Cytoskeletal Changes in Hypoxic Pulmonary Endothelial Cells Are Dependent on MAPK-activated Protein Kinase MK2*

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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 MAPK p38, which is activated in pulmonary endothelial cells in response to hypoxia. Our results indicate that kinase MK2, which is a substrate of p38, becomes activated by hypoxia, leading to the phosphorylation of one of its substrates, HSP27. Because 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 phosphomimicking 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.

Hypoxia causes injury in a variety of organs and has been associated with many lung diseases including the acute respiratory distress syndrome, 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 adherence 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 MAPK1 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, immediately downstream of p38, is known to phosphorylate the small heat shock protein HSP27 (17). Because 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 is blocked by overexpressing dominant negative MK2 in endothelial cells. Furthermore, overexpressing a phosphomimicking 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, 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 coverslips. At various degrees of confluence, cells in serum-free medium were subjected to different treatments, e.g. hypoxia and/or kinase inhibitors. To control for the effect of coverslip coating, only cells plated on the same substrate were compared and analyzed in a particular experiment. At the end of the treatment, the coverslips were rinsed twice with phosphate-buffered saline (PBS) and fixed for 10 min with 4% formaldehyde. Next, the coverslips were washed twice with PBS and then permeabilized for 10 min with 0.4% Triton-X-100 in PBS. The cells were stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR) for 20 min. The coverslips were then washed with PBS, mounted with Citifluor, and examined using a Zeiss fluorescence microscope. The amount of filamentous actin formed was quantified by using image analysis software from 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 noprecipitated enzyme. Specific MK2 activity was assayed using the

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; RPMEC, rat pulmonary artery microvascular endothelial cells; HSP27, heat shock protein 27; PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

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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, containing 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 mixture, 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). In brief, cells were lysed in 8 M urea, 0.5% CHAPS, 60 mM dithiothreitol, 2% Pharmalyte™ 4–7. Equal amounts of protein from cell lysates obtained from different treatment groups were then mixed with IPG™ (Amersham Biosciences) rehydration buffer and used to rehydrate Immobiline™ strips (pH 4–7 linear gradient; Amersham Biosciences). 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% bromphenol blue, and 10 mg/ml dithiothreitol and then with the same buffer containing 25 mg/ml iodoacetamide instead of dithiothreitol. After equilibration, each strip was overlaid on a single-well 10–20% gradient SDS-polyacrylamide 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). The immunoreactive bands

FIG. 1. Hypoxia causes filamentous actin to switch from a web-like distribution to parallel stress fibers. Stress fiber formation was maximal at 1 h of hypoxia and returned to normal by 4 h of hypoxia (A). There was also an overall increase in filamentous actin, which was statistically significant after 1 h 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 under “Experimental Procedures.” *, p < 0.05 versus mean of normoxic control.
Hypoxia stimulates MK2 activity in RPMEC. MK2 was immunoprecipitated, and its kinase activity was assayed as described under "Experimental Procedures." Maximum increase was observed at 1 h of exposure. *, p < 0.05 versus mean of normoxic control.

FIG. 2.

FIG. 3. Hypoxia increases HSP27 phosphorylation by 30 min of exposure. Samples from different cell groups were analyzed by two-dimensional electrophoresis as described under "Experimental Procedures." Numbered positions refer to putative phosphorylated forms: 1, nonphosphorylated; 2, monophosphorylated; 3, biphosphorylated; 4, triphosphorylated HSP27. The increase in phosphorylation is followed by the disappearance of triphospho-HSP27 at 1 h of hypoxia and a return to base-line expression at 2 and 4 h of hypoxia.

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MK2 was shown to phosphorylate and activate MK2, and because 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 under "Experimental Procedures." As shown in Fig. 1A, MK2 activity increased in hypoxia with a maximum increase observed after 1 h of hypoxia. The time course of activation of MK2 was very similar to that of p38 activation by hypoxia as observed in an earlier report (16). The increased MK2 activity was significant.

