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Anti-apoptotic protein survivin plays a significant role in tubular morphogenesis of human coronary arteriolar endothelial cells by hypoxic preconditioning

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Abstract Brief exposure of endothelial cells to oxidative stress induced by hypoxia followed by reoxygenation enhances tube formation. Our study provides evidence that hypoxic preconditioning accelerates tubular morphogenesis along with the activation of reactive oxygen species-inducible nuclear transcription factor-κB (NF-κB), phosphatidylinositol 3-kinase (PI3-kinase) and broad-spectrum anti-apoptotic protein survivin in human coronary arteriolar endothelial cells (HCAEC). The formation of tubular morphogenesis was inhibited by using the PI3-kinase and NF-κB antagonists LY294002 and SN50 respectively. The activation of survivin by hypoxic preconditioning was also inhibited by LY294002 and SN50 along with increased apoptosis in HCAEC. These data demonstrate a crucial role of PI3-kinase/Akt/NF-κB/survivin signaling in tubular morphogenesis of HCAEC triggered by hypoxic preconditioning. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Angiogenesis; Human coronary arteriolar endothelial cell; Tubular morphogenesis; Apoptosis; Survivin; Nuclear factor-κB; Hypoxia; Reoxygenation; Phosphatidylinositol 3-kinase; Reactive oxygen species; SN50; LY294002; Oxidative stress; Malonaldehyde; Matrigel

1. Introduction

Angiogenesis is regulated and controlled by several known and as yet unknown factors. It is an important process in inflammation, solid tumor growth and several other pathological phenomena. Short exposure to hypoxia/reoxygenation, either directly or indirectly, produces oxidative stress which is associated with angiogenesis or neovascularization. This process is thought to be regulated by several growth factors (epidermal, transforming-α, β-fibroblast, vascular endothelial (VEGF)). Induction of angiogenic factors is triggered by various stresses. For instance, tissue hypoxia exerts its pro-angiogenic action through various angiogenic factors, the most notable being VEGF, which has been mainly associated with initiating the process of angiogenesis through the recruitment and proliferation of endothelial cells.

Mechanisms controlling angiogenesis and tubular morphogenesis in rat myocardium and human microvascular endothelial cells respectively by nuclear transcription factor NF-κB are well-established [1–4]. NF-κB appears to be a critical regulator for gene expression induced by diverse stress signals including mutagenic, oxidative and hypoxic stresses [3,4]. Activation of NF-κB is likely to be involved in the induction of gene expression associated with ischemic as well as hypoxic adaptation [5]. NF-κB is present as a predominant form in unstimulated cells as a heterodimer of two proteins, p50 and p65 complexed to an inhibitory subunit called IκB that prevents migration of the heterodimer to the nucleus and DNA binding. Under stress, this inhibitory subunit becomes phosphorylated and dissociated from NF-κB, allowing migration of the heterodimer to the nucleus where it binds to DNA and increases gene transcription [6]. Recently, phosphorylation of IκB-α by phosphatidylinositol 3-kinase (PI3-kinase) was demonstrated which directly caused the release of IκB-α from the NF-κB complex [7]. A recent study documented that myocardial adaptation to ischemia by repeated brief episodes of ischemia and reperfusion translocated and activated the DNA binding capacity of NF-κB [8]. NF-κB is an oxidative stress responsive transcription factor and plays a significant role in the process of apoptosis [9]. This nuclear transcription factor appears to function both as a pro- and as an anti-apoptotic factor depending upon cell type and mode of stress [10].

Survivin, a recently described inhibitor of apoptosis, also plays a significant role in the process of cell proliferation in human hepatocellular carcinoma [11]. It was demonstrated that angiopoietin-1 (Ang-1), presumably acting via the Tie-2 tyrosine kinase receptor, is capable of preventing endothelial cell apoptosis by activating Akt, a critical survival messenger. In addition, Ang-1 also can up-regulate the broad-spectrum apoptosis inhibitor, survivin [12]. Thus, inhibition of endothelial cell apoptosis by survivin expression may have significant clinical significance in the realm of angiogenesis by improving endothelial cell viability.

