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Original Research Communication

Effects of Hypoxia/Reoxygenation on Angiogenic Factors and Their Tyrosine Kinase Receptors in the Rat Myocardium

PARTHA S. RAY, HIROAKI SASAKI, TATIANA ESTRADA-HERNANDEZ, LI ZU, and NILANJANA MAULIK

ABSTRACT

The process of angiogenesis is initiated primarily as a consequence of hypoxic stimulation at the cellular and molecular level. Although several angiogenic growth factors have been identified, at present a detailed understanding of the interplay among inducing stimuli, growth factors, and their respective molecular targets remains to be evaluated. Here we report the effects of progressively increasing durations of moderate hypoxia on the protein expression profiles and tissue distribution patterns of the vascular endothelial growth factor system and the angiopoietin/Tie system in the adult rat myocardium. The relative temporal trends of expression of the various components of these two systems, as well as apparent relationships between Flk-1 and angiopoietin-2 and between Flt-1 and Tie-1, suggest a probable sequence of involvement during myocardial angiogenesis, as proposed in our model. Such relationships may potentially be utilized in formulating strategies for sequential gene therapy to achieve clinically relevant myocardial angiogenesis. Antioxid. Redox Signal. 3, 89–102.

INTRODUCTION

THE THERAPEUTIC INDUCTION of collateral vascularization in the ischemic heart is fast emerging as a highly attractive treatment modality in the realm of cardiovascular medicine (28). To develop better and more effective therapeutic strategies using the powerful concept of inducing new vessel growth by employing vascular growth factors, it is essential to further our understanding of the molecular mechanisms and chain of events underlying the fascinating process of angiogenesis. Among the various triggers of angiogenesis, tissue hypoxia has been identified as being a particularly important stimulus for the induction of new vessel growth (11). Tissue hypoxia exerts such a proangiogenic action through various angiogenic factors, the most notable being vascular endothelial growth factor (VEGF), which has been associated chiefly with initiating the process of angiogenesis through the recruitment and proliferation of endothelial cells. Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2) are the endothelial specific tyrosine kinase receptors of VEGF through which its effects are primarily mediated (5, 20). Two other angiogenic factors, the angiopoietins 1 and 2 (Ang-1 and Ang-2), have been found to regulate the maturation of new blood vessels from the proliferated endothelial cells (38). Tie-1 and Tie-2 comprise another family of endothelial specific receptor tyrosine kinases, Ang-1 and Ang-2 being the specific ligands for Tie-2.

Department of Surgery, University of Connecticut Health Center, Farmington, CT 06030-1110.

The fact that VEGF, Flt-1, and Flk-1 expression is up-regulated in response to hypoxia *in vitro* and *in vivo* (13, 15, 21, 36) and to ischemia *in vivo* (1, 9, 16) it well established, although there are conflicting reports with regard to Flk-1 *in vitro*, suggesting the involvement of adenosine acting as a paracrine mediator through the A2 receptor (2, 34).

The observed *in vivo* effects of ischemia on the above factors may logically be attributed primarily to its hypoxia component. However, the effects of hypoxia on such expression in the adult myocardium *in vivo* have not yielded consistent results, nor has expression at the protein level been confirmed. Furthermore, the influence, if any, of hypoxia on the myocardial expression characteristics of the Tie family of receptors and/or the ligands Ang-1 and Ang-2 either *in vitro* or *in vivo* has not been reported.

The existence of a discrete factor with proangiogenic activity was first reported almost three decades ago (8). Since then, many angiogenic growth factors have been identified and their importance demonstrated in various experimental models of angiogenesis, but a detailed understanding of the interplay among inducing stimuli, growth factors, and their respective molecular targets remains to be elucidated, especially with reference to the adult mammalian heart. In the present study we sought to determine the possible cooperative involvement of the angiopoietin/Tie system in conjunction with the VEGF system in initiating the angiogenic response at the molecular level under hypoxic control. As an initial study to understand better the molecular mechanisms by which hypoxia can induce an angiogenic response in the adult rat myocardium *in vivo*, we sought to determine how stimulation in the form of progressively increasing durations of hypoxia/reoxygenation would simultaneously influence the myocardial tissue protein levels of VEGF, Flk-1, Flt-1, Ang-1, Ang-2, Tie-1, and Tie-2. This would provide not only valuable insight into how the level of a particular protein changes with increasing durations of hypoxic exposure, but also how it relates to the level of expression of the other proteins at the same time point of evaluation. In addition, correlative studies were undertaken to determine the tissue distribution patterns of each protein with increasing duration of hypoxia. Previous studies have concentrated on determining the myocardial mRNA levels of the above factors. Although the detection of enhanced transcription of specific mRNAs is highly suggestive in itself, it remains inconclusive in indicating the nature of the final protein products actually translated. Hence, in our study, we sought to confirm the expression profile characteristics of the various factors at the protein level.

The aim of this study was to find out whether a nonlethal moderate hypoxic challenge is capable of increasing protein levels of these important angiogenic factors and their receptors in the adult rat myocardium. Such an approach would further help to identify which angiogenic growth factors are expressed by adult myocardium when subjected to a hypoxic/ischemic challenge and therefore help to identify which factors are more important in stimulating and maintaining myocardial angiogenesis. This is the first report that investigates the temporal relationship between protein expression of the VEGF system and Ang/Tie system in response to systemic hypoxia in adult myocardium and that addresses the differences in tissue distribution patterns arising thereof.

