

Hypoxia induces Wee1 expression and attenuates hydrogen peroxide-induced endothelial damage in MS1 cells

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Abbreviations: cdc2, cell division cycle 2; cdk1, cycle dependent kinase 1; HIF-1 α , hypoxia inducible factor-1 α ; Hsp, heat shock protein

Abstract

In an oxygen-depleted environment, endothelial cells initiate an adaptive pattern of synthesis, which may enable them to survive hypoxic crises. Using high-resolution two-dimensional gel electrophoresis in conjunction with mass spectroscopy, we obtained a 24 differential display of proteins in the pancreatic endothelial cell line, MS-1, at four time points following induction of hypoxia. The induction of Wee1 under hypoxia was confirmed both at the mRNA and protein levels. The phosphorylation of cell division cycle 2, which is downstream of Wee1, was also increased after hypoxic exposure. In addition, pre-exposure to hypoxia attenuated a decrease in hydrogen peroxide-induced cell number. The induction of bax (a pro-apoptotic protein) and reduction of bcl (an anti-apoptotic protein) after hypoxia stimulus were also attenuated by hypoxic pre-exposure. Moreover, hydrogen peroxide-induced morphologic damage did not appear in the wild-type Wee1-expressing cells. Taken together, our results suggest that Wee1 may have important role in hypoxia-induced pathophysiological situations in endothelial cells.

Keywords: cell hypoxia; endothelial cells; hydrogen peroxide; hypoxia inducible factor 1, α subunit; Wee1 protein, human

Introduction

Endothelial cells play an integral role in the maintenance of vascular homeostasis. As the cell type directly in contact with the blood, endothelial cells must adapt to changing conditions within the blood vessels and synthesize regulators of leukocyte and vascular smooth muscle cell behavior. Medical conditions, such as atherosclerosis, varicose veins, pulmonary hypertension, and tumor angiogenesis, may induce vascular hypoxia (Eyries *et al.*, 2008; Han *et al.*, 2008; Kubo *et al.*, 2008; Osada-Oka *et al.*, 2008a; Rankin *et al.*, 2008), and the molecular aspects of hypoxia have been studied in order to explain the pathology underlying these diseases; however, many questions remain.

Hypoxic conditions may profoundly affect endothelial cells through a cascade of reactions involving neutrophils (Sumagin *et al.*, 2006, 2008; Butler *et al.*, 2008; Carpenter and Alexander, 2008; Hu *et al.*, 2008) and smooth muscle cells (Michiels *et al.*, 2000; Aley *et al.*, 2008; Jernigan *et al.*, 2008; Mayr *et al.*, 2008; Osada-Oka *et al.*, 2008a, 2008b; Sheshgiri *et al.*, 2008). Hypoxia regulates the expression of many genes, and the list of hypoxia-related genes is growing (Yoshida *et al.*, 2006; Eckardt *et al.*, 2007; Said *et al.*, 2007; Wang *et al.*, 2007; Chen *et al.*, 2008; Furuta *et al.*, 2008; Yang *et al.*, 2008). These molecular changes appear to favor endothelial cell survival of hypoxic crises. Many genes involved in tumor biology are regulated by levels of oxygen. In some human tumor types, hypoxia inducible factor-1 α (HIF-1 α) is overexpressed compared with corresponding normal tissues (Zhong *et al.*, 1999; Furlan *et al.*, 2007; Fan *et al.*, 2008; Hamaguchi *et al.*, 2008; Liang *et al.*, 2008; Masamune *et al.*, 2008; Sayed *et al.*, 2008; Simiantonaki *et al.*, 2008).