This study was undertaken to investigate the role of survivin in angiogenesis and the significance of the inhibition of apoptosis during this process. This study utilized human coronary arteriolar endothelial cells (HCAEC) as an in vitro angiogenesis model to examine the effect of hypoxic preconditioning on cell migration or tubular morphogenesis, survivin expression and the extent of apoptosis. The involvement of transcription factor NF-κB and PI3-kinase was also examined to establish the molecular mechanism of the expression of survivin and their role in the process of tubular morphogenesis by increasing endothelial cell viability. In this study, we

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demonstrate for the first time the involvement of NF-κB and PI3-kinase in the upregulation of the expression of the anti-apoptotic protein survivin with concomitant increase in the HCAEC viability and tubular morphogenesis by hypoxic preconditioning.

2. Materials and methods

2.1. Endothelial cell culture

HCAEC were obtained from BioWhittaker (Walkersville, MD, USA) and they were serially passaged. Cells were maintained in a culture medium, EGM-2.

2.2. Hypoxia/reoxygenation

Confluent HCAEC were plated on plastic 100 mm dishes supplemented with cell medium, pH 6.7 and subjected to different durations of hypoxia (4, 6 and 8 h) in an air-tight chamber under constant nitrogen atmosphere for the respective durations, pre-gassed with 95% N_2–5% CO_2, pO_2 level <32 mm Hg. After the respective duration of hypoxia cells were exposed to reoxygenation for 24 h. HCAEC maintained under normoxic conditions at pO_2 160 mm Hg served as controls. To determine whether SN50 (NF-κB inhibitor obtained from Calbiochem, San Diego, CA, USA) and LY294002 (PI3-kinase inhibitor obtained from Sigma, St. Louis, MO, USA) have any effect on the expression of survivin or the extent of apoptosis and tubular morphogenesis, cells were incubated with 18 μM SN50 (8) and 5 μM LY294002 [13] for 1 h prior to induction of hypoxia.

2.3. Qualitative analysis of tube formation by HCAEC

The endothelial cells (5×10^4) were seeded onto Matrigel (Becton Dickinson Labware, Bedford, MA, USA) in a culture slide chamber with four wells. The chambers with the cells were exposed to hypoxia and reoxygenation in a closed incubator as discussed above. The cells were exposed to SN50 and LY294002 before even hypoxic exposure. After various durations of hypoxia followed by 18 h of reoxygenation the reorganization of the HCAEC and the extent of tube formation was recorded by phase contrast microscope (magnification $\times200$) with a digital camera.

2.4. Detection of apoptosis-induced DNA strand breaks using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL)

Coronary arteriolar endothelial cells, after exposure to various experimental protocols in situ, were fixed in 3.7% formaldehyde solution (pH 7.4). We used the Fragment End Labeling (FragEL™, Oncogene Research Products, Cambridge, MA, USA) DNA fragmentation detection kit to detect apoptosis. In this assay terminal deoxynucleotidyl transferase (TdT) binds to expose 3′-OH ends of DNA fragments generated in response to apoptotic signals and catalyze the addition of biotin-labeled and unlabeled deoxynucleotides. Biotinylated nucleotides are detected using a streptavidin–horseradish peroxidase (HRP) conjugate. Diaminobenzidine reacts with the labeled sample to generate an insoluble colored substrate at the site of DNA fragmentation. Counterstaining with methyl green aids in the morphological evaluation and characterization of normal and apoptotic cells.

2.5. Nuclear extract preparation for gel shift assay

Endothelial cells were washed with ice-cold phosphate-buffered saline, scraped from the plates and suspended in buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9], 10 mM KCl, 0.1 mM EDTA [ethylenediaminetetraacetic acid], 0.1 mM EGTA [ethylene glycol-bis(2-amino-ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 1 mM DTT [dithiothreitol], and 0.5 mM PMSF [phenylmethylsulfonyl fluoride]). The cells were allowed to swell on ice for 15 min, then Nonidet P-40 was added to a final concentration of 0.5%. After centrifugation, the pellet was washed with buffer A. The nuclear pellet was resuspended in ice-cold buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT, 1 μg/ml of pepstatin, 1 μg/ml of leupeptin) and allowed to swell on ice for 1 h. The nuclear extract was obtained after centrifugation at $12\,000\times g$ for 20 min. The supernatant was kept at −70°C for further use [8].

