Cytoskeletal Changes In Hypoxic Pulmonary Endothelial Cells Are Dependent On The MAP Kinase-Associated Protein Kinase MK2

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Abbreviations: RPMEC, rat pulmonary artery microvascular endothelial cells; MAPKAPK2, mitogen-activated protein kinase-associated protein kinase 2; HSP27, heat shock protein 27; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Summary

Exposure to hypoxia causes structural changes in the endothelial cell layer that alter its permeability and its interaction with leukocytes and platelets. One of the well-characterized cytoskeletal changes in response to stress involves the reorganization of the actin cytoskeleton and the formation of stress fibers. This report describes cytoskeletal changes in pulmonary microvascular endothelial cells in response to hypoxia, and potential mechanisms involved in this process. The hypoxia-induced actin redistribution appears to be mediated by components downstream of the MAP kinase p38, which is activated in pulmonary endothelial cells in response to hypoxia. Our results indicate that the kinase MK2, which is a substrate of p38, becomes activated by hypoxia, leading to the phosphorylation of one of its substrates, HSP27. Since HSP27 phosphorylation is known to alter actin distribution in response to other stimuli, we postulate that it also causes the actin redistribution observed in hypoxia. This notion is supported by the observations that similar actin redistribution occurs in cells overexpressing constitutively active MK2 or phospho-mimicking HSP27 mutant. Overexpressing dominant negative MK2 blocks the effects of hypoxia on the actin cytoskeleton. Taken together these results indicate that hypoxia stimulates the p38-MK2-HSP27 pathway leading to significant alteration in the actin cytoskeleton.
Introduction

Hypoxia causes injury in a variety of organs and has been associated with many lung diseases including the acute respiratory distress syndrome (ARDS), pulmonary embolism, and ischemia-reperfusion injury. Hypoxia has been shown to increase the permeability of the endothelial barrier both in vitro (1-4) and in vivo (5). Moreover, hypoxia increases endothelial adhesiveness to neutrophils (6,7). In that respect, endothelial cells respond to hypoxia in a manner similar to their response to inflammation. However, as opposed to the response of endothelial cells to inflammatory products, which has been extensively explored, the signal transduction pathways involved in the endothelial response to hypoxia remain poorly understood. Recent reports have demonstrated activation of the stress-activated MAP kinase p38 in response to hypoxia (8-16). For example, we have described the activation of p38 in hypoxic pulmonary microvascular endothelial cells, and implicated it as one of the mechanisms of activation of the reactive oxygen producing enzyme xanthine oxidase (16). The enzyme MK2 is immediately downstream of p38, and is known to phosphorylate the small heat shock protein HSP27 (17). Since HSP27 interacts with actin and modulates cytoskeletal organization (18,19), we investigated whether the MK2 pathway is activated by hypoxia, and whether this process can lead to cytoskeletal changes. Our findings indicate that MK2 is indeed activated by hypoxia in RPMEC, and that HSP27 phosphorylation is increased, concomitantly with reorganization of the actin cytoskeleton. The effect of
hypoxia on the actin cytoskeleton is mimicked by overexpressing constitutively active MK2, and blocked by overexpressing dominant negative MK2, in endothelial cells. Furthermore, overexpressing a phospho-mimicking mutant HSP27 in endothelial cells causes reorganization of the actin cytoskeleton similar to the actin redistribution caused by hypoxia.

**Experimental Procedures**

*Cell Culture:* RPMEC were a gift from Dr. Una Ryan (Avant Immunotherapeutics, Needham, MA), and have been well characterized by us and others (20). These cells exhibit typical endothelial cobblestone morphology, and stain positively with antibodies against von Willebrand factor. For hypoxic exposure, cells were placed in humidified airtight incubation chambers (Billups-Rothenberg, Del Mar, CA) and gassed with 3% O₂, 5% CO₂, and balance N₂. Normoxic cells were placed in a tissue culture incubator maintained at 5% CO₂ and 37 °C.

*Actin Cytoskeleton Examination:* Cells were seeded on poly-L-lysine- or collagen-coated cover slips. At various degrees of confluence, cells in serum-free media were subjected to different treatments, e.g., hypoxia, and/or kinase inhibitors. To control for the effect of cover slip coating, only cells plated on the same substrate were compared and analyzed in a particular experiment. At the end of the treatment, the cover slips were
rinsed twice with phosphate-buffered saline (PBS), and fixed for 10 minutes with 4% formaldehyde. Next the cover slips were washed twice with PBS, then permeabilized for 10 minutes with 0.4% triton-X-100 in PBS. The cells were stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR) for 20 minutes. The cover slips were then washed with PBS, mounted with Citifluor and examined using a Zeiss fluorescence microscope. The amount of filamentous actin formed was assessed by quantifying rhodamine-phalloidin fluorescence using the image analysis software, IP Lab Scanalytics (Fairfax, VA).