EXPERIMENTAL PROCEDURES

In vivo hypoxia/reoxygenation model

All animals in this study received humane care in compliance with the principles of laboratory animal care formulated by the National Society for Medical Research and *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (publication no. NIH 85-23, revised 1985). The experimental protocol was performed after receiving approval from the Institutional Animal Care Committee. Twenty-four unrestrained conscious male Sprague–Dawley rats weighing 275–300 g were randomly divided into eight groups as outlined in Fig. 1. Rats were subjected to a hypoxic challenge (10% O_2/90% N_2) of different durations (groups 1–7) followed by a 24 h-period of reoxygenation in an anesthesia chamber with an in-flow aperture for the

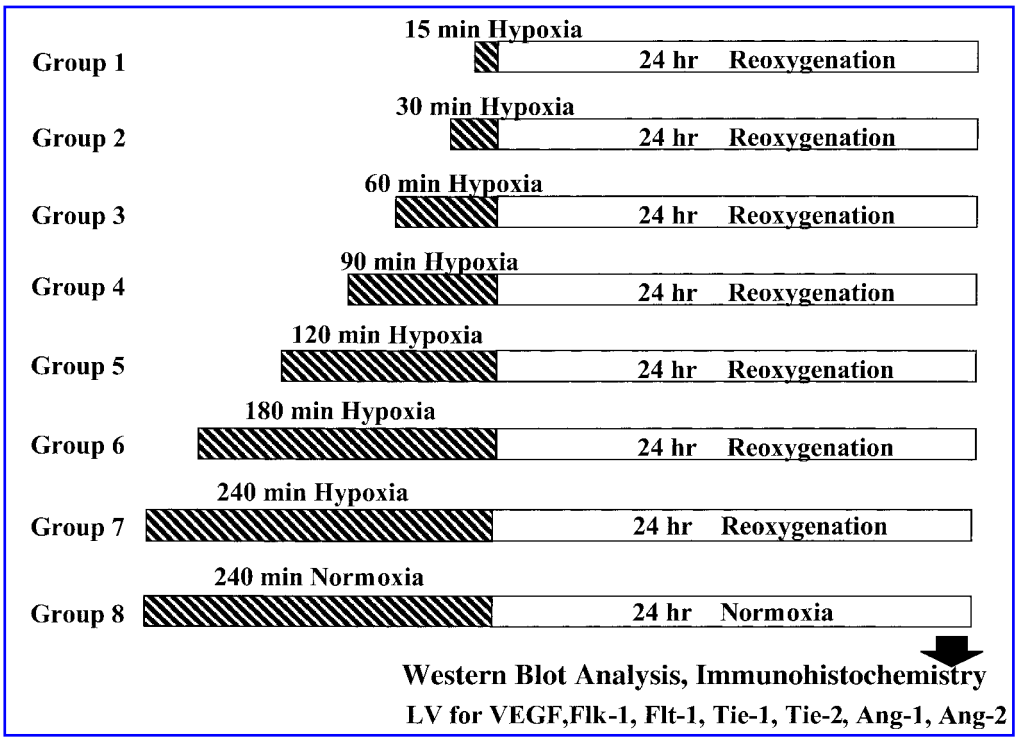

FIG. 1. Outline of experimental protocol. Groups 1–7 were experimental groups where the duration of hypoxia used was as indicated. Group 8 served as control.

gas mixture and an out-flow aperture connected to a gas absorption cannister (A.M. Bickford Inc., Wales Center, NY, U.S.A.). Animals in group 8, which served as control, were maintained in a normoxic environment to match the maximal hypoxic duration.

The level of O_2 saturation in the gas mixture used to achieve the normobaric hypoxemic hypoxia has been previously reported in an *in vivo* model (25). Hypoxic stress is known to inhibit protein synthesis. Because the major emphasis in our study was to examine expression of various factors at the protein level, we chose to use hypoxic conditions that have previously been shown not to inhibit protein synthesis (31). Furthermore, to be able to delineate fully the ability of hypoxia to affect such protein expression, it is necessary to allow sufficient time for translational events to occur. Hence, our protocol included a 24-h reoxygenation period after the period of hypoxic exposure to enable the evaluation of the effects of short durations of hypoxia on specific myocardial protein levels. This would not have been possible if the hypoxic period had been allowed to extend up until the rats were killed.

A KE-25 galvanic cell O_2 sensor (Kent Scientific, Litchfield, CT, U.S.A.) was used to monitor continuously the $\%O_2$ concentration, the signal being amplified through a TRN-005 Amplifier (Kent Scientific). The O_2 concentration was gradually lowered from an ambient $\%O_2$ of 23% down to 10% over the course of 30 min. Once stabilization was obtained, the in-flow rate was adjusted so as to maintain the $\%O_2$ at $10 \pm 0.5\%$ for the various durations outlined in the figure. After the period of hypoxic exposure, rats were returned to normal housing conditions for a 24-h period. Rats were then anesthetized with sodium pentobarbital (80 mg/kg body weight, intraperitoneal injection; Abbott Laboratories, North Chicago, IL, U.S.A.) and anticoagulated with heparin sodium (500 IU/kg body weight, intravenous injection; Elkins–Sinn Inc., Cherry Hill, NJ, U.S.A.). After sufficient depth of anesthesia was ensured, thoracotomy was performed and hearts were excised and immediately immersed in ice-cold lactated Ringer's solution (Baxter Healthcare Corp., Deerfield, IL, U.S.A.). A transverse section ~2 mm in thickness was obtained from the midventricular region and immersed in or-

nithine carbamyltransferase compound (Miles Inc., Elkhardt, IN, U.S.A.) contained in a metal embedding tray before being fresh-frozen in liquid nitrogen and stored at −70°C for later immunohistochemical studies. The remaining left ventricular tissue was then isolated, snap-frozen in liquid nitrogen, and stored at −70°C until later processing for western blots as described below.