Protein concentration was estimated as above. NF-κB oligonucleotide (AGTTGAGGGGACTTTCCCAGG) (2.5 μl, 20 ng/μl) was labeled using T4 polynucleotide kinase as previously described. The binding reaction mixture contained in a total volume of 20.2 μl, 0.2 μl DTT (0.2 M), 1μl BSA (bovine serum albumin, 20 mg/ml), 4 μl poly(dI-dC) (0.5 μg/μl), 2 μl buffer D^+, 4 μl buffer F, 2 μl [^{32}P]oligo (0.5 ng/μl) and 7 μl extract containing 10 μg protein. Composition of buffer D^+ was 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% Nonidet P-40 while buffer F contained 20% Ficoll 400, 100 mM HEPES, pH 7.9, and 300 mM KCl. Incubation was carried out for 20 min at room temperature. 10 μl of the solution was loaded onto a 4% acrylamide gel and separated at 80 V until the dye hit the bottom. After electrophoresis, gels were dried and exposed to Kodak X-ray film at −70°C.

The purity of the nuclear extracts was examined using lactate dehydrogenase as a cytosolic marker. The supershift assays and the competition assays were performed to measure that the signal was specific for NF-κB. For the assays, anti-p65 or anti-p50 antibodies were added, separately or together, to the reaction mixtures immediately after addition of radiolabeled probe. For the competition assays, increasing fold molar excess of unlabelled NF-κB oligonucleotide was added into separate reaction mixtures.

2.6. Western blot analysis for survivin

For immunodetection of survivin, endothelial cells after various experimental protocols were harvested in lysis buffer (1.0% Igepal CA.630, 0.5% sodium deoxycholate, 0.1% SDS [sodium dodecyl sulfate]). Pure cell lysates obtained after centrifugation were used for protein analysis. Total protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce, Rockville, IL, USA). Cell lysates (40 μg) were run on polyacrylamide electrophoretic gels (SDS–PAGE) typically using 15% (acrylamide to bisacrylamide ratio). Separated proteins were electrophoretically transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) using a semidry transfer system (Bio-Rad, Hercules, CA, USA). Protein standards (Bio-Rad) were run in each gel. The blots were blocked in Tris-buffered saline/Tween-20 (TBS-T containing 20 mM Tris base, pH 7.6, 137 mM NaCl, 0.1% Tween-20) supplemented with 5% BSA for 1 h. Blots were incubated for 2 h with the specific primary rabbit antibodies (Novus Biologicals, Littleton, CO, USA) against survivin (1:1000). Blots were then incubated for 1 h at room temperature with 1:4500 diluted HRP-conjugated secondary antibodies (Boehringer Mannheim, Indianapolis, IN, USA) which were goat anti-rabbit IgG. Direct reprobing with anti-β-actin antibody as an internal control for Western blot was also performed. The blot was directly reprobed after washing with PBS-T for 10 min at room temperature, and then reprobed with a mouse monoclonal anti-β-actin as an internal control antibody (clone AC-150; Sigma, St. Louis, MO, USA) with a dilution of 1:5000 in blocking solution after detection of the primary target. The secondary antibody used for β-actin was HRP-conjugated goat polyclonal anti-mouse IgG (Transduction Laboratories, Lexington, KY, USA). After three washes of 5 min each, blots were treated with Enhanced Chemiluminescence reagent (ECL from Amersham, Life Science, Arlington Heights, IL, USA) and the required proteins were detected by autoradiography for variable lengths of time with Kodak X-Omat film. All the samples were tested for non-specific labeling. Negative and positive controls were run to validate the results.