**MK2 Kinase Assay:** After exposure of cells to normoxia or hypoxia, activation of MK2 was assayed by measuring the activity of the immunoprecipitated enzyme. Specific MK2 activity was assayed using the MK2 assay kit from Upstate Biotechnology (Lake Placid, NY). In brief, the cells were washed and lysed, and then MK2 was immunocomplexed with agarose-conjugated anti-MK2 antibody by rocking overnight at 4 °C. The immunocomplex was then brought down by centrifugation and the pellet was washed. Next, MK2-specific peptide substrate was added along with [γ-32P]ATP and incubated with the immunoprecipitated kinase with vigorous shaking at 30 °C. Then, the complex was brought down by centrifugation and the supernatant, which contains the peptide substrate, was spotted on p81 phosphocellulose paper, and washed with phosphoric acid and acetone to remove unincorporated label. Finally, the p81 paper was transferred to scintillation vials containing scintillation cocktail, and the samples were counted on a Packard beta counter.
**Two-dimensional Electrophoresis and Immunoblotting:** Isoelectrofocusing was performed in a Multiphor 2 unit according to manufacturer’s instructions (Amersham Biosciences, Inc., Piscataway, NJ). In brief, cells were lysed in 8 M urea, 0.5% CHAPS, 60 mM DTT, 2% Pharmalyte™ 47. Equal amount of protein from cell lysates obtained from different treatment groups were then mixed with IPG™ (Amersham Biosciences, Inc., Piscataway, NJ) rehydration buffer and used to rehydrate Immobiline™ strips (pH 4-7 linear gradient; Pharmacia, Piscataway, NJ.). After isoelectrofocusing, the strips were equilibrated with 2% SDS, 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 0.002% bromophenol blue, 10 mg/mL DTT, and then with the same buffer containing 25 mg/mL iodoacetamide instead of DTT. After equilibration, each strip was overlaid on a single well 10-20% gradient SDS-PAGE gel and electrophoresed according to Laemmli (21). After electrophoresis, the gel was blotted onto an Immobilon-P membrane by electrophoretic transfer. The membrane was then washed, blocked with 5% milk and probed with an antibody against HSP27 (Upstate Biotechnology, Lake Placid, NY). The immunoreactive bands were visualized using anti rabbit secondary antibody conjugated to horseradish peroxidase and a chemiluminescent substrate according to manufacturer’s instructions (Super Signal, Pierce, Rockford, IL).

**Transfection of Endothelial Cells:** Constitutively active mutant, and dominant negative mutant MK2 constructs, as well as phosphomimicking mutant HSP27 constructs were generated as described (22-24). In brief, these constructs were made in the pcDNA3 vector (Invitrogen, Carlsbad, CA), in which the cytomegalovirus promoter drives the
eukaryotic expression of the corresponding protein. The vectors used for transfection were either pcDNA3 vector alone, pcDNA3-MK2EE to express constitutively active MK2 in which the residues, T205E, T317E, were mutated, and pcDNA3-MK2KR to express dominant negative MK2 in which the residue, K76R, was mutated. The vector pcDNA3-HSP27PM was used to express HSP27 in which the residues, S15D, S78D, S82D, were mutated to mimic phosphorylated HSP27. These vectors were introduced (5 µg) into endothelial cells by electroporation. Stable cell lines were obtained by selection with geneticin, and resistant colonies were isolated, expanded, then screened for the level of MK2 activity or for human HSP27 expression level.

Statistical Analysis: Statistical analysis was carried out using SPSS software (Chicago, IL). Student’s \( t \) test was used to analyze differences between two groups. When comparisons between multiple groups were carried out, one-way analysis of variance was employed. Statistical significance was considered at \( P < 0.05 \).