RESULTS

Hypoxia Alters the Actin Cytoskeleton in Pulmonary Endothelial Cells—Hypoxia is known to cause changes in the cytoskeleton that 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% O2) 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 (Fig. 1A). The change is observed as early as 30 min after exposure to hypoxia, becomes more significant by 1 h, and begins to reverse itself by 4 h of exposure to hypoxia (Fig. 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 under "Experimental Procedures." As shown in Fig. 1B, there was a significant increase in filamentous actin by 1 h of hypoxia (150% as compared with normoxic control cells), with a return to base line by 4 h 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 MAPK 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). Because p38 kinase has been shown to phosphorylate and activate MK2, and because 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 under "Experimental Procedures." As shown in Fig. 2, MK2 activity increased in hypoxia with a maximum increase observed after 1 h of hypoxia. The time course of activation of MK2 was very similar to that of p38 activation by hypoxia as observed in an earlier report (16). The increased MK2 activity 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.
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 (Fig. 1), consistent with a role for MK2 in mediating that effect.

HSP27 Is Phosphorylated in Hypoxic Endothelial Cells—Because 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 whether endogenous HSP27 phosphorylation is increased in hypoxic RPMEC, cell lysates from normoxic and hypoxic (3% O2) samples were analyzed by two-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 triphosphorylated) can be resolved by two-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 triphosphorylated HSP27. The different spots shown in Fig. 3 reflect putative nonphosphorylated as well as mono-, bi-, and triphosphorylated forms of HSP27, with the triphospho-HSP27 being most acidic and migrating farthest to the right. As shown in Fig. 3, by 30 min of hypoxia, the relative amount of phospho-HSP27 increased significantly compared with normoxia. By 1 h of hypoxia, there was a significant decrease in the triphospho-HSP27 (Fig. 3). The distribution of phospho- and nonphospho-HSP27 began to return to normal by 2 and 4 h of hypoxia (Fig. 3). The decrease in triphospho-HSP27 by 1 h 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 min, which began to return to baseline by 4 h of hypoxia. A comparison of the time course of actin redistribution (Fig. 1) with the time course of HSP27 phosphorylation (Fig. 3) reveals that stress fiber formation begins by 30 min of hypoxic exposure, concomitant with HSP27 phosphorylation (Fig. 3). Stress fibers are thickest by 1 h 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 h of hypoxia, which coincides with the reversal of HSP27 phosphorylation as indicated in Fig. 3.

MK2 Activity Is Correlated with Actin Cytoskeleton Reorganization—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
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(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 under “Experimental Procedures.” As shown in Fig. 4, cells overexpressing the constitutively active form of MK2 displayed significantly greater activity compared with mock-transfected cells. Overexpressing dominant negative MK2 mutant did not affect base-line MK2 activity (data not shown).

Stably transfected cells overexpressing constitutively active MK2 and dominant negative MK2 were plated on collagen-coated coverslips, and then their actin cytoskeleton was examined as described above. Overexpression of constitutively active MK2 caused an increase in stress fiber formation (Fig. 5A) and filamentous actin (Fig. 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 (Fig. 6A). In the cells overexpressing dominant negative MK2, no increase in filamentous actin was observed in response to hypoxia (Fig. 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 whether phosphorylation of HSP27 has an effect on actin distribution, RPMEC were transfected with the empty vector alone (mock-transfected) or with phosphomimicking HSP27 mutant in which phosphorylatable amino acids were replaced by negatively charged aspartates as described under “Experimental Procedures.” After selecting stable transfectants with geneticin and isolating and expanding clones, the cells were grown on coverslips and labeled with rhodamine-phalloidin as described above. Overexpression of the phosphomimicking HSP27 mutant in endothelial cells caused an increase in stress fibers and filamentous actin in normoxic cells (Fig. 5). Thus, formation of stress fibers in endothelial cells correlates with negatively charged amino acids, which mimic phosphorylated amino acids in HSP27.
DISCUSSION

Hyoxia is associated with many lung diseases including acute respiratory distress syndrome, 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 MAPK 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 Ref. 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, interleukin-1α production is increased in hypoxic endothelial cell cultures (27). Vascular endothelial growth factor 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 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 the result of transcriptional processes. One example of a nontranscriptional 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 nontranscriptional endothelial response to hypoxia, namely the phosphorylation of the reactive oxygen-producing enzyme, xanthine oxidase, and subsequent up-regulation 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 MAPK 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 MAPK that becomes activated in response to different stimuli such as ultraviolet radiation and hyperosmolality. 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 intense research (for review, see Ref. 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 vascular endothelial growth factor (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 had described previously (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 h 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 and motility of endothelial cells, as well as increased adhesiveness of inflammatory cells. Experiments are currently under way 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 may present targets for drug development against diseases such as pulmonary edema, in which the endothelial barrier is altered.