2.7. Measurement of malonaldehyde (MDA) formation by HPLC

The cells were exposed to various durations of hypoxia and reoxygenation; the cells were harvested, washed with PBS containing sodium metabisulfite at 4°C, counted and weighed. The cell pellets were immediately homogenized by a Brinkman Polytron (Westbury, NY, USA) in 2 ml 10% TCA (trichloroacetic acid) containing 5.9 mM sodium metabisulfite, vortexed on ice for 10 min and centrifuged at $3000\times g$ for 10 min at 4°C. TCA supernatants were immediately derivatized with 100 μl 2,4-dinitrophenylhydrazine (3.1 mg/ml 2 N HCl), and then extracted with pentane. MDA formation was then measured using HPLC [14].

2.8. Statistical analysis

Data were obtained from at least six independent cell cultures. For statistical analysis, a two-way analysis of variance (ANOVA) followed by Scheffé's test was first carried out using Primer Computer Program (McGraw-Hill, 1988) to test for any differences between groups. If differences were established, the values were compared using Student's

t-test. The values are expressed as mean ± S.E.M. The results were considered significant if $P < 0.05$.

3. Results

3.1. Tubular morphogenesis of HCAEC after hypoxic preconditioning: effect of SN50 and LY294002

HCAEC plated on the surface of Matrigel after 4, 6 and 8 h of hypoxia followed by reoxygenation form tube-like structures. The formation of a capillary network of tubular structure was extremely prominent when the cells were exposed to 8 h of hypoxia followed by 12 h of reoxygenation (Fig. 1D vs. Fig. 1B,C). Modulation of the NF-κB- and PI3-kinase-dependent angiogenesis in the system was examined with SN50 (18 μM) and LY294002 (5 μM), inhibitors of NF-κB and PI3-kinase, respectively. The cells were pre-incubated with either SN50 or LY294002 and then were exposed to hypoxia and reoxygenation. Cells pretreated with SN50 and LY294002 displayed inhibition of hypoxia/reoxygenation-mediated tubular morphogenesis of HCAEC (Fig. 1E,F). NF-κB and PI3-kinase appeared to be directly involved in hypoxia/reoxygenation-mediated HCAEC tubular morphogenesis.

