxel and colchicine on hypoxic cardiomyocyte viability.** LDH content in cell culture supernatants usually indicates the severity of cell injury. After hypoxia, LDH contents in culture supernatants were significantly elevated, indicating an increased LDH leakage (Fig. 3). Release of LDH in the H group at 1, 3, and 6 h after hypoxia was significantly higher than that at 0 h after hypoxia. The LDH content was significantly higher in the colchicine- and 15 μmol/l paclitaxel-treated groups compared with hypoxia alone. Five micromoles/liter of paclitaxel did not significantly affect LDH leakage from hypoxic cells, although LDH leakage was significantly decreased in response to 10 μmol/l paclitaxel compared with the untreated hypoxic cells after 3 and 6 h of hypoxia. Thus cell viability is significantly reduced by colchicine and high doses of paclitaxel (>10 μmol/l) when cells are subjected to hypoxia (other measures of cell survival suggest that there is no significant difference in the lower cell survival with 15 μmol/l paclitaxel and colchicine under hypoxic conditions; data not shown). These results parallel the disruption seen to microtubular structure as described above for 15 μmol/l paclitaxel and colchicine under hypoxic conditions.

The results of glycolysis, LDH content, and ATP levels in normoxic cardiomyocytes treated with paclitaxel and colchicine for 12 h are shown in Fig. 4. LDH content in cardiomyo-
cytes treated with 10 and 15 μmol/l colchicine or 15 μmol/l paclitaxel was significantly higher than that in the control group (Fig. 4A). PK, HK, PFK, and ATP contents in cardiomyocytes treated with 15 μmol/l colchicine were significantly lower than those in the untreated control group (Fig. 4, B–E).

Effect of paclitaxel and colchicine on content of key glycolytic enzymes and energy metabolism in hypoxic cardiomyocytes. ATP content fell for all groups between 1 and 6 h in culture when subjected to hypoxia. ATP content (Fig. 5A) was significantly decreased after hypoxia in the H group, and ATP content was significantly lower in the colchicine treatment group at each sample time compared with the hypoxia-alone group. The results were more variable in the paclitaxel groups such that ATP content was significantly higher for the 10 μmol/l treatment compared with hypoxia alone and in general was higher than ATP levels with other paclitaxel dosages. The ATP value for the lower dose of paclitaxel was significantly higher than the hypoxia group only after 1 h of hypoxia, and the ATP values for both the lower doses after 1 h of hypoxia were similar to those measured at 0 h. In contrast, the higher dose of 15 μmol/l paclitaxel showed significantly lower ATP cell content at 3 and 6 h compared with the hypoxia-alone treatment but higher than ATP levels seen in the colchicine treatment group.

In the colchicine treatment group the fall in ATP was concomitant with a significant rise in ADP between 1 and 6 h compared with the hypoxia-alone group, which also showed increased ADP content (Fig. 5B), and ADP content was significantly increased after hypoxia in the H group. Similarly, the treatments with both low and middle doses of paclitaxel had significantly lower levels of ADP compared with hypoxia alone and again at 1 h were still similar to the levels seen at 0 h, whereas the high paclitaxel dose had significantly elevated levels at 3 and 6 h compared with hypoxia alone, although slightly lower ADP content than cells cultured with colchicine.

The trends in the data were very similar for PK, HK, PFK, and lactate (Fig. 5, C–F), although the cellular content of HK and PFK was half that of PK. Also, the cellular content of PK, HK, PFK, and lactate was significantly higher in the H group at 1, 3, and 6 h after hypoxia than in the H group at 0 h after hypoxia. Within 1 h of culturing under hypoxic conditions the levels of all three had dramatically increased for all treatment groups. In the cultures with colchicine or 15 μmol/l paclitaxel PK, HK, PFK, and lactate contents were significantly lower than in the untreated hypoxia cultures, and these levels decreased with longer culture times (15 μmol/l paclitaxel maintaining similar or slightly lower values compared with the colchicine group). In contrast, the PK, HK, PFK, and lactate