Results

_Hypoxia alters the actin cytoskeleton in pulmonary endothelial cells:_ Hypoxia is known to cause changes in the cytoskeleton, which alter the motility and permeability of the endothelial barrier. Using rhodamine-conjugated phalloidin, which binds to filamentous actin, we assessed the distribution of actin in normoxic and hypoxic cells. Our results indicate that exposure of endothelial cells to hypoxia (3% O\(_2\)) causes a shift in
filamentous actin from a web-like structure (in normoxic cells) to parallel stress fibers. The latter become thicker and increase in number with exposure time (Figure 1A). The change is observed as early as 30 minutes after exposure to hypoxia, becomes more significant by 1 hour, and begins to reverse itself by 4 hours of exposure to hypoxia (Figure 1A). In addition to the reorganization of actin filaments, we observed an overall increase in filamentous actin in response to hypoxia. To quantify filamentous actin, rhodamine-phalloidin fluorescence was assessed in micrographs of coverslips from several experiments using image analysis software as described in Experimental Procedures. As shown in Figure 1B, there was a significant increase in filamentous actin by 1 hour of hypoxia (150% as compared to normoxic control cells), with a return to baseline by 4 hours of exposure. In conclusion, these results indicate that hypoxia exerts a rapid and significant change in the actin cytoskeleton.

**Hypoxia stimulates MK2 in rat pulmonary microvascular endothelial cells:** Recent reports have demonstrated the activation of the p38 MAP kinase in various cell types in response to hypoxia (8-16). Our laboratory has recently demonstrated an important role for p38 in mediating xanthine oxidase activation in response to hypoxia (16). Since p38 kinase has been shown to phosphorylate and activate MK2, and since MK2 is known to be involved in actin remodeling (17), we examined the involvement of this latter kinase in mediating the effects of hypoxia on the actin cytoskeleton. First, we tested whether MK2 becomes activated in hypoxic (3% O₂) RPMEC. MK2 was immunoprecipitated from cell lysates with an agarose-conjugated antibody, and its activity was measured as
described in Experimental Procedures. As shown in Figure 2, MK2 activity increased in hypoxia with a maximum increase observed after 1 hour of hypoxia. The time course of activation of MK2 was very similar to that of p38 activation by hypoxia we observed in an earlier report (16). The increased MK2 activity was also blocked by pre-incubation with a p38 kinase inhibitor (not shown). Hence, MK2, which is downstream of p38, appears to be activated in response to p38 activation in hypoxia. The time-course of activation of MK2 is similar to that of actin cytoskeleton reorganization in hypoxia (Figure 1), consistent with a role for MK2 in mediating that effect.

**HSP27 is phosphorylated in hypoxic endothelial cells:** Since HSP27 is a known substrate of MK2, and has been shown to regulate the actin cytoskeleton, we tested the possibility of HSP27 phosphorylation in hypoxia. To check if endogenous HSP27 phosphorylation is increased in hypoxic RPMEC, cell lysates from normoxic and hypoxic (3% O₂) samples were analyzed by 2-dimensional electrophoresis, followed by immunoblotting with an antibody against HSP27. Phosphorylation causes a protein to become more acidic thus reducing its isoelectric point. Hence, differently phosphorylated HSP27 (non-, mono-, bi-, or tri-phosphorylated) can be resolved by 2-dimensional electrophoresis (25). Although phosphospecific antibodies have been used to study changes in HSP27 phosphorylation by SDS-PAGE, these antibodies usually recognize one phosphoepitope and do not discriminate between mono-, bi- or tri-phosphorylated HSP27. The different spots shown in Figure 3 reflect putative non-phosphorylated as well as mono-, bi-, and tri-phosphorylated forms of HSP27, with the tri-phospho
HSP27 being most acidic and migrating farthest to the right. As shown in Figure 3, by 30 minutes of hypoxia, the relative amount of phospho HSP27 increased significantly compared to normoxia. By 1 hour of hypoxia, there was a significant decrease in the tri-phospho HSP27 (Figure 3). The distribution of phospho and non-phospho HSP27 began to return to normal by 2 and 4 hour of hypoxia (Figure 3). The decrease in tri-phospho HSP27 by 1 hour was reproducible in different experiments, and its significance is currently being investigated. Possibilities include rapid aggregation or degradation of the most heavily phosphorylated form. In conclusion, hypoxia caused a rapid increase in HSP27 phosphorylation by 30 minutes, which began to return to baseline by 4 hours of hypoxia. Comparing the time course of actin redistribution (Figure 1) to the time-course of HSP27 phosphorylation (Figure 3) reveals that stress fiber formation begins by 30 minutes of hypoxic exposure, concomitant with HSP27 phosphorylation (Figure 3). Stress fibers are thickest by 1 hour of hypoxia at a time when less HSP27 is available to inhibit stress fiber formation. Finally, stress fibers become thinner, and the actin resumes a normal distribution by 4 hours of hypoxia, which coincides with the reversal of HSP27 phosphorylation as indicated in Figure 3.