Wee1 encodes a nuclear protein, a tyrosine kinase belonging to the Ser/Thr family, which catalyzes the inhibitory tyrosine phosphorylation of cell division cycle 2 (cdc2)/cyclin B kinases (McGowan and Russell, 1995; Okamoto and Sagata, 2007). By shielding the nucleus from activated cytoplasm cdc2 kinase, Wee1 may coordinate the

transition between DNA replication and mitosis (Yamada *et al.*, 2004; De Schutter *et al.*, 2007; Perry and Kornbluth, 2007). Phosphorylation of Wee1 is dependent upon mitotic cell cycle dependent kinase 1 (cdk1) activity (Stumpff *et al.*, 2004; Harvey *et al.*, 2005). The molecular mechanisms that regulate Wee1 are poorly understood; hence, a more detailed knowledge of these mechanisms will be an important step toward understanding what determines whether a cell undergoes mitosis.

Results

Hypoxia induces Wee1 expression

The expression of HIF-1 α serves as a cellular marker for hypoxic conditions. In order to examine hypoxic systems, we investigated the expression of HIF-1 α after exposure to hypoxia (Figure 1A). Hypoxia also induced a time-dependent activation of the vascular endothelial growth factor (VEGF) promoter downstream of HIF-1 α (Figure 1B), suggesting that our hypoxic system was working appropriately. HIF-1 α is known to translocate to the