Western blot analysis

To quantify the abundance of the angiogenic factors VEGF, Ang-1, Ang-2, and their receptors, we performed western blot analysis using various specific primary antibodies. Heart tissues exposed to various durations of hypoxia followed by straight 24 h of reoxygenation were homogenized and suspended (5 mg/ml) in sample buffer (10 m*M* HEPES, pH 7.3, 11.5% sucrose, 1 m*M* EDTA, 1 m*M* EGTA, diisopropyl fluorophosphate, 0.7 mg/ml pepstatin A, 10 mg/ml leupeptin, 2 mg/ml aprotinin). The homogenates were centrifuged at 3,500 rpm, and the cytosolic fractions were used for protein analysis. The total protein concentration was determined by using bicinchoninic acid protein assay kit (Pierce, Rockville, IL, U.S.A.). The cytosolic proteins (10 μg) were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The resolving gel concentration for VEGF, Ang-1, and Ang-2 was 10% and for Tie-1, Tie-2, Flk-1, and Flt-1 was 8% of the acrylamide to bis ratio. The separated proteins were electrophoretically transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA, U.S.A.) using a semidry transfer system (Bio-Rad, Hercules, CA, U.S.A.). Protein standards (Bio-Rad) were run in each gel. The blots were blocked in Tris-buffered saline/Tween-20 (containing 20 m*M* Tris base, pH 7.6, 137 m*M* NaCl, 0.1% Tween-20) supplemented with 5% bovine serum albumin (BSA) for 1 h. Blots were incubated for 2 h with the specific primary rabbit antibodies (Santa Cruz Biotech, Santa Cruz, CA, U.S.A.) against VEGF (1:200), Tie-1 (1:500), Tie-2 (1:500), Flk-1 (1:1,000) and Flt-1 (1:1,000). Blots being analyzed for assessment of Ang-1 and Ang-2 were incubated with the appropriate primary goat antibodies (Santa Cruz) diluted 1:200. Blots were then incubated for 1 h at room temperature with 1:10,000 diluted horseradish peroxidase (HRP)-conjugated secondary antibodies (Boehringer Mannheim Corp., Inc), which were goat anti-rabbit IgG for all except Ang-1 and Ang-2, where rabbit anti-goat IgG was used. Direct reprobing with anti-β-actin antibody as an internal control for western blot was also performed. The blot is directly reprobed after washing with phosphate-buffered saline (PBS)/Tween-20 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, U.S.A.) at a dilution of 1:5,000 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, U.S.A.). After three washes of 5 min each, blots were treated with Enhanced Chemi-Luminescence (ECL from Amersham) reagent and the required proteins were detected by autoradiography for variable lengths of time with Kodak X-Omat film. All the samples were tested for nonspecific labeling. Negative and positive controls were run to validate the results.

Immunohistochemistry

Serial sections of fresh-frozen heart tissue were obtained by using a cryostat, mounted on poly-L-lysine-coated slides, and allowed to air-dry for 30 min before fixing in 100% acetone for 15 min. Sections were washed three times for 5 min each PBS in between each step. To achieve endogenous peroxidase inactivation, sections were immersed in 2% H_2O_2 in methanol for 25 min. The blocking step involved a 1-h incubation period in 5% goat serum in PBS for all sections except those that were to be analyzed for Ang-1 and Ang-2 activities, which were incubated in 1% BSA instead. An additional avidin/biotin blocking step was performed by using a commercial kit (Vector Labs, Burlingame, CA, U.S.A.) according to the manufacturer's instructions. All primary antibodies were obtained from Santa Cruz Biotech. Secondary antibodies, strepta-

vidin HRP, the peroxidase substrate kits Vector Nova Red and Vector VIP, and Nuclear Fast Red for counterstaining were all obtained from Vector Labs. Harris' hematoxylin and Permount monitoring medium were obtained from Fisher Scientific Co. (Pittsburgh, PA, U.S.A.). Sections were incubated overnight with appropriate primary antibody diluted 1:50 in 5% goat serum in PBS except those that were being analyzed for Ang-1 and Ang-2 immunoreactivity, in which case the specific antibody was diluted 1:50 in 1% BSA in PBS. For assessing VEGF activity, the primary antibody dilution used was 1:100. Thereafter slides were incubated in biotinylated secondary antibody solution (goat anti-rabbit IgG diluted 1:200 in 5% BSA) for 1 h followed by incubation in streptavidin HRP (diluted 1:100 in PBS) for 45 min. The biotinylated secondary antibody used in the Ang-1 and Ang-2 assays were rabbit anti-goat IgG diluted 1:200 in 1% BSA in PBS. For visualizing VEGF, Ang-1, and Ang-2, Vector Nova Red was used as the peroxidase substrate to produce a reddish-brown reaction product, and sections were briefly counterstained with Harris' hematoxylin before coverslipping with permanent mounting medium. Flk-1, Flt-1, Tie-1, and Tie-2 were visualized using Vector VIP as the peroxidase substrate to yield a violet reaction product, counterstaining being achieved with Nuclear Fast Red prior to permanent mounting. Sections were viewed under an Olympus BH-2 microscope, and images were captured with an Olympus DP-10 digital camera.

RESULTS

Analysis of VEGF system

Western blot: Endothelial cells express both monomeric and dimeric forms of VEGF. Under the conditions of this study, VEGF migrated in sodium dodecyl sulfate–polyacrylamide gel electrophoresis as a dimer of ~40 kDa and a monomer of 20 kDa only (Fig. 2). The expres-

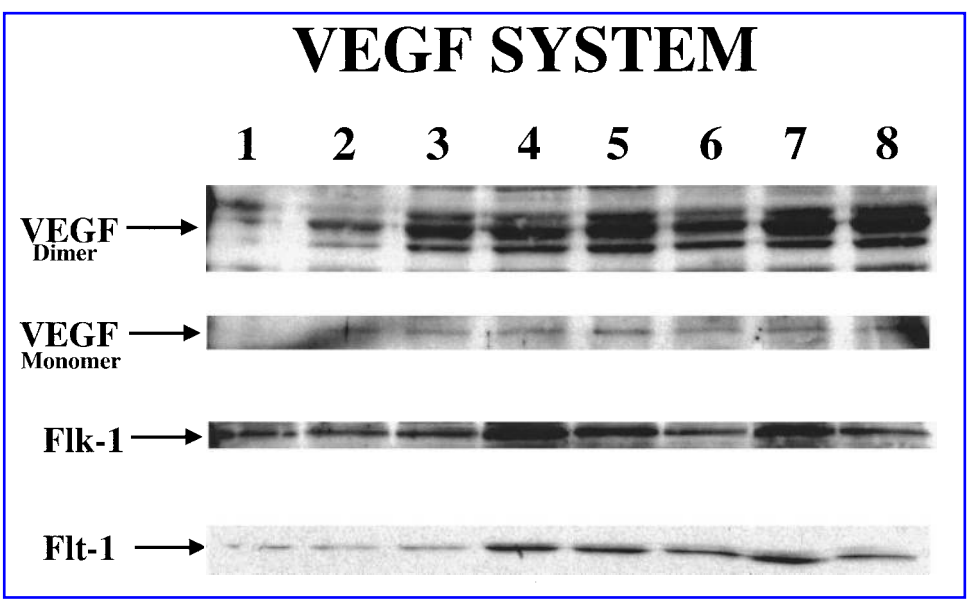

FIG. 2. Representative western blot analysis for VEGF system. This includes VEGF dimer as well as monomer and its receptors Flk-1 and Flt-1. VEGF dimer and VEGF monomer proteins were expressed as 40-kDa and 20-kDa bands, whereas Flk-1 and Flt-1 proteins were expressed as 150-kDa and 200-kDa bands, respectively. Rats were exposed to different durations of hypoxic challenge, and after 24 h of reoxygenation rats were killed and total protein was isolated from rat myocardium as described under Experimental Procedures. Similar results were obtained in three independent experiments performed in triplicate. Lane 1, baseline; lane 2, 15 min of hypoxia; lane 3, 30 min of hypoxia; lane 4, 60 min of hypoxia; lane 5, 90 min of hypoxia; lane 6, 2 h of hypoxia; lane 7, 3 h of hypoxia; lane 8, 4 h of hypoxia. All the animals after hypoxia were exposed to 24 h of reoxygenation.