3.2. Activation of the DNA binding activity of NF-κB during hypoxic preconditioning

The binding activity of NF-κB increased significantly when

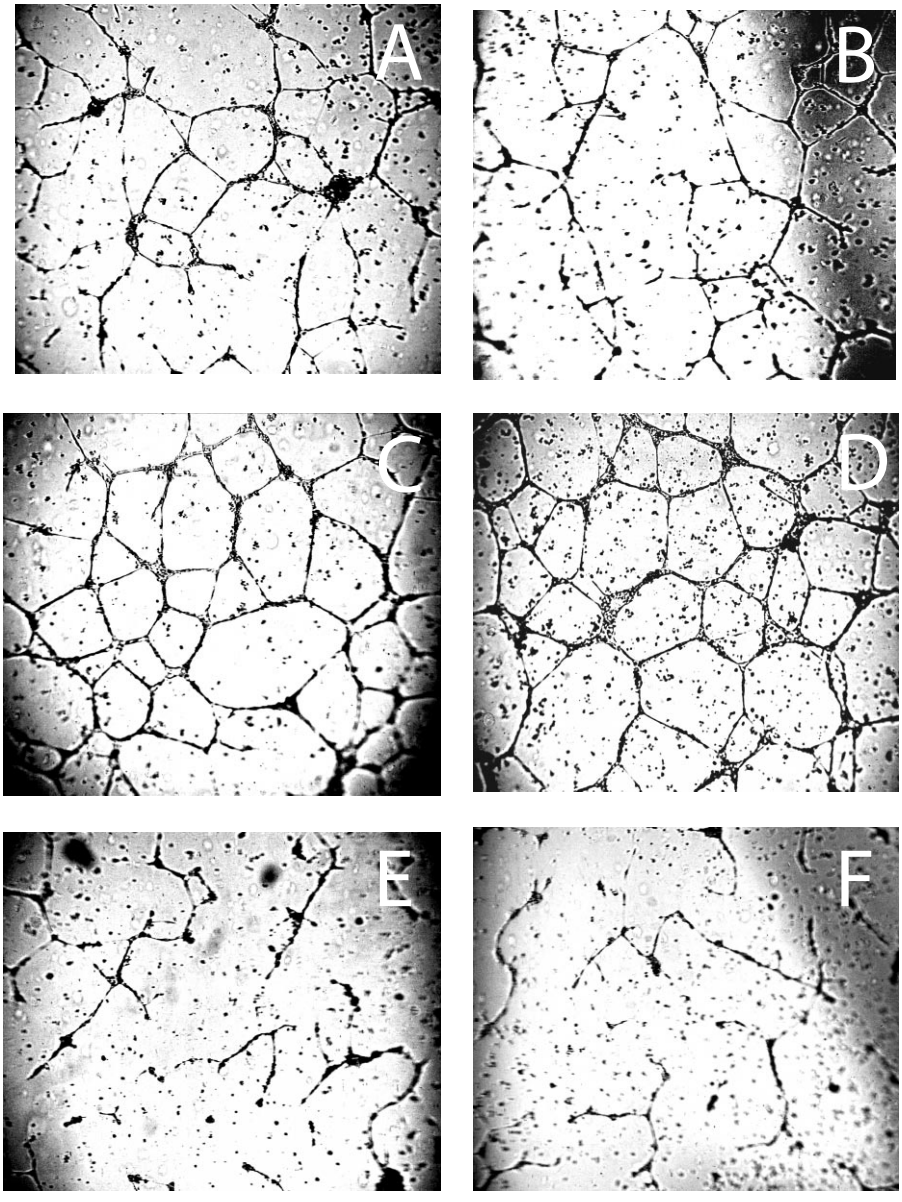

Fig. 1. Development of tube-like structures by HCAEC. Cells were plated onto Matrigel when they were confluent. A: Baseline control, normoxic. B: Cells exposed to 4 h hypoxia followed by 12 h of reoxygenation. C: Cells exposed to 6 h hypoxia followed by 12 h reoxygenation. D: Cells exposed to 8 h of hypoxia followed by 12 h of hypoxia. E: Cells pretreated with NF-κB inhibitor SN50 followed by 8 h of hypoxia and 12 h of reoxygenation. F: Cells pretreated with PI3-kinase inhibitor LY94002 followed by 8 h hypoxia and 12 h of reoxygenation. Note the inhibition of tube formation by SN50 and LY294002. The tube formation is very prominent when the cells were exposed to 8 h of hypoxia followed by 12 h of reoxygenation.

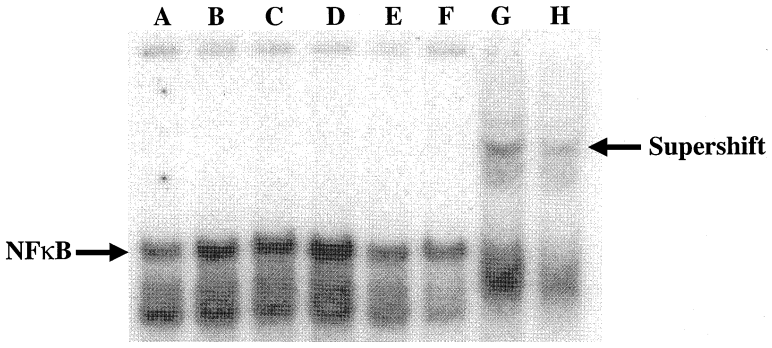

Fig. 2. Effect of various durations of hypoxia followed by 2 h of reoxygenation on the DNA binding activity of NF-κB. Lane A: Control baseline. Lane B: Cells exposed to 4 h hypoxia followed by 2 h of reoxygenation. Lane C: 6 h hypoxia followed by 2 h of reoxygenation. Lane D: 8 h of hypoxia followed by 2 h of reoxygenation. Lane E: cells pretreated with SN50 followed by 8 h of hypoxia and 2 h of reoxygenation. Lane F: Cells pretreated with LY294002 followed by 8 h of hypoxia and 2 h of reoxygenation. Lanes G and H: Baseline samples were used to perform supershift assay with a polyclonal antibody recognising NFkB p65 subunit proteins.