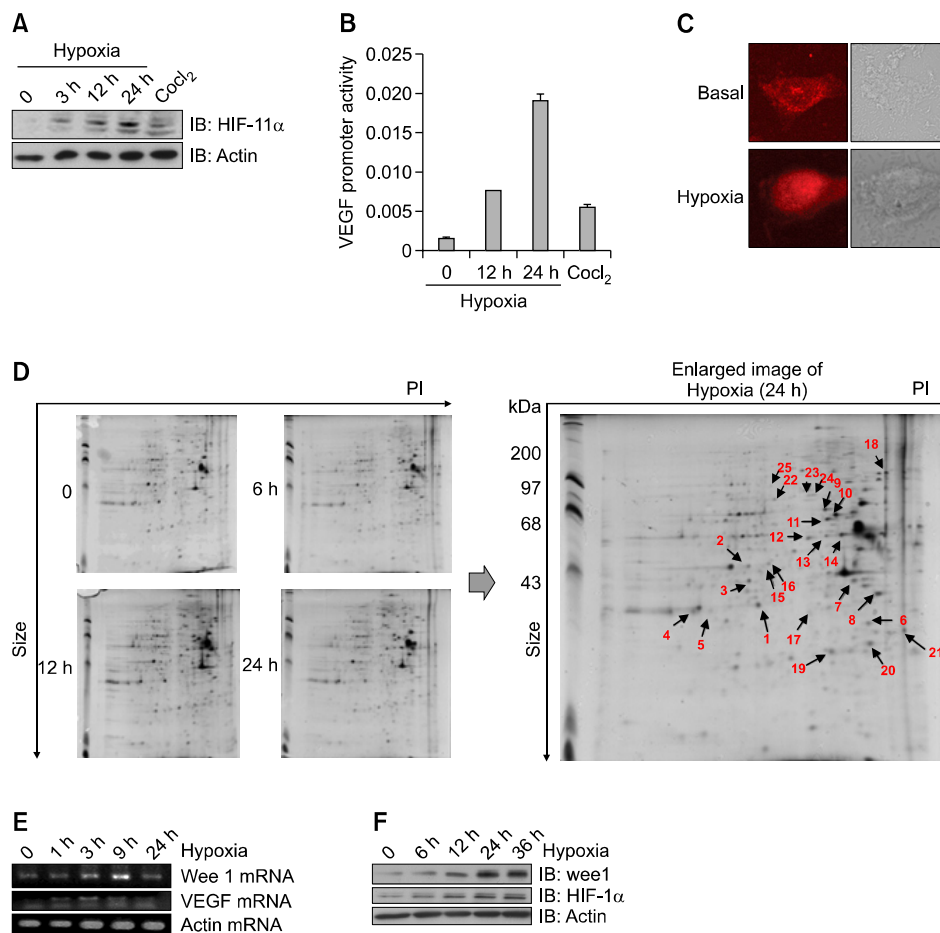


Figure 1. Hypoxia induced Wee1 expression. (A) Induction of HIF-1 α by hypoxia in MS-1 cells. Cells were exposed for the indicated times in a hypoxic chamber (0.1% O₂) and then blotted with HIF-1 α antibody. The lower panel demonstrates equivalent loading of total actin in the whole cell lysates. (B) The effect of hypoxia on the VEGF promoter. The promoter plasmid was transiently transfected and maintained for 24 h in MS-1 cells. After hypoxia (0.1% O₂) for indicated times, the promoter activity was monitored. CoCl₂ was treated and used as a chemical hypoxic inducer. (C) Effect of hypoxia on sub-cellular localization of HIF-1 α . Before hypoxic treatment, MS-1 cells were preincubated for 30 min with hypoxia culture medium. The HIF-1 α signal was then measured after staining with antibody. (D) Spectrometric analysis of hypoxic samples. Two-dimensional gels showing the silver-stained signal obtained from 200 μ g of MS-1 cell before and at measured intervals after induction of hypoxia. The right image is the enlarged images of the 24 h spot in the 2D gels. The arrows indicate the identification of the protein by spot-picking, tryptic digestion, and MALDI-TOF. (E) Image of total RNA was prepared from MS-1 cells and amplified by RT-PCR using Wee1/VEGF-specific primers. Actin mRNA was used as a positive control. The PCR products were separated on 2% agarose gels and visualized with UV. (F) Effect of hypoxia on Wee1 expression. The MS-1 cells were exposed to hypoxia for the indicated times. Expression of Wee1 was then examined by blotting with antibodies to HIF-1 α and Wee1. The lower panel demonstrates equivalent loading of total actin in the whole cell lysates.

Table 1. List of summary of MS analysis

Spot	Protein name	Function
2	Enolase	Glycolysis
17	Aminoacylase	
9	Mortalin-2	Chaperone mediator of protein folding
11	Heat shock protein 70	
12	Protein disulfide-isomerase ER60 precursor	
13	Chaperone subunit 8	
14	Heat shock protein 60 kDa protein 1	
30	Glucose-regulated protein 78 (Grp78/BiP)	Bind phospholipids
1	Annexin I	
6	Annexin V	
7	Beta-actin	Cell mobility or structure
19	Actin-gamma	
20	Chain A, structure of bovine-actin-profilin complex	
21	Tropomyosin	
25	Beta-fodrin	
26	Gelsolin	
32	Nebulette	
33	Vinculin	
34	Collagen alpha	
37	Plastin	
39	Vimentin	
3	Elongation factor TU	Others
4	Proteasome activator complex subunit 2	
5	Acetyl cholinesterase collagen-like-tail subunit isoform V	
8	Eukaryotic translation initiation factor 2	
15	Eukaryotic translation elongation factor 1 gamma	
18	Nucleolin	
22/31	Vault protein	
24	Wee1 homolog	

nucleus in hypoxic conditions. To confirm this in another way, we investigated the subcellular location of HIF-1 α . We found that the majority of HIF-1 α was located in the nucleus in hypoxic conditions (Figure 1C). These findings confirm the effectiveness of hypoxia in our experimental system. Two-dimensional protein expression maps were generated for the triplicate samples of MS-1 cells before and at measured intervals after induction of hypoxia (Figure 1D). Gel images were checked manually to minimize gel-to-gel variations. Following the shift to hypoxia, 44 spots indicated the differential expression. Spots that were 1.5-fold up- or down-regulated by hypoxia were defined as differentially expressed. Twenty-four proteins were identified and categorized (Table 1). Identified