REFERENCES

1. Ogawa, S., Shreeniwas, R., Butura, C., Brett, J., and Stern, D. M. (1990) Adv. Exp. Med. Biol. 281, 303–312
2. Ogawa, S., Gerlach, H., Espósito, C., Pasagian-Macaulay, A., Brett, J., and Stern, D. (1990) J. Clin. Invest. 85, 1090–1098
3. Ogawa, S., Koga, S., Kuwabara, K., Brett, J., Morrow, B., Morris, S. A., Bilezikian, J. P., Silverstein, S. C., and Stern, D. (1992) Am. J. Physiol. 262, C546–C554
4. Partridge, C. A. (1995) Am. J. Physiol. 268, L52–L58
5. Stelzner, T. J., O'Brien, R. F., Sato, K., and Weil, J. V. (1988) J. Clin. Invest. 82, 1840–1847
6. Ichikawa H., Flores, S., Kvetlys, P. R., Wolf, R. E., Yoshikawa, T., Granger,
MK2 Mediates Cytoskeletal Changes in Hypoxia

D. N., and Aw, T. Y. (1997) Circ. Res. 81, 922–931
7. Pinskiy, D. J., Naka, Y., Liao, H., Oz, M. C., Wagner, D. D., Mayadas, T. N., Johnson, R. C., Hynes, R. O., Heath, M., Lawson, C. A., and Stern, D. M. (1996) J. Clin. Invest. 97, 493–500
8. Conrad, P. W., Rust, R. T., Han, J., Millhorn, D. E., and Beitner-Johnson, D. (1999) J. Biol. Chem. 274, 23570–23576
9. Seko, Y., Takahashi, N., Tohe, K., Kadowaki, T., and Yazaki, Y. (1997) Biochem. Biophys. Res. Commun. 239, 840–844
10. Conrad, P. W., Millhorn, D. E., and Beitner-Johnson, D. (2000) Cell. Signal. 12, 463–467
11. Kacimi, R., Chentoufi, J., Honbo, N., Long, C. S., and Karliner, J. S. (2000) J. Mol. Cell. Cardiol. 32, 139–150
12. Welsh, D. J., Peacock, A. J., MacLean, M., and Harnett, M. (2001) Am. J. Respir. Crit. Care Med. 164, 289–299
13. Das, M., Bouchez, D. M., Moore, M. J., Hopkins, D. C., Nemenoff, R. A., and Stermark, K. R. (2001) J. Biol. Chem. 276, 15431–15440
14. Marrais, E., Genade, S., Huisman, B., Strijdom, J. G., Moodman, J. A., and Lochner, A. (2001) J. Mol. Cell Cardiol. 33, 769–778
15. Carini, R., De Cesari, M. G., Splendore, R., Vay, D., Domenicotti, C., Nitti, M. P., Paola, D., Pronzato, M. A., and Albane, E. (2001) Hepatology 33, 131–139
16. Kayyali, U. S., Donaldson, C., Huang, H., Abdelnour, R., and Hassoun, P. M. (2001) J. Biol. Chem. 276, 14359–14365
17. Kotlyarov, A., Yannoni, Y., Fritz, S., Laass, K., Telliez, J. B., Pitman, D., Lin, L. L., and Gaestel, M. (2002) Mol. Cell. Biol. 22, 4827–4835
18. Schneider, G. B., Hamano, H., and Cooper, L. F. (1996) J. Cell. Physiol. 177, 575–584
19. Huot, J., Houle, F., Marceau, F., and Landry, J. (1997) Circ. Res. 80, 383–392
20. Cote, C. G., Yu, F. S., Zulueta, J. J., Vosatka, R. J., and Hassoun, P. M. (1996) Am. J. Physiol. Lung. 271, L869–L874
21. Laemmli, U. K. (1970) Nature 227, 680–685
22. Engel, K., Schultz, H., Martin, F., Kotlyarov, A., Platth, K., Hahn, M., Heinemann, U., and Gaestel, M. (1995) J. Biol. Chem. 270, 27213–27221
