Potential candidates for ischemic preconditioning-associated vascular growth pathways revealed by antibody array

Our understanding of the phenomenon of myocardial vascular growth is very limited even though various studies have been conducted in several different models, because the focus in each has been on a select few very number of proteins as the possible growth factors. In the present study, we used the ischemic preconditioning (IP) model in the form of four in vivo repetitive cycles of coronary artery occlusion, each followed by reperfusion as the model to stimulate vascular growth, and performed the protein profiling using high-throughput antibody array technology. Rats were divided into two groups: control + left anterior descending coronary artery (LAD) occlusion (CMI), and IP+ LAD occlusion (IPMI). The antibody array experiment performed to compare the expression of 512 proteins between the IPMI and CMI samples revealed significant upregulation of growth proteins like TGF-α, BMX, granulocyte-monocyte colony-stimulating factor, signal transducer and activator of transcription 3, α- and β-catenins, ubiquitin-conjugating enzyme UbcH6, nesilin, and PKC-α. β-JNK1 and c-Src tyrosine kinase were expectedly found to be downregulated. Western blot experiments validated the changes in expression of these proteins. Therefore, this study puts forward the above-mentioned proteins as valid participants in the vascular growth signals that are known to be triggered by ischemic preconditioning of heart.

myocardial infarction; signal transduction

REPETITIVE TRANSIENT ISCHEMIA-REPERFUSION is well known to confer a protective effect, referred to as ischemic preconditioning (IP), on the myocardium against subsequent prolonged ischemia (9). Neovascularization, the natural physiological process of formation of new blood vessels, is extremely important to ameliorate the function of the heart that undergoes ischemic stress. This process includes formation of collateral arteries (arteriogenesis) and formation of capillaries (angiogenesis) by sprouting from preexistent ones. Development of coronary collateral circulation is the heart’s own bypass mechanism by which it retains the blood supply to the ischemic tissue (3). It is also now well known that IP of the heart can trigger the repair process of angiogenesis (6). Discerning the myriad pathways that are possibly associated with this vascular growth process is therefore of utmost importance to develop targeted drugs and bring the therapy from bench to bedside.

Past studies have proven that vascular endothelial growth factor (VEGF), an angiogenic mitogen, is a highly specific growth factor for vascular endothelial cells both in vitro and in vivo. Previous studies, including ours, also have shown that transient ischemia upregulates VEGF mRNA in cardiac tissues, suggesting that VEGF mediates neovascularization during myocardial ischemia (6). In this study, Bcl-2 and survivin expression also were increased and VEGF-mediated vascular permeability was controlled because of suppression of c-Src. However, information regarding the effect of IP on the augmentation of angiogenic processes in terms of change in expression of other angiogenic genes/proteins remains scant. The existence of a discrete factor with proangiogenic activity was first reported almost three decades ago (5). 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. Therefore, in the present study we conducted protein profiling using the high-throughput antibody array technique to add to our understanding of the mechanism of vascular growth observed in the previous study (6). Once this experiment was performed, the proteins that were differentially regulated in this study were examined for their known biological relevance and short-listed for further validation on the basis of their potential role in vascular growth.

MATERIALS AND METHODS

Animals. All animals used in this study received humane care in compliance with the principles of laboratory animal care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The experimental protocol was approved by the Institutional Animal Care Committee of the University of Connecticut Health Center (Farmington, CT).