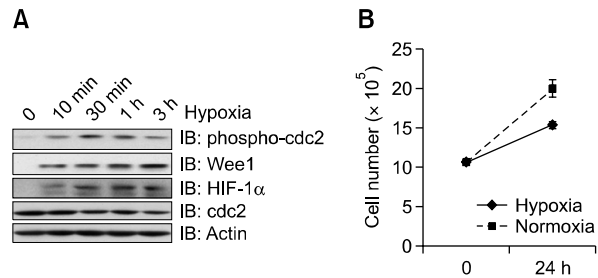


Figure 2. Cdc2 phosphorylation (Tyr¹⁵) in hypoxia. (A) Effect of hypoxia on cdc2 phosphorylation. To induce hypoxic conditions, MS-1 cells were incubated for the indicated times in a hypoxic chamber. The phosphorylation of cdc2 was analyzed with western blotting. The lower panel demonstrates equivalent loading of total cdc2 and actin in the whole cell lysates. (B) Number of MS-1 cells after hypoxia. Cells were exposed to hypoxia for the indicated times. The results are expressed as the mean \pm SEM for three separate experiments.

proteins were categorized as being involved with glycolysis, chaperone mediaion, or cell mobility. These proteins were already known to be involved with hypoxia, suggesting the relevance of our data. To validate the MS results, the level of Wee1 mRNA was examined (Figure 1E). The level of VEGF mRNA was also examined and used as a positive control for hypoxic conditions. Concurrently, the level of Wee1 protein in the hypoxic cells was measured and shown to increase (Figure 1F). The protein level of HIF-1 α was used as a positive control for hypoxia. These findings support a specific role for Wee1 in hypoxia-mediated signaling events.

Cdc2 was phosphorylated under hypoxia

To trace the effects of Wee1 in hypoxia-mediated signaling, we measured the phosphorylation of cdc2, a Wee1 downstream molecule. Exposure of cells to hypoxia for 10 min induced phosphorylation of cdc2 (Tyr¹⁵; Figure 2A), confirming Wee1 activation in hypoxia-induced signaling. We then monitored cell numbers following exposure to hypoxia for 24 h, and found a reduction in cell proliferation (Figure 2B), which indicated that both Wee1 and cdc2 were involved in the hypoxia-induced signaling pathway.

Hypoxia attenuates the reduction in cell viability induced by hydrogen peroxide

Pre-exposure of cells to hypoxia (24 h) attenuated the hydrogen peroxide-induced suppression of cell viability (Figure 3A). While 1 mM of hydrogen peroxide suppressed cell viability by approximately 80%, this suppression was attenuated to 50%–60% in cells pre-exposed to hypoxia. Bcl-2 is the member of the Bcl family of apoptosis regulator protein. It is thought to be involved in resistance to