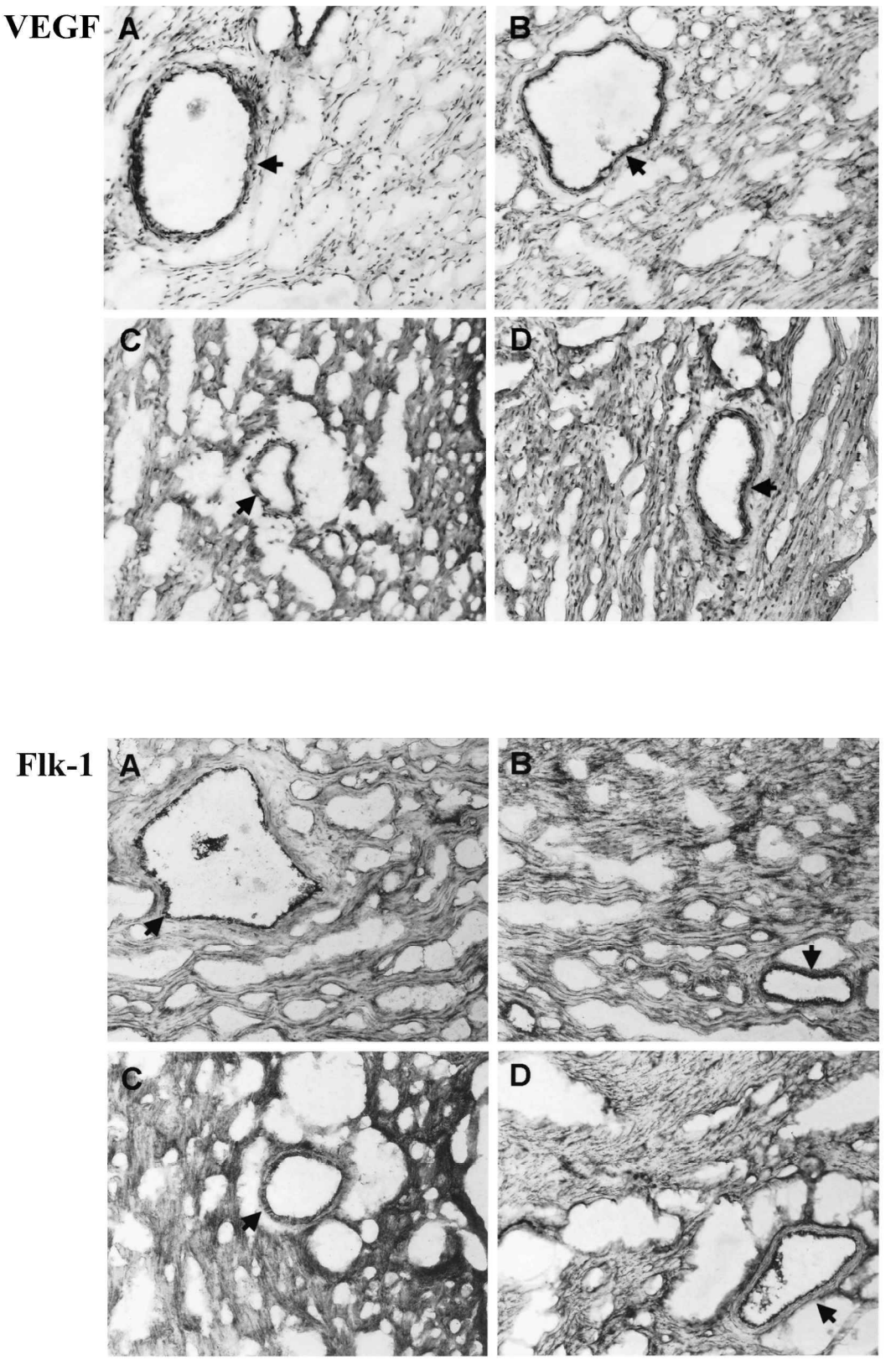

FIG. 3. Immunohistochemistry for VEGF system. Representative sections of rat ventricular myocardium immunostained for VEGF, Flk-1, and Flt-1 reactivities are shown. (**A**) Myocardial tissue sections from rat in normoxic control group. (**B, C, and D**) Myocardial tissue sections from rats in 1-h, 2-h, and 3-h hypoxic groups, respectively. Arrows point to coronary arteries. Original magnification, 200×.