![Fig. 4. Effects of paclitaxel and colchicine on cultured normothermic cardiomyocyte LDH and ATP content and key glycolytic enzymes. A: LDH content of cardiomyocytes (IU/g protein, means ± SD) treated with different concentrations of paclitaxel and colchicine after 12 h in culture. B: ATP content of cardiomyocytes under the same conditions (mg/g protein, means ± SD). C–E: content of pyruvate kinase (PK), hexokinase (HK), and phosphofructokinase (PFK) in cardiomyocytes under the same conditions (IU/g protein, means ± SD); n = 10. *P < 0.05 vs. H group.](http://ajpheart.physiology.org/)

contents for the hypoxia-alone group and the 5 and 10 μmol/l paclitaxel groups all showed elevated levels at 3 h compared with 1 h and then subsequent falls at 6 h; the 10 μmol/l paclitaxel group had significantly higher levels at 1 and 3 h compared with hypoxia alone, but this difference was not apparent at 6 h. The 5 μmol/l paclitaxel group generally had lower but nonsignificant values compared with the hypoxia groups. It should be noted that the fall in lactate content between 1 and 6 h seen in the colchicine and 15 μmol/l paclitaxel treatment groups (Fig. 5F) are in general consistent with the increase in LDH, although in this case colchicine has a much more dramatic effect than 15 μmol/l paclitaxel (cf. Figs. 3 and 5).

The maximum lactate accumulation following 6 h of hypoxia was ≈6 mmol/l in our study. To test the effect of lactate on microtubular structure, cells were cultured for 6 h at 37°C with 3 or 6 mmol/l lactate (Nanjing Jiancheng Biotech; final concentration). Microtubular structural changes were observed by laser confocal scanning microscopy (Fig. 6). The results showed that there was no significant influence on microtubule structure after 6 h of culturing with either 3 or 6 mmol/l lactate. Measurements of microtubulin content using fluorescence showed no significant differences between control and lactate-treated cultures (Fig. 6D).

Effect of microtubule network changes on HIF-1α endonuclear aggregation in hypoxic cardiomyocytes. Normal-thermic cardiomyocytes were double labeled with α-tubulin and HIF-1α and showed a lack of HIF-1α expression (Fig. 7A). In the colchicine-treated cells, HIF-1α appeared to be restricted to areas around the nucleus and staining intensity decreased with longer times in culture (Fig. 7, B–D, HC). In contrast, HIF-1α staining in the hypoxia-alone or paclitaxel-treated cells showed relatively intense staining after 1 h in culture. The staining, while more intense around the nucleus, was also more evenly distributed within the cytoplasm (Fig. 7, B–D, HC). HIF-1α staining intensity also decreased with longer cultures, such as seen in the hypoxia and colchicine groups. The higher HIF-1α endonuclear aggregation in the 10 μmol/l paclitaxel group also appeared to be associated with the better preservation of α-tubulin staining and structure compared with the other treatment groups.

Cells with a higher expression of MAP4 (cf. Fig. 8, A and B), when cultured under hypoxic conditions, showed increased staining of HIF-1α and endonuclear aggregation compared with nontransfected cells (cf. Fig. 8, C and D). This was associated with increased staining of α-microtubulin and stabilized microtubular structures in cardiomyocytes under hypoxic conditions. RNA interference of α-microtubulin expression significantly reduced the staining of HIF-1α and endonuclear aggregation as well as reducing staining of α-microtubulin (cf. Fig. 8, H and I).
Effect of microtubule network changes on HIF-1α mRNA and protein contents. HIF-1α protein level in normoxic cardiomyocytes was generally low throughout the duration of the culture period and was less than half that seen for hypoxic cells (Fig. 9A). HIF-1α protein content significantly increased with hypoxia, and this increase was significantly augmented by 10 μmol/l paclitaxel, which showed a peak in protein content after 3 h of hypoxia. The values for the 5 μmol/l paclitaxel group were initially higher than those of the hypoxia group at 1 h, but by 6 h this value was significantly lower. HIF-1α protein levels in the 15 μmol/l paclitaxel group were significantly lower after 3 and 6 h in culture. Protein levels in the colchicine group were all significantly lower than those of the untreated hypoxic cells and decreased with longer times in culture. Interestingly, RNA levels for HIF-1α were not significantly different in any of the treatments and were unchanged from those of normoxic cardiomyocytes.