Surgical procedure. Male Sprague-Dawley rats weighing 250–300 g (ages 8–10 wk) were anesthetized with ketamine hydrochloride (100 mg/kg ip) and xylazine (10 mg/kg ip). Cefazolin (25 mg/kg ip) was administered as a preoperative antibiotic cover. After tracheotomy and initiation of ventilation (room air, Harvard Apparatus Rodent Ventilator model 683), the heart was exposed through a left lateral thoracotomy (4th intercostal space). A 6-0 polypropylene suture was passed with a tapered needle under the left anterior descending coronary artery (LAD) just below the tip of the left auricle, and a nontraumatic occluder was applied on the artery. The myocardium was preconditioned by undergoing a short duration of temporary regional ischemia (5 min) followed by a period of reperfusion (10 min), which was repeated four times (Fig. 1). Myocardial infarction (MI) was produced by permanent LAD occlusion. In the nonpreconditioned group, the rats underwent the LAD ligation after the chest...
was opened for 60 min without the preconditioning procedure. After completion of all surgical protocols, the chest wall was reclosed. After application of buprenorphine (0.1 mg/kg sc) and weaning from the respirator, the rats were placed on a heating pad while recovering from anesthesia. Forty-eight hours after MI, the hearts were perfused to demarcate the risk area including infarcted area from the left ventricular myocardium as described previously (6). The hearts that underwent similar infarction by LAD occlusion [control (CMI); LAD (CMI): 45 ± 3%; IP + LAD (IPMI): 48 ± 4%; IP + LAD (IPMI): 45 ± 3%] were considered for further evaluations. The peri-infarcted myocardial tissue sample was excised and rapidly snap-frozen in liquid nitrogen.

Antibody array experiment. Antibody array slides (lot 4020056; BD Biosciences Clontech, Palo Alto, CA) consisting of 512 individual antibodies spotted in duplicate were used. Two such slides comprised one experiment. Crude heart tissue from the left ventricle was used, and the protein was extracted using the manufacturer’s protocol. In brief, 100 mg of frozen tissue were homogenized in 1 ml of extraction/labeling buffer. The suspension was centrifuged at 10,000 g for 30 min, and the supernatant was transferred. Protein concentration was measured using the Pierce BCA protein assay reagent kit, and the samples were diluted to 1.1 mg protein/ml. Cy3 and Cy5 dyes (Amersham Biosciences, Piscataway, NJ) were each diluted with 110 g of each of the dyes and the unbound dye was removed from the samples by filtration through PD-10 columns (Amersham Biosciences). Labeling efficiency was estimated from dye absorbance and was typically 2.5–3.5 molecules of dye per protein molecule. Cy5-labeled protein (100 μg) from the IPMI-treated sample was mixed with 100 μg of Cy3-labeled protein from the CMI sample. Similarly, 100 μg of Cy5-labeled protein from the IPMI-treated sample were mixed with 100 μg of Cy5-labeled protein from the CMI sample. Protein (20 μg) from each of these two mixes was incubated separately with antibody microarray slides for 30 min and washed in sequence with the seven buffers provided in the kit. The slides were dried and scanned immediately using Scanarray Express from Packard Bioscience (now Perkin-Elmer Life Sciences).

Antibody array data analysis and statistics. The images for each slide separately scanned for Cy3 and Cy5 labels were merged and analyzed using Imagene software (BioDiscovery) to produce text files with signal intensities. These files were then inputted into GeneSpring software (Silicon Genetics) to calculate the relative change and draw the scatter plot. Spots were considered valid only if their duplicates did not differ by >20% and the signal was at least more than twice that of the background. A standard two-dye normalization protocol (Lowess normalization) was used. A relative change >1.30 or <0.75 was considered significant.

Western blot analysis. Total protein (50 μg) in the Clontech extraction buffer was added to an equal volume of sodium dodecyl sulfate (SDS) buffer and boiled for 10 min before being separated on 7–15% SDS-polyacrylamide gels in running buffer [25 mM Tris, 192 mM glycine, 0.1% (wt/vol) SDS, pH 8.3] at 200 V. The Precision Plus Protein Kaleidoscope standards (10 μl; Bio-Rad Laboratories, Hercules, CA) were used as molecular mass standards. The gel was transferred onto nitrocellulose membrane (Bio-Rad Laboratories) at 100 V for 1 h in transfer buffer [25 mM Tris base, 192 mM glycine, 20% (vol/vol) methanol, pH 8.3]. After the membranes were blocked for 1 h in Tris-buffered saline [TBS-T: 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% (vol/vol) Tween 20] containing 5% (wt/vol) nonfat dry milk, blots were incubated overnight at 4°C with the various primary antibodies. The antibodies were purchased from BD Transduction Laboratories and were used at manufacturer-recommended dilutions. Membranes were washed three times in TBS-T before incubation for 1 h with horseradish peroxidase-conjugated secondary antibody diluted 1:2,000 in TBS-T and 5% (wt/vol) nonfat dry milk.