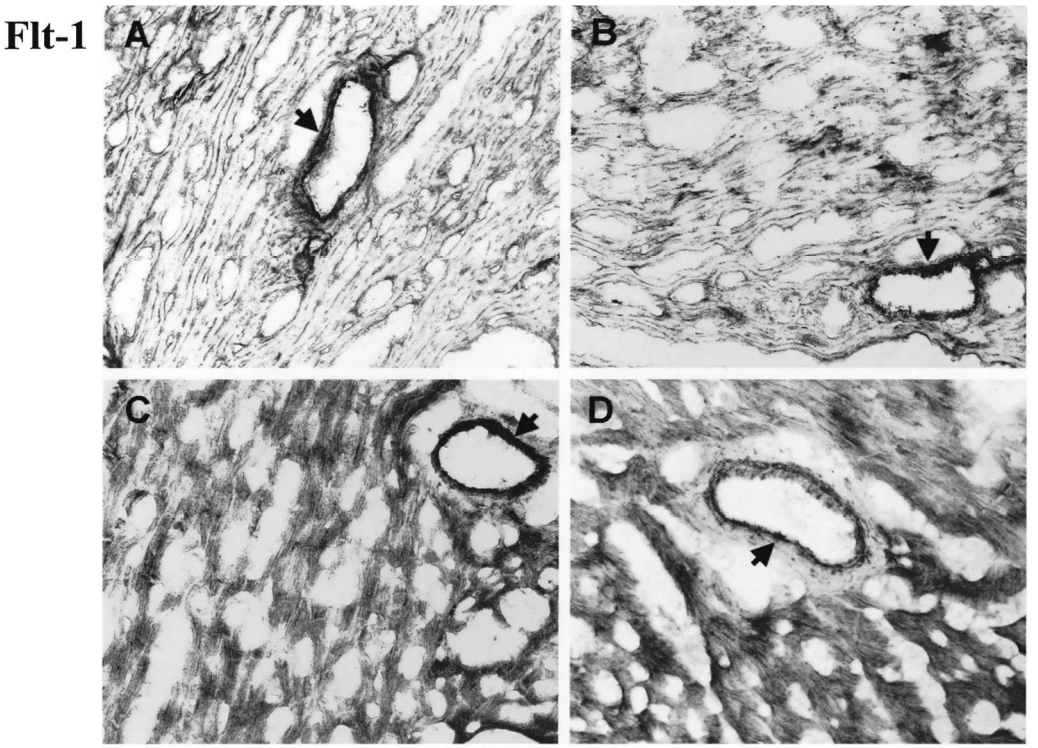

FIG. 3. *Continued from page 94.*

sion patterns of the two VEGF forms seem similar, although the level of the expression pattern seems significantly higher in the case of the VEGF dimer. In three different experiments performed in triplicate, VEGF expression was significantly increased (~50% compared with the control) within 30 min of hypoxic challenge and remained up-regulated at the same level even after 4 h of hypoxia. The expression of Flk-1 protein was increased after 1 h of hypoxia (41.1% compared with the corresponding control; Fig. 2, Flk-1, lane 4) and remained expressed until 3 h of hypoxia (lane 7). Expression of the other VEGF receptor Flt-1 was significantly elevated when compared with the degree of the induction of Flk-1 receptor by hypoxic challenge after 1 h of hypoxic exposure (3.25-fold compared with 41.1% only; Fig. 2, Flt-1, lane 4). This trend of the expression pattern of Flt-1 persisted even after 4 h of hypoxia (lane 8).

Immunohistochemistry. Immunohistochemical analysis of VEGF revealed a diffuse pattern of distribution throughout the ventricular myocardium with strong localization around the coronary arterial wall where both coronary endothelium and vascular smooth muscle appeared to stain positive for VEGF (Fig. 3, VEGF). Hearts obtained from rats that had been subjected to hypoxia followed by a 24-h period of reoxygenation displayed a progressive increase in intensity of staining for VEGF with increasing durations of hypoxia. Although higher in intensity as compared with control, the distribution pattern remained diffuse and there were no observable areas of localization around capillaries. However, VEGF remained strongly localized around the coronary arteries.

The tissue distribution patterns of the VEGF receptors Flk-1 (Fig. 3, Flk-1) nd Flt-1 (Fig. 3, Flt-1) were different from that of VEGF in that, in addition to displaying strong localization around the coronary arteries, they both displayed intense staining along capillaries. Around the coronary arteries, Flk-1 was sharply localized almost exclusively to the coronary endothelium, whereas staining for Flt-1 was not as sharp in comparison. The intensity of staining for both receptors increased

with increasing durations of hypoxia, but that of Flt-1 tended to persist, whereas that of Flk-1 tended to decrease slightly after 2 h of hypoxia.

Analysis of Ang/Tie system

Western blot. Induction of the expression of Ang-1 protein was observed within 30 min of hypoxia (1.21-fold compared with the baseline control; Fig. 4, Ang-1, lane 3); however, the Ang-1 protein level was slightly decreased after 1 h of hypoxia (lane 4), with the significant induction of Ang-2 protein at the same time point (31%, Fig. 4, Ang-2, lane 4). Again after 1 h of hypoxia the induction of the expression of Ang-1 was gradually increased and remained up-regulated even after 4 h of hypoxia (Fig. 4, Ang-1, lane 8) in contrast to the steady decrease in the expression level of Ang-2 protein. A significant increase in the expression pattern of Tie-1 protein was observed after 1 h of hypoxia (5.3-fold compared with the control; Fig. 4, Tie-1, lane 4); it persisted even after 90 min of hypoxia (5.86-fold compared with the control; lane 5) and thereafter gradually decreased to resemble the basal appearance by 3 h of hypoxia (lane 7). In the case of Tie-2, the significant abundance of the same was found as early as 30 min of hypoxia (1.34-fold when compared with the control; Fig. 4, Tie-2, lane 3); this high level of protein induction was maintained even after 3 h of hypoxia (lane 7).

Immunohistochemistry: Immunostaining for Ang-1 and Ang-2 was diffusely present throughout the ventricular myocardium and resembled the pattern of VEGF distribution except around the coronary arteries, where they were less intense by comparison (Fig. 5). Immunoreactivity to Ang-1 was increased after hypoxic exposure noticeably around the coronary vessels in contrast to its baseline pattern. Intensity of Ang-1 staining did appear to be slightly decreased after 1 h of hypoxia (Fig. 5, Ang-1, B), coinciding with the strong presence of Ang-2 at the same time point (Fig. 5, Ang-2, B). Thereafter, Ang-1 staining displayed a progressive increase in intensity in contrast to the steady decrease in Ang-2 immunoreactivity with increasing duration of hypoxia.

Basal Tie-1 and Tie-2 distribution patterns were similar in that both were strongly local-