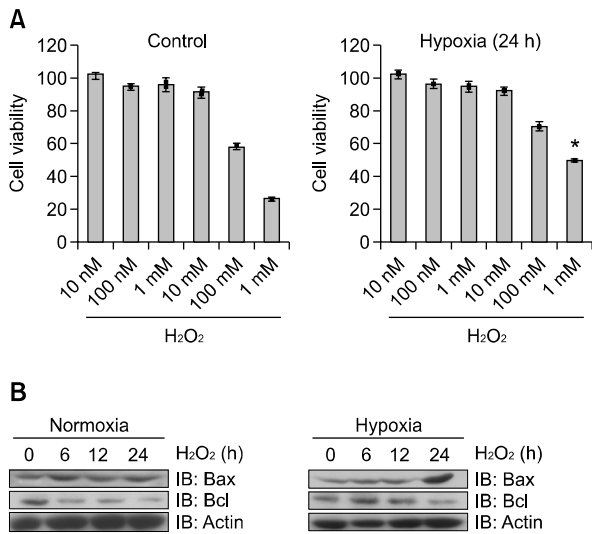


Figure 3. Hypoxia attenuates the reduction in cell viability induced by hydrogen peroxide. (A) Viability of MS-1 cells after hydrogen peroxide exposure. Cells were exposed to hydrogen peroxide either with or without hypoxic preconditioning. The results are expressed as the percentage of absorbance (MTT) relative to the control. Each value represents the mean \pm SEM for three separate experiments. Statistical analyses was conducted in comparison to control values; $P < 0.001$. (B) Effect of hypoxia on hydrogen peroxide-mediated apoptosis-related protein expression. Before hydrogen peroxide treatment, MS-1 cells were exposed to hypoxia for indicated times. Anti-bax and bcl expression were then examined after hydrogen peroxide treatment, and protein expression was examined by blotting with bax and bcl antibodies. The lower panel demonstrates equivalent loading of total actin in the whole cell lysates.

cancer treatment, so regarded as anti-apoptosis protein. Bax is a pro-apoptotic protein Bcl family protein. It promotes apoptosis by competing Bcl protein. This hypoxic pre-exposure also suppressed the induction of bax, and delayed the downregulation of bcl after hydrogen peroxide treatment. While bax was induced after 6 h of exposure to hydrogen peroxide in normoxic conditions, this increase appeared at 24 h in cells pre-exposed to hypoxia (Figure 3B). The expression of bcl began to decrease at 6 h in normoxia. This pattern was delayed under conditions of pre-exposure hypoxia. Thus, Wee1 signaling due to hypoxia may be involved in protection from oxidative stress.

Wee1 suppression of hydrogen peroxide-induced cellular injury

To confirm the role of Wee1 in hydrogen peroxide-mediated signaling, we compared the decrease in hydrogen peroxide-induced cell numbers between the wild-type Wee1 and mutant Wee1-expressing cells. After treatment with hydrogen peroxide for different times and at different doses, the number of cells was counted. The wild-type expressing

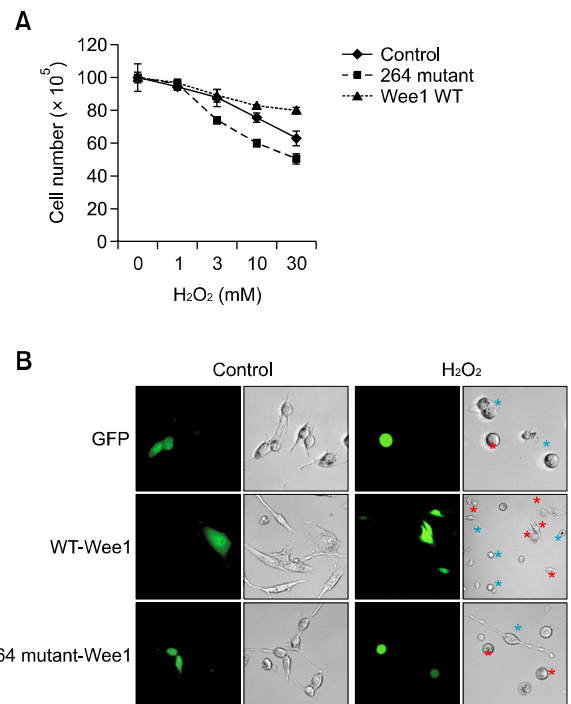


Figure 4. Wee1 expression prevents hydrogen peroxide-induced cellular injury. (A) Number of cells after Wee1 expression under hydrogen peroxide. Cells were transiently transfected with either the wild-type or mutant Wee1 and then maintained for 24 h, prior to hydrogen peroxide stimulation. Cells were exposed to hydrogen peroxide for 24 h with indicated doses. The results are expressed as the mean \pm SEM for three separate experiments. (B) Morphologic changes of MS-1 cells after Wee1 expression. Cells were transiently transfected with either Green Fluorescent Protein-tagged wild type or mutant Wee1, prior to hydrogen peroxide exposure. Images were obtained with confocal microscopy. Green color indicates GFP-transfected cell, red color indicate non-transfected cell.

cells, but not those with the mutant gene, resisted the anti-proliferative effects of hydrogen peroxide in a time- and dose-dependent manner (Figure 4A). In parallel experiments, we found that hydrogen peroxide did not alter the morphology of cells which expressed green fluorescent protein (GFP)-tagged wild-type Wee1, but severely shrank the morphology of cells which expressed GFP-tagged mutant Wee1 (Figure 4B). The morphology of cells which expressed both wild-type and mutant Wee1 did not change under control conditions. This result supports the hypothesis that the physiologic role of Wee1 is to protect cells in response to hydrogen peroxide.