the cells were exposed to hypoxia and reoxygenation (Fig. 2B–D). The DNA binding activity was increased by 35% after 4 h of hypoxic insult and 45% after 6 h hypoxia compared to the baseline control. The 8 h hypoxic cells (Fig. 2D) showed a 66% increase in the DNA binding activity of NF-κB when compared to the normoxic, baseline control endothelial cells. This increased DNA activity of NF-κB was significantly decreased when SN50 and LY294002, inhibitors of NF-κB and PI3-kinase, respectively, were used (as shown in Fig. 2E,F). To confirm NF-κB binding activity with p65, we performed supershift assay with a polyclonal antibody recognizing NF-κB p65 subunit proteins as shown in lanes G and H of Fig. 2.

3.3. Activation of the expression of the anti-apoptotic protein survivin during hypoxic preconditioning

Western blot analysis revealed a prominent 16.5 kDa survivin band which was significantly induced in HCAEC exposed to hypoxia/reoxygenation (Fig. 3). Survivin was found to be increased by 25% after 4 h of hypoxia whereas this broad-spectrum anti-apoptotic protein was increased 5.1- and 7.4-fold in 6 h and 8 h hypoxic cells, respectively, when compared to the control baseline values. However, treatment with SN50 as well as LY294002 reduced the induction of survivin significantly (Fig. 3E,F) and restored the baseline level. This clearly establishes the involvement of NF-κB and PI3-kinase in the regulation of expression of the anti-death protein survivin.

3.4. Hypoxic preconditioning-mediated oxidative stress as measured by MDA

Oxygen-derived free radicals play a crucial role in the pathophysiology of reoxygenation-mediated injury and hypoxia/reoxygenation causes the development of oxidative stress to HCAEC. MDA production is a presumptive marker for the development of oxidative stress. As depicted in Fig. 4, the MDA concentration in HCAEC was increased from 196.7 ± 34.3 to 350 ± 63.1 pmol/g after 4 h of hypoxia followed by 2 h reoxygenation. The level of oxidative stress as measured by the concentration of MDA formation was significantly reduced to 93.1 ± 9.3 and 100 ± 10 pmol/g. These values were reduced below the baseline values when the duration of hypoxia was increased to either 6 or 8 h, followed by 2 h reoxygenation (Fig. 4). The amount of oxidative stress was significantly increased, as expected, in SN50- and LY294002-treated cells even after 8 h of hypoxia followed by 2 h reoxygenation (390 ± 22 and 412 ± 25 pmol/g). This demonstrates that NF-κB and PI3-kinase inhibition increases oxidative stress and thereby increases the extent of apoptosis.

3.5. The extent of apoptosis during hypoxic preconditioning

As expected, the extent of apoptosis increased with increasing oxidative stress as observed by the enhanced MDA production in the hypoxic cells. HCAEC exposed to 4 h hypoxia followed by 2 h reoxygenation ($23.7 \pm 2.5\%$ vs. $5.3 \pm 1.8\%$)

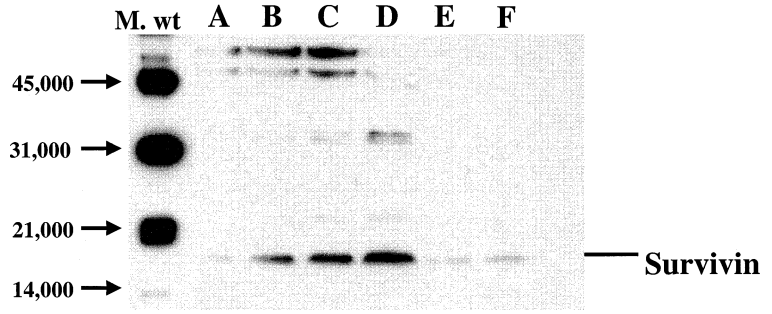

Fig. 3. Western blot analysis for survivin expression. Lane A: Control baseline. Lane B: Cells exposed to 4 h hypoxia followed by 24 h of reoxygenation. Lane C: 6 h hypoxia followed by 24 h of reoxygenation. Lane D: 8 h of hypoxia followed by 24 h of reoxygenation. Lane E: Cells pretreated with SN50 followed by 8 h of hypoxia and 24 h of reoxygenation. Lane F: Cells pretreated with LY294002 followed by 8 h of hypoxia and 24 h of reoxygenation.