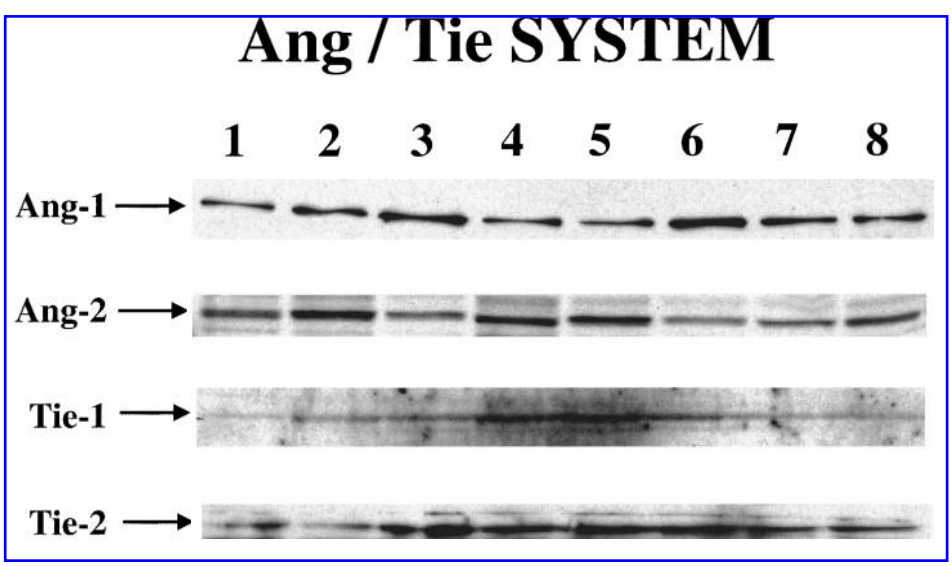

FIG. 4. Representative western blot analysis for Ang/Tie system. This includes Ang-1 as well as Ang-2 and their receptor Tie-2. Tie-1 was also studied. Ang-1 and Ang-2 proteins were expressed as 60-kDa and 62-kDa bands, respectively, whereas Tie-2 and Tie-1 proteins were expressed as 140-kDa bands. Rats were exposed to different durations of hypoxic challenge, and after 24 h of reoxygenation rats were killed and total protein was isolated from rat myocardium as described under Experimental Procedures. Similar results were obtained in three independent experiments performed in triplicate. Lane 1, baseline; lane 2, 15 min of hypoxia; lane 3, 30 min of hypoxia; lane 4, 60 min of hypoxia; lane 5, 90 min of hypoxia; lane 6, 2 h of hypoxia; lane 7, 3 h of hypoxia; lane 8, 4 h of hypoxia. All the animals after hypoxia were exposed to 24 h of reoxygenation.

ized around coronary arteries and both displayed reticular staining along capillaries (Fig. 5). A sharp increase in Tie-1 immunoreactivity was observed after 1 h of hypoxia (Fig. 5, Tie-1, B), which gradually decreased to resemble the basal appearance after 3 h of hypoxia (D). Tie-2 staining, on the other hand, displayed a modest increase by 1 h of hypoxia (Fig. 5, Tie-2, B), becoming very intense after 2 and 3 h of hypoxia (C and D).

DISCUSSION

Our results present the first comprehensive evaluation of the effects of systemic hypoxia on the protein expression and distribution profiles of the VEGF system and the Ang/Tie system in adult rat myocardial tissue. The relative time course of protein expression in response to increasing durations of hypoxia, as indicated from our experiments, seems to suggest a probable sequence or order of involvement of the various receptors and their ligands in angiogenesis. Hypoxia has been found to be the strongest inducer of VEGF both *in vitro* and *in vivo* (29, 32). Recent investigations have yielded contrasting results with respect to hypoxic and ischemic modulation of myocardial VEGF expression (1, 9, 12, 15, 16, 18, 21). In our study, hypoxia was found to increase the levels of both monomer and dimer forms of VEGF. The increase in expression of the homodimer was more pronounced when compared with that of the VEGF monomer. On the basis of this finding, it is suggested that the VEGF dimer plays the predominant role in angiogenesis. The abundance of VEGF protein after hypoxic exposure apparent on immunoblotting was further substantiated by immunohistochemistry, where the progressive increase in VEGF intensity was clearly visible. VEGF expression continued to be strong and persistent even after 4 h of hypoxia, stressing the important and continuous nature of its role in angiogenesis. This would seem to suggest that it is important not only in the initial stages of angiogenesis, but also in the subsequent maturation and maintenance of the new vasculature. The biological functions of VEGF, triggered by external stimuli, are initiated through the activation of intracellular signal transduction cascades involving specific kinases. It is reported that a rapid increase in VEGF expression is due to the presence of hypoxia-inducible factor-sensitive elements located in the VEGF promoter, which up-regulates the transcription of VEGF (27). Furthermore, endothelial cells detect external angiogenic stimuli via oncogene activation (22). Formation of a receptor complex between VEGF and its tyrosine kinase receptor Flk-1 activates c-src, which plays an important role in coordinating the effects of VEGF on cell adhesion and cell motility (3, 10, 14). The physiological role of VEGF in increasing vascular permeability is also exerted through the Flk-1 receptor and involves mitogen-activated protein kinase activation mainly mediated by protein kinase C (6, 35).

Another endothelial cell-specific tyrosine kinase receptor of VEGF is Flt-1. The only ligand so far known for Flk-1 is VEGF, whereas Flt-1 has, in addition to VEGF, another ligand called placenta-derived growth factor. Little is known regarding the expression patterns of the VEGF receptors, Flk-1 and Flt-1. In our systemic whole-body hypoxic model, we found significant abundance of Flk-1 after 30 min of hypoxia, which remained elevated even after 3 h of hypoxic exposure. This expression was markedly reduced after 4 h of hypoxia, suggesting a role in the initial rather than later stages of the angiogenic process. This would be consistent with the known effects of endothelial cell migration and proliferation mediated by VEGF through this receptor, which are important early steps in angiogenesis. In comparison, induction of Flt-1 protein expression is increased after only 1 h of hypoxia, but continues to be elevated even after 4 h of hypoxia. This is indicative of a functional role potentially geared more toward the later components of the angiogenic process. We have also studied the protein expression of Tie-1 and Tie-2 receptors under the same experimental conditions. Tie-1 and Tie-2 constitute a family of endothelial cell-specific receptor tyrosine kinases. These are different from the receptors for VEGF in that they possess matrix association motifs in their extracellular domains (4, 29). An increased abundance of Tie-1 protein was found after 1 h of hypoxic challenge, and its protein