Discussion

The primary finding of this study is that Wee1 mediates some of the adaptive synthetic pattern which may enable cells to survive a hypoxic crisis.

The role of Wee1 has previously been evaluated in control cell cycle arrest and DNA replication checkpoints (Watanabe *et al.*, 1995). In response to DNA damage, Wee1 can inactivate the cdc2/cyclin B complexes in order to activate the G₂ DNA damage checkpoints and inhibit the G₂/M transition in the cell cycle in order to prevent cells from progressing through to mitosis prior to completion of repair. This subsequently results in an irreversible alteration to the genome that can have profound effects on cellular viability and coordinated cell proliferation and development (Igarashi *et al.*, 1991; Raleigh and O'Connell, 2000; Stumpff *et al.*, 2004). In the present study, we showed that phosphorylation of cdc2 was induced by hypoxic stimuli, suggesting that Wee1 may promote the cell cycle checkpoint pathway for hypoxic conditions in endothelial cells.

Hypoxia, as well as heavy metals, oxygen-free radicals, inflammation, and ischemia, may also induce heat shock proteins (Hsps), or chaperone proteins. As molecular chaperones, the Hsps stabilize intracellular proteins to facilitate their biosynthesis and folding. Induction of Hsp70 by hypoxia, for example, may increase resistance of the heart to ischemic injury (Zhong *et al.*, 2000). Hsp90 is one of the most abundant and ubiquitous molecular chaperones. In the fission yeast, Wee1 was identified as a Hsp90 substrate (Aligue *et al.*, 1994). These facts suggest that Wee1 may have important role in hypoxia through regulating molecular chaperone, such as Hsp90.

In conclusion, we have demonstrated that hypoxia induces Wee1, and that this protein protects endothelial cells against hypoxic damage. Further studies will be focused on elucidating the role of Wee1 in hypoxia-related pathologic conditions, such as in tissue ischemia caused by atherosclerosis.

Methods

Reagents

Antibodies to HIF-1 α , Wee1, bax, bcl, actin, and cdc2 were purchased from Cell Signaling Technology Inc. (New England Biolabs, Beverly, MA). Anti-phospho-cdc2 (Tyr¹⁵) was obtained from Stressgen Bioreagents Corporation (Victoria, B.C., Canada). Horseradish peroxidase-conjugated secondary antibodies were obtained from the Kirkegaard and Perry Lab (Gaithersburg, MD). Cobalt-chloride was purchased from Sigma-Aldrich (St. Louis, MO) and hydrogen peroxide was obtained from Calbiochem (San Diego, CA).

Cell cultures

Mouse pancreatic endothelial (MS-1) cells were cultured in

a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM; GIBCO™, Auckland, NZ) containing 0.584 g/L of L-glutamate and 4.5 g/L of glucose, and F-12 medium containing 0.146 g/L of L-glutamate, 1.8 g/L of glucose, 100 g/ml of gentamicin, 2.5 g/L of sodium carbonate, and 10% heat-inactivated fetal bovine serum.