**MK2 activity is correlated with actin cytoskeleton reorganization:** In order to assess the involvement of MK2 in mediating hypoxia-stimulated alteration of the actin cytoskeleton, the level of MK2 activity was modulated in endothelial cells. As no specific inhibitors of MK2 are available, the activity of MK2 was modulated by expressing different forms of the enzyme in cells. RPMEC were transfected with the
empty vector alone (mock-transfected), with constitutively active MK2, or with dominant negative MK2. After selecting stable transfectants with geneticin, and isolating and expanding clones, MK2 activity was measured as described in Experimental Procedures. As shown in Figure 4, cells overexpressing the constitutively active form of MK2 displayed significantly greater activity compared to mock-transfected cells. Overexpressing dominant negative MK2 mutant did not affect the baseline MK2 activity (data not shown).

Stably transfected cells overexpressing constitutively active MK2, and dominant negative MK2, were plated on collagen-coated cover-slips, and then their actin cytoskeleton was examined as described above. Overexpression of constitutively active MK2 caused an increase in stress fiber formation (Figure 5A) and filamentous actin (Figure 5B) in normoxic cells resembling the effect of hypoxia. On the other hand, overexpressing dominant-negative MK2 inhibited the formation of stress fibers in response to hypoxia (Figure 6A). In the cells overexpressing dominant negative MK2, no increase in filamentous actin was observed in response to hypoxia (Figure 6B). Thus, the formation of actin stress fibers correlated with MK2 activity, and disrupting MK2 activity blocked stress fiber formation in response to hypoxia. These results are consistent with a role for MK2 in mediating the effects of hypoxia on the endothelial cytoskeleton.

**Overexpressing phosphomimicking HSP27 mutant increases stress fiber formation:** To test if phosphorylation of HSP27 has an effect on actin distribution, RPMEC were
transfected with the empty vector alone (mock-transfected), or phospho-mimicking HSP27 mutant, in which phosphorylatable amino acids were replaced by negatively charged aspartates as described in Experimental Procedures. After selecting stable transfectants with geneticin, and isolating and expanding clones, the cells were grown on cover slips and labeled with rhodamine-phalloidin as described above. Overexpression of phosphomimicking HSP27 mutant in endothelial cells caused an increase in stress fibers and filamentous actin in normoxic cells (Figure 5A, B). Thus, formation of stress fibers in endothelial cells correlates with negatively charged amino acids in HSP27 mimicking phosphorylated HSP27.

Discussion

Hypoxia is associated with many lung diseases including ARDS, pulmonary embolism and ischemia-reperfusion injury. The pulmonary microvascular endothelium is an obvious target of hypoxia because of its key anatomical location in the alveolar capillary gas exchange unit. In this report, we examined the role of downstream components of the p38 MAP kinase pathway in effecting structural changes in endothelial cells exposed to hypoxia. Our findings indicate that the kinase MK2 becomes activated in hypoxia leading to HSP27 phosphorylation. These changes are accompanied by alterations in the filamentous actin cytoskeleton. A causal link between MK2 activation, HSP27 phosphorylation and actin redistribution is supported by experiments in which the activity of MK2 and HSP27 were modulated by overexpressing different forms of these proteins.
The endothelium constitutes a barrier that controls the flow of fluids and materials from the blood to tissues, and it regulates blood vessel tone, homeostasis, growth, and response to injury (for review, see 26). In response to injury, the structure and function of the endothelium become altered in a manner that affects the physiology of the blood vessel and the involved organ in general. For instance, the permeability of the endothelial barrier has been shown to increase in response to hypoxia, both in vitro (1-3), and in vivo (5). Furthermore, hypoxia promotes the production of cytokines and growth factors by the endothelial layer. For example, IL-1α production is increased in hypoxic endothelial cell cultures (27). The vascular endothelial growth factor (VEGF) is a classic hypoxia-induced angiogenic factor that mediates vascular remodeling in the lung (28,29). Hypoxia impairs endothelial anti-thrombogenic potential (2) as well as the ability of the endothelium to regulate vascular tone (26). For instance, the synthesis of the vasodilator prostacyclin is decreased in hypoxic pulmonary artery rings as well as in cultured endothelial cells from neonatal calves (30). Nitric oxide (NO) is another endothelium-derived vasodilator regulated in hypoxia. Work from our laboratory has demonstrated regulation of the constitutive as well as the inducible form of nitric oxide synthase (eNOS and iNOS, respectively) in response to hypoxia (31,32). Hypoxia stabilizes the iNOS mRNA expression induced by cytokine treatment (32), suggesting that hypoxia may alter the effects of inflammatory cytokines (33).