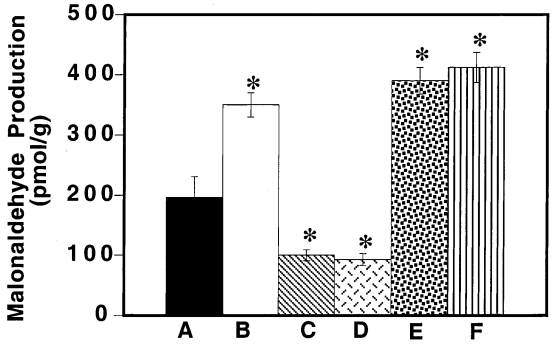

Fig. 4. Effects of various durations of hypoxia/reoxygenation on MDA content of HCAEC. A: Control baseline. B: Cells exposed to 4 h hypoxia followed by 2 h of reoxygenation. C: 6 h hypoxia followed by 2 h of reoxygenation. D: 8 h of hypoxia followed by 2 h of reoxygenation. E: Cells pretreated with SN50 followed by 8 h of hypoxia and 2 h of reoxygenation. F: Cells pretreated with LY294002 followed by 8 h of hypoxia and 2 h of reoxygenation. MDA was estimated in cells using HPLC as described in Section 2. Results are mean ± S.E.M. of six different sets. Each result was run in duplicate. $*P < 0.05$ compared to control.

revealed a significant number of apoptotic cells compared to baseline control. However, when the hypoxic period was increased to 6 h or 8 h followed by the same 2 h of reoxygenation, the number of apoptotic cells was decreased to baseline (6 ± 2% and 7.2 ± 1.9 vs. 5.3 ± 0.8%) control value (Fig. 5). On the other hand, the number of apoptotic cells was significantly increased with the treatment of SN50 and LY294002 (40.5 ± 3% and 42.5 ± 4%, respectively).

4. Discussion

In this study, we have demonstrated the activation and regulation of survivin, an anti-apoptotic protein, during HCAEC survival and tubular morphogenesis induced by hypoxic preconditioning. These findings constitute, to the best of our knowledge, the first direct demonstration of the role of PI3-kinase and NF-κB in regulating the anti-death candidate,

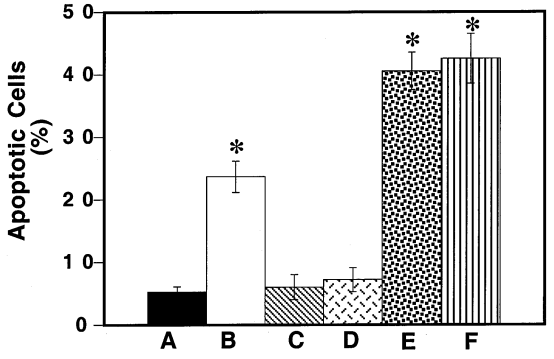

Fig. 5. Evaluation of endothelial apoptosis using TUNEL staining. Bar graph showing average number of endothelial cells undergoing apoptosis. Results are expressed as mean ± S.E.M. of six different sets. A: Control baseline. B: Cells exposed to 4 h hypoxia followed by 2 h of reoxygenation. C: 6 h hypoxia followed by 2 h of reoxygenation. D: 8 h of hypoxia followed by 2 h of reoxygenation. E: Cells pretreated with SN50 followed by 8 h of hypoxia and 2 h of reoxygenation. F: Cells pretreated with LY294002 followed by 8 h of hypoxia and 2 h of reoxygenation. $*P < 0.05$ compared to control.