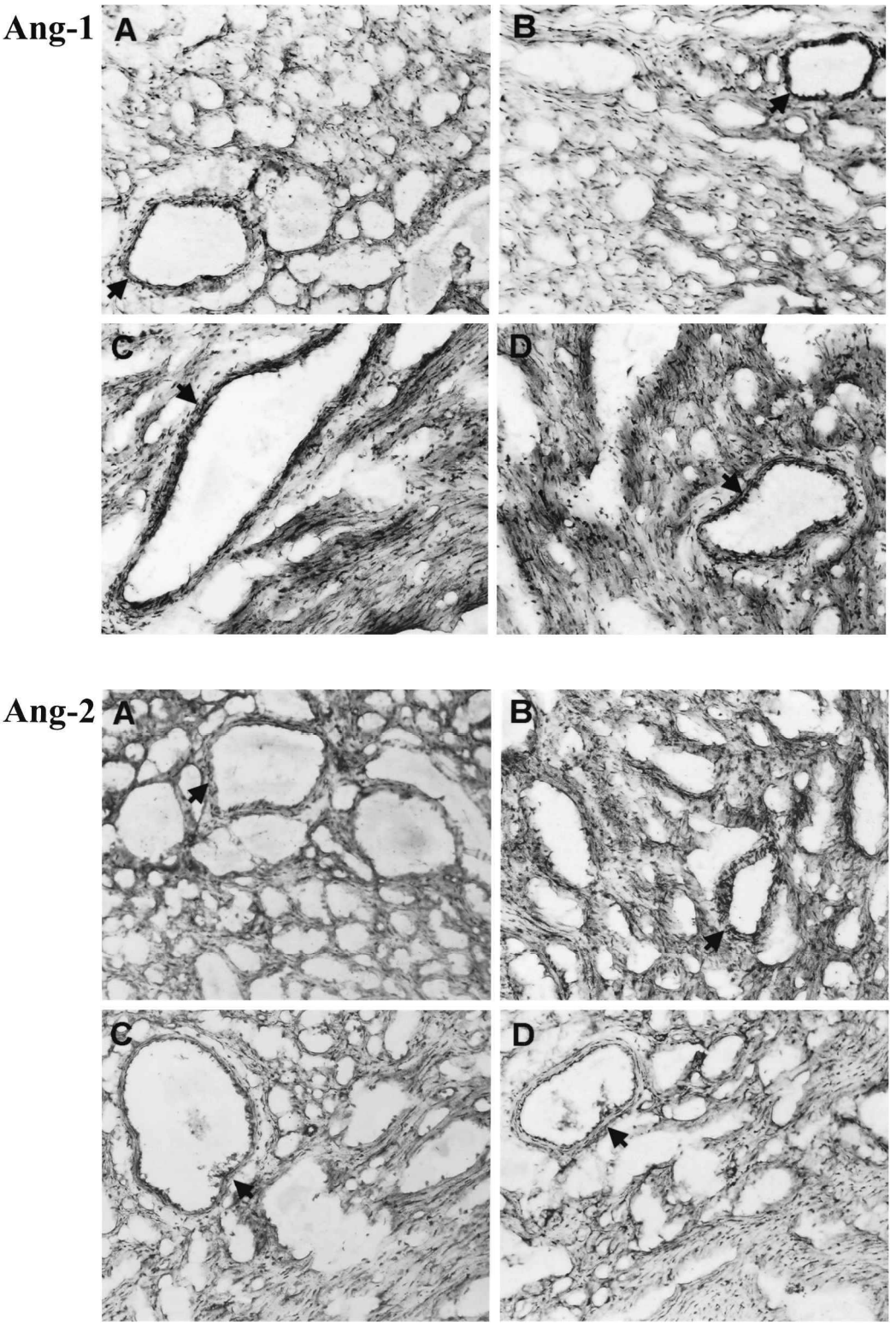

FIG. 5. Immunohistochemistry for Ang/Tie system. Representative sections of rat ventricular myocardium immunostained for Ang-1, Ang-2, Tie-1, and Tie-2 reactivities are shown. (**A**) Myocardial tissue sections from rat in normoxic control group. (**B, C, and D**) Myocardial tissue sections from rats in 1-h, 2-h, and 3-h hypoxic groups, respectively. Arrows point to coronary arteries. Original magnification, 200×.