23. Inogalla, T., Ehrnsperger, M., Previle, X., Kotlyarov, A., Lutsch, G., Ducasse, C., Paul, C., Wieske, M., Arrigo, A. P., Buchner, J., and Gaestel, M. (1999) J. Biol. Chem. 274, 18947–18956
24. Winnep, R., Kracht, M., Ritter, B., Wilhelm, A., Chen, C. Y., Shyu, A. B., Muller, M., Gaestel, M., Resch, K., and Holtmann, H. (1999) EMBO J. 18, 4969–4980
25. Benndorf, R., Hayess, K., Ryazantsev, S., Wieske, M., Behlke, J., and Lutsch, G. (1994) J. Biol. Chem. 269, 20760–20764
26. Stermark, K. R., and Mechem, R. P. (1997) Annu. Rev. Physiol. 59, 89–144
27. Shreininaw, R., Koga, S., Karakurum, M., Pinsky, D., Kaiser, E., Bressett, J., Wolitzky, B. A., Norton, C., Pleininski, J., Benjamin, W., Burns, D. K., Goldstein, A., and Stern, D. (1992) J. Clin. Invest. 90, 2333–2339
28. Erukhmonov, J. A., Tang, Z. L., Johnson, B. A., Donahoe, M. P., Razzack, J. A., Gibson, R. F., Lee, W. M., Wasserloos, K. J., Watkins, S. A., and Pitt, B. R. (2000) Am. J. Respir. Crit. Care Med. 162, 288–294
29. Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992) Nature 359, 843–845
30. Badesch, D. B., Orton, E. C., Zapp, L. M., Westcott, J. Y., Hester, J., Voelkel, N. F., and Stermark, K. R. (1989) Am. J. Respir. Crit. Care Med. 140, 489–498
31. Liao, J. K., Zulueta, J. J., Yu, F. S., Peng, H. B., Cote, C. G., and Hassoun, P. M. (1995) J. Clin. Invest. 96, 2661–2666
32. Zulueta, J. J., Sawhney, R., Kayyali, U., Fogel, M., Donaldson, C., Huang, H., Lanzillo, J. J., and Hassoun, P. M. (2002) Am. J. Respir. Cell Mol. Biol. 26, 22–30
33. Pitt, B. R., and St Croix, C. M. (2002) Am. J. Respir. Cell Mol. Biol. 26, 6–9
34. Wang, G. L., and Semenza, G. L. (1993) J. Biol. Chem. 268, 21513–21518
35. Guillen, K., and Krasnow, M. A. (1997) Cell 89, 9–12
36. O'Rourke, J. F., Dachs, G. U., Gleedle, J. M., Maxwell, P. H., Pugh, C. W., Stratford, I. J., Wood, S. M., and Ratcliffe, P. J. (1997) Oncol. Res. 9, 327–332
37. Herlaar, E., and Brown, Z. (1999) Mol. Med. Today 5, 439–447
38. Lee, J. C., Kumar, S., Griswold, D. E., Underwood, D. C., Votta, B. J., and Adams, J. L. (2000) Immunopharmacology 47, 185–201
39. Klemenz, R., Andres, A. C., Frohli, E., Schafer, R., and Auyama, A. (1993) J. Cell Biol. 120, 639–645
40. Wang, G. L., and Semenza, G. L. (1993) J. Biol. Chem. 268, 221–229
41. Jakob, U., Gaestel, M., Engel, K., and Buchner, J. (1993) J. Biol. Chem. 268, 1517–1520
42. Ehrnsperger, M., Graber, S., Gaestel, M., and Buchner, J. (1997) EMBO J. 16, 2189–2196
43. Aufricht, C., Ardito, T., Thulin, G., Kaszgarian, M., Siegel, N. J., and Van Why, S. K. (1998) Am. J. Physiol. 274, F215–F222
44. Ridder, C. F., van Ommen, E. J., and van Steenhoven, A. A. (1983) J. Biomech. 16, 339–348