The mechanisms involved in vascular responses to hypoxia are likely to be quite complex. Some of these responses involve the activation of transcription through the
action of transcription factors such as hypoxia-induced factor-1 (HIF-1) (34-36). Other events, however, are considered too rapid to be due to transcriptional processes. One example of a non-transcriptional hypoxic response by the endothelium is the mobilization of P-selectin, and its release from membranous organelles, which allows it to bind and activate neutrophils (7). Recent work from our laboratory identified another non-transcriptional endothelial response to hypoxia, namely the phosphorylation of the reactive oxygen-producing enzyme, xanthine oxidase, and subsequent upregulation of the enzymatic activity (16). In these experiments, the rapid phosphorylation and activation of xanthine oxidase was found to be mediated by the kinases casein kinase II and p38 (16).

Activation of p38 MAP kinase has been described in various cell types in response to hypoxia (8-15). Our laboratory has recently demonstrated the activation of p38 by hypoxia in rat pulmonary microvascular endothelial cells as well (16). p38 is a stress-activated MAP kinase that becomes activated in response to different stimuli such as ultraviolet radiation and hyperosmolarity. Because of its involvement in either mediating the effects or regulating the expression of many growth factors and cytokines that are important in inflammation, p38 has been the subject of intensive research (for review, see 37). Inhibitors of p38 have been developed for use in a wide variety of inflammatory diseases from arthritis to lung disease (38). Once p38 is activated, it can phosphorylate a variety of substrates, including the kinase MK2. Upon its phosphorylation by p38, MK2 becomes activated, and in turn, phosphorylates different
substrates, such as the small heat shock protein HSP27. Expression of HSP27 is particularly high in the lung (39,40). Like other heat-shock proteins, HSP27 can function as a chaperone, and is known to stabilize proteins such as citrate synthase and alcohol dehydrogenase (41,42). Of particular interest is the ability of HSP27 to interact with actin and to reduce actin polymerization into filaments (25). Upon phosphorylation, HSP27 changes its polymer state such that it loses its function as a chaperone and no longer blocks the polymerization of actin, thus resulting in the stabilization of actin fibers (25). HSP27 has also been implicated in the stabilization of actin fibers in vivo in ischemic rat kidneys (43). Indeed p38 has been implicated in mediating actin reorganization through altering HSP27 phosphorylation in response to oxidative stress and VEGF (19,44).

After demonstrating that p38 is activated in RPMEC by hypoxia (16), we tested the hypothesis that downstream components of the p38 pathway might mediate cytoskeletal change in hypoxia. First, we demonstrated that activation of MK2 by hypoxia follows a time-course that closely mirrors the time-course of p38 activation by hypoxia that we previously described (16). HSP27, a substrate of MK2, also becomes phosphorylated within the same time frame. Furthermore, actin stress fibers, which are known to be regulated by HSP27 phosphorylation, become thicker and more abundant in response to hypoxia. These events peak within 1 hour of exposure to hypoxia. A causal link between these events is supported by the results obtained with different MK2 and HSP27 constructs. Overexpressing constitutively active MK2 caused actin redistribution
similar to that observed in hypoxic cells. Conversely, overexpressing dominant-negative MK2 inhibited the hypoxia-stimulated actin redistribution. These results support the notion that hypoxia causes redistribution of the actin cytoskeleton through activation of MK2. Furthermore, overexpressing a phosphomimicking mutant HSP27 increased filamentous actin and stress fiber formation, consistent with a direct role for HSP27 phosphorylation in mediating reorganization of the actin cytoskeleton.