Hypoxic cardiomyocytes previously transfected in order to increase MAP4 showed significantly higher HIF-1α protein levels compared with hypoxia alone. MAP4 transfection increased protein level to values very close to those seen with 10 μmol/l paclitaxel (cf. Fig. 9, A and B). As shown above, RNA levels remained near those seen under normoxic culture conditions and were not significantly different. Conversely, interference RNA transcripts caused low expression of α-microtubulin and significantly decreased HIF-1α protein levels, albeit not to the levels seen in normothermic cells (Fig. 9C), without altering HIF-1α RNA levels.

HIF-1α protein levels affected by paclitaxel and colchicine after 12 h of normothermic culture showed that the different concentrations of paclitaxel and colchicine had no significant influence on HIF-1α protein levels (Fig. 10).

DISCUSSION

Microtubules are an important cytoskeletal component. It has been demonstrated that microtubules play an important role in the maintenance of cellular physiological structure, function, and metabolism (4, 10, 15a, 24, 26, 27, 30, 41). In cardiomyocytes, microtubules either are free or occur as polymerized dimers in the cytoplasm. A dynamic balance between the two states is crucial for cell stability, contractility, and other physiological functions (1, 15a, 23, 29, 33, 35, 39). However, there is no direct evidence indicating that the microtubular structural changes in hypoxic cardiomyocytes are capable of affecting glycolysis of cardiomyocytes during early hypoxia. We thus postulated that microtubular structural changes in hypoxic cardiomyocytes are capable of affecting glycolysis of cardiomyocytes during early hypoxia. We thus postulated that microtubular structural changes regulate the glycolysis pathway and energy metabolism of cardiomyocytes.

To test this hypothesis, we first consolidated altered microtubular structures of cardiomyocytes with the microtubule-stabilizing agent paclitaxel and the microtubule-depolymerizing agent colchicine and then observed their effects on microtubular structures and energy metabolism in cultured cardiomyocytes. Colchicine may depolymerize microtubules and suppress polymerization of free microtubule proteins. Paclitaxel can stabilize polymerized microtubules and counteract the microtubule-depolymerizing ac-
Fig. 7. Effect of paclitaxel and colchicine on the microtubular network and hypoxia-inducible factor (HIF)-1α endonuclear aggregation in hypoxic cardiomyocytes. A: cells were labeled with α-tubulin and HIF-1α and then visualized with secondary antibodies conjugated to FITC (green) and tetramethylrhodamine isothiocyanate (TRITC, red), and then the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue). B–D: cells were double labeled for α-tubulin and HIF-1α after 1, 3, and 6 h of hypoxia, respectively. Columns from left to right show a representative cell from the hypoxic (H), paclitaxel (HT; 5, 10, and 15 μmol/l), and colchicine (HC) groups. Top: HIF-1α staining. Bottom: merged images of α-tubulin, HIF-1α and nuclear staining. Figure shows that stabilized microtubular structures of hypoxic cardiomyocytes can increase HIF-1α endonuclear aggregation and, conversely, disrupted microtubular structures of hypoxic cardiomyocytes can decrease HIF-1α endonuclear aggregation.
tion of colchicine (1, 7, 29, 40). Our previous studies and others reported that paclitaxel, colchicine, or other microtubule-intervening agents failed to influence the activity and physiological function in normal rat cardiomyocytes within certain concentrations and time ranges (33, 35, 40, 41). The present study indicated that hypoxia was associated with microtubule depolymerization and a breakdown of RMS in cardiomyocytes, the effects of which persisted throughout the duration of hypoxia. Microtubule depolymerization and low expression of α-tubulin significantly aggravated microtubular structural damage, causing