survivin. The results clearly demonstrated the involvement of NF-κB and PI3-kinase as essential components of the survivin signaling pathway. Recent experimental evidence has suggested that regulation of endothelial cell apoptosis may be an essential mechanism to maintain angiogenesis in vivo [15]. This is also documented in our experimental finding that during SN50 and LY294002 treatment (Fig. 1E,F) a significant increase in cell apoptosis was observed which was accompanied by a decrease in tubular morphogenesis. Therefore, a direct relationship does exist between endothelial cell apoptosis and tubular morphogenesis. In this model, survivin, a recently described crucial regulator in the molecular mechanism of apoptosis, played a significant role in the reduction of cell apoptosis. Survivin is the smallest member of the IAP gene family. It is expressed in several apoptosis-regulated fetal tissues, including lung, liver, heart, and gastrointestinal tract [11]. Activation of survivin was found to inhibit caspase-3 activity [16]. Therefore inhibition of endothelial cell apoptosis during angiogenesis may occur simultaneously through various pathways, which might involve mitochondrial integrity by bcl-2 [17] and suppression of caspase activity by survivin [18,19].

Interestingly, our results indicate that hypoxic preconditioning induced adaptive modification of the arteriolar endothelial cells by oxidative stress where reactive oxygen species play a role as signaling molecules and thereby are able to reduce cellular injury. The lipid peroxidation product MDA is a presumptive marker for oxidative stress and an indicator of free radical production. As shown in our study, 4, 6 and 8 h of hypoxia followed by 2 h reoxygenation resulted in the development of short-lived oxidative stress. This stress increased appreciably after 4 h hypoxia followed by 2 h of reoxygenation and then came down steadily and progressively. It is interesting to note that after 6 and 8 h of hypoxia followed by 2 h of reoxygenation, the MDA level was decreased to below the baseline value. Corroborating these findings, the up-regulation of the anti-death candidate survivin increased to 5.1- and 7.5-fold of the baseline control. Therefore, it is tempting to speculate that such up-regulation of survivin followed by reduced apoptosis may have resulted from adaptation of the HCAEC to the oxidative stress. The event of adaptation required almost 24 h in our experimental model. In addition, it is also possible that some other stress proteins are being activated in response to hypoxic stress and mediate the beneficial effects of hypoxia/reoxygenation.

Another interesting finding was the inhibition of DNA binding activity of NF-κB by the PI3-kinase inhibitor LY294002, in HCAEC. This was accompanied by severe oxidative stress as documented by increased MDA production and activation of apoptosis. It was suggested that both the regulatory and the catalytic subunits of PI3-kinase play a role in NF-κB activation by the tyrosine phosphorylation-dependent pathway [20]. The function of PI3-kinase in NF-κB activation may also involve its phosphorylated lipid products. These lipid mediators may regulate the activity of protein kinases, e.g. PKCζ [21]. This isoform has already been implicated in NF-κB activation [22]. Recent studies have also indicated that PKCζ is a downstream target of PI3-kinase in vivo. Here we showed that the PI3-kinase antagonist LY294002 and the NF-κB antagonist SN50 act cooperatively to inhibit hypoxia/reoxygenation-mediated HCAEC survival. This suggests the importance of the PI3-kinase/Akt pathway

along with NF-κB in survival signaling. Several lines of evidence exist to indicate the involvement of Ang-1 and VEGF in the activation of the survival-promoting PI3-kinase/Akt pathway in promoting survival signal [23–26]. One of the potential targets of PI3-kinase is the serine/threonine kinase, Akt. Activation of Akt triggers survival signaling that eventually inhibits apoptosis. The pro-apoptotic factor Bad was recently found to be phosphorylated by activated Akt. Phosphorylation of Bad dissociates from Bcl-xl. Released Bcl-xl thus promotes cell survival. Recently, Akt was also found to phosphorylate caspase-9 and thereby diminishes its activity. Therefore the PI3-kinase pathway may promote cell survival and angiogenesis. It may control survival and death by various multiple downstream mechanisms. Thus, one of the multiple downstream mechanisms for survival signaling may be PI3-kinase–Akt–NF-κB–survivin.

In conclusion, we have demonstrated the role of PI3-kinase in cooperation with the redox-regulated transcription factor NF-κB in hypoxic preconditioning-mediated HCAEC tubular morphogenesis. Also, activation of this pathway decreases cell death through the induction of the broad-spectrum anti-apoptotic protein survivin.

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