Fig. 1. Schematic showing the experimental ischemic preconditioning protocol. CMI, control myocardial infarction; LAD, left anterior descending coronary artery; IPMI, ischemic preconditioning followed by myocardial infarction; I, ischemia (5 min); R, reperfusion (10 min).

Fig. 2. Scatter plot of the antibody array experiment showing upregulation and down-regulation of proteins and their relative (fold) change (IPMI vs. CMI) as analyzed using GeneSpring software. STAT3, signal transducer and activator of transcription 3; GM-CSF, granulocyte-monocyte colony-stimulating factor.
Western blots were developed with the ECL Detection Reagents 1 and 2 (Amersham Biosciences) and exposed to Kodak X-OMAT film.

RESULTS AND DISCUSSION

In exactly the same preconditioning model, improvements in cardiac function, reduction in infarct size, changes in microvascular density, improved blood flow, and reduction in extent of apoptotic cell death (cardiomyocyte and endothelial) were observed in the IPMI group (6).

In our present study, the set of proteins differentially expressed (relative change $>1.30$ or $<0.75$) in the IPMI sample was studied using public protein databases such as LocusLink and SwissProt for their biological functions. Among the proteins of interest, the antibody array experiment had revealed the relative changes in the overexpressed proteins transforming growth factor-$\beta$ (TGF-$\beta$), BMX, granulocyte-monocyte colony-stimulating factor (GM-CSF), signal transducer and activator of transcription 3 (STAT3), $\alpha$- and $\beta$-catenins, the ubiquitin-conjugating enzyme UbcH6, nexilin, Rad50, and PKC-$\epsilon$ and $\lambda$ to be 1.9, 1.8, 1.5, 1.7, 1.7, 2.8, 2.6, 2.9, 1.3, and 1.4 respectively. The relative changes for underexpressed proteins c-Src tyrosine kinase (CSK) and JNK1 were 0.7 each (Fig. 2). The upregulation of TGF-$\beta$, BMX, GM-CSF, $\beta$-catenin, UbcH6, and nexilin was validated using Western blot analysis (Fig. 3).

TGF-$\beta$ is a multifunctional peptide that controls proliferation, differentiation, and other functions in many cell types. When injected subcutaneously in newborn mice, it causes formation of granulation tissue (induction of angiogenesis and activation of fibroblasts to produce collagen) at the site of

Fig. 3. Representative images of the proteins TGF-$\beta$, BMX, GM-CSF, nexilin, UbcH6, and $\beta$-catenin chosen to be validated through Western blot analysis. All experiments were performed at least twice, and the representative images show only 2 of a minimum of 3 samples studied in each category (IPMI and CMI). One control representative blot with GAPDH antibody is also shown per membrane used.
injection (12). GM-CSF is a cytokine that stimulates the growth and differentiation of hematopoietic precursor cells from various lineages, including granulocytes, macrophages, eosinophils, and erythrocytes. In one study, subcutaneous application of GM-CSF was shown to stimulate arteriogenesis and functional improvement in the brain (2). Monocytes play a role in both angiogenesis and arteriogenesis and maybe the common denominator for both pathways of vascular growth (13). GM-CSF, being the survival factor of monocytes, and TGF-β, a monocyte chemoattractant, are strong arteriogenic factors (13). Therefore, upregulation of TGF-β and GM-CSF in this study, concomitant with increased capillary density and blood flow in our previous study (6), suggests that these proteins must be playing a significant role in vascular growth and development in our ischemic model of cardiac preconditioning.