Fig. 8. Changes in HIF-1α and α-microtubulin staining following microtubule-associated protein 4 (MAP4), enhanced green fluorescent protein (EGFP), and RNA interference (RNAi) transfection in hypoxic cardiomyocytes. A and B: MAP4 staining in normothermic cardiomyocytes and after MAP4 transfection, respectively, after 39 h in culture. C–E: fluorescence staining for HIF-1α (red) and α-microtubulin (green; merged images + DAPI nuclear staining) for cells that were hypoxic or MAP4 or EGFP transfected, respectively, after 3 h of hypoxia. F and G: α-microtubulin fluorescence staining of normothermic cardiomyocytes and after RNA interference of α-microtubulin, respectively. H–J: HIF-1α and α-microtubulin fluorescence staining results for hypoxia alone, RNA interference, and the interference control group (HK), respectively, after 3 h of hypoxia.
reticular microtubules to disappear. Paclitaxel (10 μmol/l) and increased expression of MAP4 maintained RMS during early hypoxia and ameliorated hypoxia-induced breakdown of RMS of cardiomyocytes. Colchicine significantly aggravated microtubule breakdown in hypoxic cardiomyocytes.

Changes of LDH reflected myocardial cell injury. The observed changes in microtubules following hypoxia paralleled changes in cell viability. The addition of colchicine or the higher dosage of paclitaxel (15 μmol/l), both of which clearly affected microtubular structure and aggregation, led to increased LDH values and reduced cell viability. Thus the severity of the cardiomyocyte injury appears to be closely related to the integrity of microtubular structures. We presumed that normal cardiomyocytes had good antidamage ca-
pacity, this ability was weakened after hypoxia, and damaged cardiomyocytes were sensitive to drugs with different concentrations.

Previous reports on liver cells also showed that microtubule-depolymerizing agents decreased ATP content, increased cell permeability, and interfered with endocytosis, while the microtubule-stabilizing agent paclitaxel counteracted the actions of microtubule-depolymerizing agents (9, 16). We investigated further the effect of paclitaxel and colchicine on energy metabolism. We found previously that under normoxic conditions changes in microtubule network structure may result in mitochondrial changes and further impede energy supply from oxidative phosphorylation. Hence, an increase in ATP content results from glycolysis enhancement and ATP hydrolysis reduction under hypoxia (with microtubule structural destruction). The results showed that 10 μmol/l paclitaxel ameliorated energy metabolism and increased ATP content and levels of lactate during early hypoxia. Although a higher dose (15 μmol/l) of paclitaxel promoted microtubulin polymerization, this excessive microtubulin polymerization disrupted the dynamic balance of normal physiological functions, thus decreasing ATP content in hypoxic cardiomyocytes. Therefore, maintaining the normal physiological structure of microtubules in hypoxic cardiomyocytes may help ameliorate their energy supply. Since energy is supplied mainly by glycolysis in hypoxic cardiomyocytes, we examined the effect of microtubule breakdown on the glycolytic pathway and the content of HK, PK, and PFK in hypoxic cardiomyocytes. The content of the key glycolytic enzymes and the content of lactic acid (the end product of glycolysis) were significantly increased if the integrity of microtubular structures was maintained for a certain period after hypoxia, resulting in significant amounts of ATP. In fact, the reticular microtubular structural changes were closely associated with the content of key glycolytic enzymes. It has been reported for other cell models that a breakdown of RMS decreases the content of key glycolytic enzymes (7, 8), and in nerve cells these enzymes may directly bind to the microtubules (22, 38). Once RMS had disappeared, the amounts of key glycolytic enzymes were significantly decreased. These enzymes and microtubules work together to facilitate organelle localization, chromosome migration, mobility of MAPs, and cell metabolism and energy supply (36).