Immunoblot analysis

The cells were lysed in 100 μ l of lysis buffer (50 mM Tris-Cl [pH 7.4], 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM ethylenediaminetetraacetic acid [EDTA], and 150 mM NaCl). The samples were then briefly sonicated, heated for 5 min at 95°C, and centrifuged for 3 min. Supernatant proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 8%) gels, and then transferred to polyvinylidene difluoride membranes. Blots were incubated with primary antibodies, and then washed 6 times in Tris-buffered saline/0.1% Tween-20 prior to a 1-h probe with horseradish peroxidase-conjugated secondary antibodies. Blots were developed using ECL (Amersham Biosciences, Buckinghamshire, UK).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Levels of VEGF, Wee1, and actin expression were determined with RT-PCR, as described below. The synthetic oligonucleotide primer sequences for VEGF, Wee1, and actin were as follows: VEGF (forward), 5'-TGTTTCGTGTCC TTCATCCTG-3', VEGF (reverse), 5'-GAAGGAGATGGCA AGGAGTG-3'; Wee1 (forward), 5'-GATGTGCCACAGACT CCTCA-3', Wee1 (reverse), 5'-CAAAGCGTTCTGCTCATC AA-3'; actin (forward), 5'-CGATGCTGGCGCTGAGTAC-3', and actin (reverse), 5'-CGTTCAGCTCAGGGATGACC-3'. Amplifications using the above-listed primers were performed in a 25- μ l reaction for 30 amplification cycles in a Gene Amp PCR system 2400 (Perkin Elmer, Wellesley, MA) with 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C.

Two-dimensional (2D)-PAGE

Cells were lysed in protein extraction buffer (7 M urea, 2 M thiourea, 4% [3-(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 100 mM dithiothreitol, and 1% phosphate [pH 3-10]), and lysates were centrifuged at 356,000 $\times g$ for 10 min in a Beckman Optima TL Tabletop Ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). The protein concentration was determined using the Bio-Rad protein assay reagent. Immobilized pH gradient strips (pH 3-10, linear, 18 cm) were rehydrated overnight in 400 μ l of lysate that contained 250 μ g of protein. Isoelectric focusing on a Multiphor II system (Amersham Biosciences, Buckinghamshire, UK) and SDS-PAGE on 10% gels using the Investigator System (Genomic Solutions, Chelmsford, MA) were performed.

In-gel trypsin digestion of protein and mass spectrometry

After gels were destained in 10% acetic acid, protein spots were excised and macerated with a scalpel. Portions (typically, 1/20) of the complete trypsin digest were co-crystallized in a matrix of α -cyano-4-hydroxycinnamic acid and analyzed by using a PerSeptive BioSystems matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. Peptides were eluted isocratically for 10 min, followed by a linear gradient (0.95%/min) to a final mobile phase composition of 63% acetonitrile/0.082% trifluoroacetic acid in H₂O. Eluting peptides were detected with an ABI 785A UV detector. Protein identities were derived using the MS spectra with the MS-Tag program.

Plasmid transfection and luciferase assays

Cells were seeded in 6-well tissue culture plates 24 h before transfection. Sub-confluent cells were transfected with VEGF promoter-luciferase using the FuGEN6 transfection reagent (Roche Diagnostics, Mannheim, Germany). After 24 h, the cells were treated with hypoxia (0.1% O₂), as appropriate. The luciferase assay was done using the luciferase assay kit (Promega, Madison, WI).

Immunofluorescence and confocal microscopy

Cells grown on glass coverslips were fixed in 4% phosphate-buffered paraformaldehyde for 15 min. Cells were permeabilized in methanol at -20°C for 4 min. After permeabilization, cells were incubated with primary antibody to Wee1 for 2 h at room temperature, washed 3 times in phosphate-buffered saline, and then incubated with secondary antibodies conjugated with 546-Alexa (red) from Molecular Probes (Invitrogen, Carlsbad, CA). Confocal scanning analysis was performed using a confocal microscope (Zeiss LSM 510, Oberkochen, Germany).

Hypoxia system

Cultured cells were transferred into a hypoxic chamber (model 1025; Forma Scientific, Marietta, OH) for the indicated times at 0.1% O₂.

Data analysis

Statistical analyses were conducted using SigmaStat (SPSS Inc., Chicago, IL). A *P*-value < 0.05 was considered statistically significant.

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