BMX has been reported to have a redundant function downstream of angiopoietin and VEGF receptors in arterial endothelium (11). In a recent study, BMX, a member of the Btk/Tec family of nonreceptor tyrosine kinases, was found to participate in PKC-ε signaling in a rabbit model of nitric oxide-induced pharmacological preconditioning (16). Two PKC isoforms, ε and λ, were found to be overexpressed in our experiment. Because many studies have already shown that the protective effects of IP are mediated by PKC, they were not validated by Western blot analysis. In another study, a constitutively active BMX was shown to be able to activate STAT3 with rapid kinetics, suggesting that the activation of STATs by BMX could be one of the ways it provides cardioprotection (14). STATs are transcriptional factors that affect the growth and apoptosis behaviors of cells. In addition, the protective role of Btk family kinases also could be attributed to their ability to activate Akt and NF-κB (10), both of which have been proven to be overexpressed in preconditioned hearts. The significantly upregulated expression of BMX along with PKC-ε and STAT3 in our study suggests that BMX provides cardioprotection even in an ischemic model of preconditioning, possibly by enhancing vascular growth.

Oxidative stress during ischemia repair can lead to DNA damage and DNA fragmentation. Rad50 has been shown to function in DNA double-strand break repairs (7) and is therefore critical not only for the maintenance of genomic integrity and cell survival but also for the formation of new vasculature from the existing ones.

β-Catenin and nexitlin are adherens junction proteins involved in cell-to-cell adhesion and proliferation. Adherens junctions mediate adhesion between cells, communicate the signal that neighboring cells are present, and anchor the actin cytoskeleton. These junctions regulate normal cell growth and behavior. Under special conditions of embryogenesis, wound healing, and tumor cell metastasis, cells form and leave epithelia. Evidence that β-catenin could be a key regulator of both cell proliferation and migration and have a role in angiogenesis was shown in a study in which β-catenin was observed in endothelial cells of newly formed and preexisting blood vessels in the infarct area in the first week after MI in rat hearts, whereas vascular endothelial cells in the noninfarcted part of the heart showed no detectable β-catenin levels in their cytoplasm (1). Nexilin is a novel F-actin-binding protein localized at cell-matrix adherens junctions. At the cell-matrix junction, integrins interact with matrix proteins at the extracellular surface and with the actin cytoskeleton at the cytoplasmic region through many peripheral membrane proteins, including nexitlin. Cell adhesions to the extracellular matrix are extremely important in directing the migration, proliferation, and differentiation of the cells and are essential for continuous contractile activity of the heart and its adaptation to stress (15). Also relevant to this discussion is the 1.4-fold upregulation of integrin β3 (not validated by Western blot).

Maintenance of cellular functions requires timely and selective degradation of key regulatory proteins. UbcH6 or ubiquitin carrier protein E1 catalyzes the covalent attachment of ubiquitin to other proteins, and ubiquitin-protein conjugates are then recognized and degraded by specific protease complexes.

It was already reported that activation of MAPK pathway together with inhibition of SAPK/JNK activity by VEGF is the key event in determining whether an endothelial cell is going to survive or will undergo programmed cell death (8). JNK is crucial to programmed cell death induced by TNF receptor, and one mechanism by which NF-κB protects cells is downregulation of the JNK cascade through the transcriptional activation of Gadd45β (4). Inhibition of apoptosis by downregulation of JNK1 may represent a major aspect of the regulatory activity of VEGF on the vascular endothelium for angiogenesis. Also, consistent with a previous study (6), c-Src was downregulated, and its significance can be explained in terms of suppression of vascular permeability.

In conclusion, the preconditioning response in terms of both increased capillary density and increased blood flow, along with other functional improvements in our previous study and upregulation of these several potential angiogenic signaling candidates in this study, clearly document ischemia-associated vascular growth in rat myocardia. Each of these potential candidates deserves dedicated further investigations to zero in on their exact roles in the pathways leading to this physiologically relevant and clinically significant process of vascular growth and development.

GRANTS
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REFERENCES