Reorganization of the actin cytoskeleton has been associated with changes in endothelial permeability, motility of endothelial cells as well as increased adhesiveness of inflammatory cells. Experiments are currently underway to further elucidate how these processes might be altered in our experimental system. In conclusion, MK2 and HSP27 are important signaling molecules in mediating endothelial responses to hypoxia, such as cytoskeletal reorganization. The components of the p38-MK2-HSP27 pathway might present targets for drug development against diseases, such as pulmonary edema, where the endothelial barrier is altered.
Acknowledgments

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Figure Legends:

Figure 1: Hypoxia causes filamentous actin to switch from a web-like distribution to parallel stress fibers. Stress fiber formation was maximal at 1 hour of hypoxia and returned to normal by 4 hours of hypoxia (A). There was also an overall increase in filamentous actin, which was statistically significant after 1 hour of hypoxia (B). To assess the changes in filamentous actin, rhodamine fluorescence was quantified in micrographs from several experiments (n=3-4 for each group) using image analysis as described in Experimental Procedures. * indicates $P < 0.05$ versus mean of normoxic control.

Figure 2: Hypoxia stimulates MK2 activity in RPMEC. MK2 was immunoprecipitated and its kinase activity was assayed as described in Experimental Procedures. Maximum increase was observed at 1 hour of exposure. * indicates $P < 0.05$ versus mean of normoxic control.

Figure 3: Hypoxia increases HSP27 phosphorylation by 30 minutes of exposure. Samples from different cell groups were analyzed by 2-d electrophoresis as described in Experimental Procedures. Numbered positions refer to putative phosphorylated forms: (1) non-phosphorylated, (2) mono-phosphorylated, (3) bi-phosphorylated, and (4) tri-phosphorylated HSP27. The increase in phosphorylation is followed by a disappearance of the tri-phospho HSP27 at 1 hour of hypoxia, and return to baseline expression at 2 and
4 hours of hypoxia.

**Figure 4:** Over-expressing MK2 in RPMEC. Cells transfected with constitutively active MK2 contained significantly higher MK2 activity compared to cells transfected with the empty vector. * indicates $P < 0.05$.

**Figure 5:** Overexpressing constitutively active MK2 or phosphomimicking mutant HSP27 causes a significant increase in stress fibers in normoxic cells (A). Filamentous actin was quantified by measuring rhodamine fluorescence in micrographs from several experiments (n=3-4 for each group) using image analysis as described in Experimental Procedures. There was a statistically significant increase in filamentous actin in cells overexpressing constitutively active MK2 and in cells overexpressing phosphomimicking HSP27 as compared to mock-transfected cells (B). * indicates $P < 0.05$ versus mean of mock-transfected controls.

**Figure 6:** Overexpressing dominant negative MK2 blocks the formation of stress fibers in response to hypoxia (A). Filamentous actin was quantified by measuring rhodamine fluorescence in micrographs from several experiments (n=3-4 for each group) using image analysis as described in Experimental Procedures. There was no statistically significant increase in filamentous actin in response to hypoxia in cells overexpressing dominant negative MK2 (B) as compared to mock-transfected cell. * indicates $P < 0.05$ versus mean of mock-transfected controls.
Figure 1

A

Normoxia

Hypoxia 30 min

Hypoxia 1 hr

Hypoxia 2 hr

Hypoxia 4 hr
Figure 1

B

[Bar chart showing relative fluorescence (% of normoxic controls) for different treatments: Normoxia, 1/2 hr, 1 hr, Hypoxia, 2 hr, 4 hr. The chart displays a marked increase in fluorescence during 1 hr of hypoxia compared to other treatment times.]
Figure 2

The graph shows the MK2 activity (1000 cpm) under different treatment conditions: Normoxia, 1/2 hr, 1 hr, 2 hr, and 4 hr. The x-axis represents the treatment time, and the y-axis represents the MK2 activity. Significant differences are indicated by asterisks (*) above the bars.
Figure 3

Normoxia

Hypoxia

30 min
1 hr
2 hr
4 hr
Figure 4

The diagram shows the comparison of MK2 activity (1000 cpm) in transfected endothelial cells when expressed as Empty Vector or MK2-Active. The graph indicates a significant increase in MK2 activity in MK2-Active cells compared to Empty Vector cells, with the latter having a lower activity level.
Figure 5

A

Control  Constitutively Active Mk2  Phosphomimicking HSP27
Figure 6

A

Control

Normoxia

Hypoxia

MK2 dominant negative
Figure 6

B

![](image)

**Cell Group**

- Normoxia Control
- Hypoxia
- Normoxia MK2 dominant -ve
- Hypoxia

**Relative Fluorescence (% of Normoxic Control)**

- 0
- 20
- 40
- 60
- 80
- 100
- 120
- 140
- 160
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