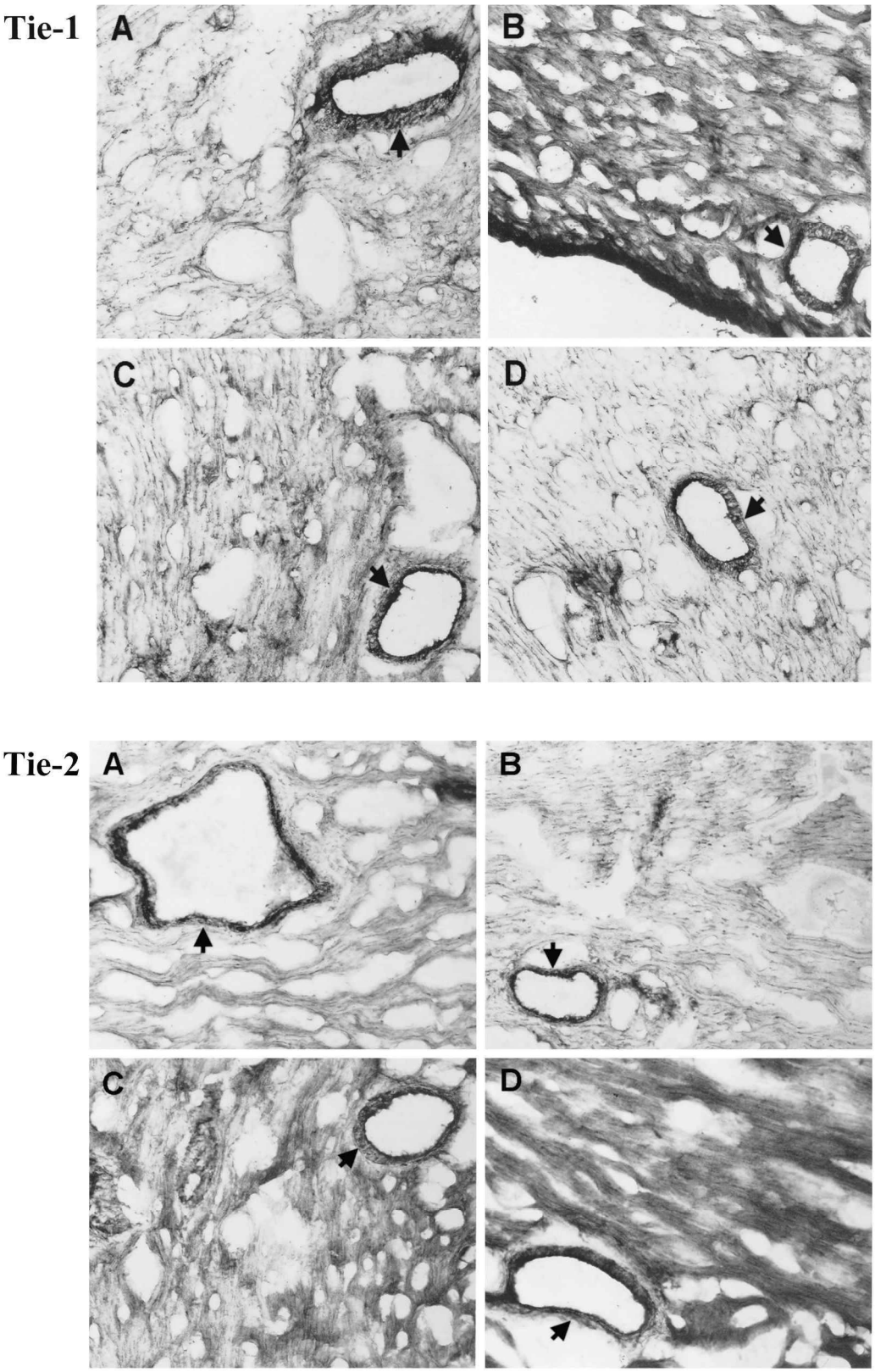

FIG. 5. *Continued from page 98.*

level remained significantly elevated until 2 h of hypoxia. After that, the abundance of this protein decreased. In contrast, the level of Tie-2 is significantly increased after 30 min of hypoxia and remains elevated even after 4 h of hypoxia in rat myocardium. The precise function of Tie-1 remains unknown, as does the identity and nature of its ligands. However, its time of expression, in relation to the other factors in our system, tends to suggest a regulatory involvement in the early to mid stages of the angiogenic process.

Although the specific ligand for Tie-1 is not yet known, the occurrence of two ligands specific for Tie-2 has been established. They are Ang-1 and Ang-2. Ang-1 and Ang-2 possess similar binding affinities for the endothelial cell tyrosine kinase receptor Tie-2 (17). The relative time frame of observed increases in Flk-1 and Flt-1 protein levels correlated relatively quite well with increases in Ang-2 and Tie-1 expression, respectively. Increased Flk-1 and increased Ang-2 provide evidence to support *in vitro* studies that demonstrated the ability of VEGF to up-regulate Ang-2 through its Flk-1 receptor via the protein kinase C and mitogen-activated protein kinase pathway (23, 37). Flt-1 phosphorylation has been shown to induce matrix metalloproteinase activity, and it is known that a specific metalloproteinase is required for the activation of soluble Tie-1 (19, 39). In addition, both hypoxia and VEGF have been shown to up-regulate Tie-1 (7). Despite the fact that a definite relation between Flk-1 and Tie-1 has not been demonstrated, our findings would support at least a partial involvement of VEGF in increasing soluble Tie-1 protein levels by matrix metalloproteinase activation through its Flt-1 receptor. However, it seems possible that additional transcriptional and/or translational up-regulation of Tie-1 occurs in response to hypoxia either directly or as an effect mediated through an intermediate factor. An investigation into the expression profile of Tie-1 and Tie-2 during the process of vascularization in the developing mouse embryo revealed that both are expressed in very early stages and closely follow the expression of the VEGF receptor Flk-1 (30). Elegant gene inactivation experiments involving the Tie-1 and Tie-2 receptors demonstrated embryonic lethality and gross structural malformations in the developing vasculature, confirming the critical importance of these receptors in the angiogenic process (4, 26, 33).

Gene inactivation experiments have also revealed the essential role of Ang-1 and Ang-2 in the process of neovascularization. Ang-2 competes with Ang-1 for binding to the Tie-2 receptor. It is well known by now that all angiopoietins (Ang-1, Ang-2, and Ang-3) bind to the Tie-2 receptors, but not to Tie-1. Ang-1 serves to promote angiogenesis, whereas Ang-2 may either promote or antagonize the angiogenic process depending upon the pathologic situation. In our experimental hypoxic model in rat myocardium, perhaps the most significant and interesting trend was the apparent relationship between Ang-1 and Ang-2 protein levels. Ang-1 expression was markedly depressed after 1 h of hypoxia, which coincided with the strong up-regulation of Ang-2 at the same time point. Thereafter, Ang-1 levels increased, but only after Ang-2 levels sharply decreased. Ang-2 has been implicated as being a natural angiostatic factor whose binding affinity for the Tie-2 receptor is comparable to that of Ang-1. However, the increased Ang-2 levels specifically in the early and mid hypoxic exposure times tend to support the argument for its playing an important role in early angiogenesis. Its increased presence may actually be stimulatory in nature. Furthermore, the surprisingly coincident, yet opposite, temporal trends in Ang-1 and Ang-2 levels suggest that although they are antagonists at the receptor level, regulation of their protein levels runs much deeper and seems to indicate modulatory control at the transcriptional and/or translational level. Whether Ang-1 expression is suppressed by Ang-2 or they are both under the control of an as-of-yet unknown factor remains to be established. A possible explanation of the above phenomenon may be the previously reported suggestion of a role of Ang-1 not in the initial phase of angiogenesis, but in the later stages of vascular development (38). There are also other reports in which the Ang-1/Tie-2 system, unlike the VEGF system, appears to be involved in later stages of angiogenesis (24). A possible mechanism by which this may be occurring is the observed inhibitory effect of Ang-

1 on the expression of Ang-2 (23). Whether a moderate hypoxic exposure is capable of imparting a state of heightened myocardial preparedness to better enable it to mount a more aggressive salvage angiogenic response in the fact of a later more severe hypoxic or ischemic challenge is an obvious question that arises in the light of our present findings. Further studies utilizing hypoxic preconditioning protocols to promote angiogenesis in experimental models of chronic myocardial infarction and heart failure will help in the characterization and identification of promising target candidates for gene therapy to achieve clinically relevant myocardial angiogenesis.

ACKNOWLEDGMENTS

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ABBREVIATIONS

Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; BSA, bovine serum albumin; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor.

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Address reprint requests to:
Dr. Nilanjana Maulik
Molecular Cardiology Laboratory
Cardiovascular Division, Department of Surgery
University of Connecticut School of Medicine
Farmington, CT 06030-1110

E-mail: nmaulik@panda.uchc.edu

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