The mechanism of microtubule changes in the glycolytic pathway in cardiomyocytes elicited by hypoxia remain unclear. Our earlier results showed that HIF-1α may be an important signaling molecule (2). Moreover, in some cells other than cardiomyocytes, microtubular structural changes directly influence HIF-1α expression or influence factors associated with endonuclear aggregation, protein expression, and degradation of HIF-1α (5, 7, 44). Previous research in other cells found that

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**Fig. 11.** Schematic diagram illustrating that microtubular structural changes influence glycolysis of cardiomyocytes during early hypoxia through regulating HIF-1α content.
HIF-1 plays a key role in the reprogramming of cell metabolism by activating transcription of genes encoding glucose transporters and glycolytic enzymes, and there should be serial signal pathways playing this role (14, 15, 19, 34). Hence, we postulated that breakdown of microtubular structures influences the content of glycolytic enzymes by influencing the expression or endonuclear aggregation of HIF-1α in hypoxic cardiomyocytes.

To test this, paclitaxel, colchicine, high expression of MAP4, and low expression of α-tubulin were used to observe the effect of stabilization or breakdown of microtubular structures on HIF-1α expression in hypoxic cardiomyocytes. The results indicated that the stability of RMS was consistent with increased endonuclear aggregation and protein content of HIF-1α under hypoxia. Under the action of hypoxia and the microtubule-depolymerizing agent RMS disappeared, or excessive microtubule polymerization occurred with the use of large doses of the microtubule-stabilizing agent, thus decreasing endonuclear aggregation and contents of HIF-1α. In addition, in the present study HIF-1α mRNA transcription did not change, suggesting that HIF-1α content in response to hypoxic microtubular structural changes may occur only at the post-transcriptional level, possibly associated with endonuclear aggregation or degradation of HIF-1α. Previous reports showed that HIF-1α protein expression was stimulated after hypoxia but that HIF-1α mRNA expression at all times of ischemic treatment and reperfusion was unchanged compared with normoxic controls. The ubiquitin-proteasome system was inhibited after hypoxia, and the degradation of HIF-1α protein was inhibited (28, 31). Previously we reported (11) that hypoxia induces microtubule depolymerization and decreases cell viability via the activation of the p38/MAPK signaling pathway and changes the phosphorylation levels of its downstream effectors, MAP4 and Op18. Moreover, many studies show that AMP-activated protein kinase (AMPK) has an intimate association with p38/MAPK (44). These signal pathways may play a role in connecting microtubule structure with HIF-1α, although more experiments are needed to verify this.

LDH content of cardiomyocytes treated with 10 and 15 μmol/l colchicine and 15 μmol/l paclitaxel for 12 h was significantly higher than that in the control group. However, glycolysis, ATP content, and microtubular structural organization in cardiomyocytes treated with 10 μmol/l colchicine and 15 μmol/l paclitaxel for 12 h were not significantly different from those in the control group. We presume the high concentration of colchicine or paclitaxel damages the cellular membrane with longer treatment times. Neonatal cardiomyocytes, as used in the present study, differ significantly from adult rat cardiomyocytes in many aspects including cytoskeleton and physiology. Our previous studies were based on neonate rat cardiomyocytes (2, 12, 13, 44), and the present study extends that work and those of others (4, 40, 41).

In summary, we show that breakdown of RMS of hypoxic cardiomyocytes when treated with colchicine or high doses of paclitaxel decreases endonuclear aggregation and protein content of HIF-1α, suppresses the content of key glycolytic enzymes, and decreases ATP content and levels of lactate. Stabilizing the RMS of hypoxic cardiomyocytes with 10 μmol/l paclitaxel facilitates endonuclear aggregation and protein content of HIF-1α, thus promoting glycolysis and ATP levels (Fig. 11).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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