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Cardiac myocytes exposed to anoxia-reoxygenation promote neutrophil transendothelial migration

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Rui, Tao, Gediminas Cepinskas, Qingping Feng, Ye-Shih Ho, and Peter R. Kvietys. Cardiac myocytes exposed to anoxia-reoxygenation promote neutrophil transendothelial migration. *Am J Physiol Heart Circ Physiol* 281: H440–H447, 2001.—The goal of the present study was to assess whether cardiac myocytes exposed to anoxia-reoxygenation (A/R) could generate a chemotactic gradient for polymorphonuclear neutrophil (PMN) transendothelial migration. Exposure of neonatal mouse cardiac myocytes to A/R induced an oxidant stress in the myocytes. Supernatants obtained from A/R-conditioned myocytes promoted mouse PMN migration across mouse myocardial endothelial cell monolayers. This increase in PMN transendothelial migration could be prevented if catalase or a platelet-activating factor (PAF) antagonist was added to the supernatants before assay. Supernatants from A/R-conditioned myocytes activated endothelial cells by inducing an intracellular oxidant stress. The oxidant stress and PMN transendothelial migration induced by supernatants from A/R-conditioned myocytes were substantially reduced when endothelial cells derived from manganese superoxide dismutase overexpressing mice were used in the assays. Supernatants from A/R-conditioned myocytes also increased endothelial cell surface levels of E-selectin and intercellular adhesion molecule-1. Our results indicate that cardiac myocytes exposed to A/R can generate a chemotactic gradient, presumably due to production and release of stable oxidants and PAF. The ability of supernatants from A/R-conditioned myocytes to promote PMN transendothelial migration was largely dependent on induction of an oxidant stress in endothelial cells. In addition, these supernatants also induced a proadhesive phenotype in the endothelial cells.

manganese superoxide dismutase-overexpressing mice; endothelial cells; adhesion molecules

REPERFUSION OF THE ISCHEMIC myocardium results in detrimental effects to the heart, ranging from arrhythmias and contractile impairments to actual necrosis (5). A substantial amount of evidence supports the contention that myocardial ischemia-reperfusion (I/R) injury is partly due to an acute inflammatory response in the affected vascular bed within the heart (5, 8, 14). This inflammatory response involves polymorphonuclear neutrophil (PMN) adhesion to coronary endothelium and subsequent infiltration into the interstitium. A significant role for invading neutrophils in the myocardial injury incurred after I/R is supported by the following lines of evidence. First, the degree of I/R-induced myocardial injury is correlated to the extent of PMN infiltration (29). Second, rendering the animals neutropenic (5) or immunoneutralization (antibodies) of adhesion molecules on PMN (CD18) or endothelial cells such as intercellular adhesion molecule-1 (ICAM-1) and P-selectin affords protection against I/R-induced myocardial injury (9, 18, 21, 28, 32). Third, genetically modified mice that are deficient in either endothelial cell (ICAM-1 or P-selectin) or PMN (CD18) adhesion molecules are more resistant than their wild-type counterparts to the inflammatory consequences of I/R (less PMN infiltration and tissue necrosis) (22, 23).

In vitro models of I/R have allowed for mechanistic studies (17) not readily performed by using in vivo models. These models involve exposure of cultured endothelial cells to anoxia or hypoxia and subsequently reoxygenating them (anoxia-reoxygenation; A/R). Supernatants obtained from endothelial cells challenged with A/R increase adhesion molecule (CD18) expression on PMN and increases PMN adhesion to naive endothelial cells (37). In addition, these models have allowed for the characterization of the time course of A/R-induced adhesion molecule expression on endothelial cells and their relative roles in A/R-induced PMN adhesion to endothelial cells (11, 24). Finally, with the use of these models, the cellular-to-molecular mechanisms (nuclear transcription factors NF-κB and AP-1) (11) as well as proinflammatory mediators produced by endothelial cells exposed to A/R have been defined (37). Although these in vitro approaches have yielded important information regarding the mechanisms involved in PMN-endothelial cell adhesive interactions, they lack one critical component that is present in vivo. This component is the presence of an interstitial-to-blood chemotactic gradient, which promotes the emi-

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gration of circulating PMN across the endothelial barrier into the interstitial compartment.

Little is known about the factors that initiate PMN recruitment into the interstitium. Analyses of cardiac lymph tissue obtained after I/R indicate that these samples of interstitial fluid can activate PMN and induce adhesion of PMN to endothelial cell monolayers (4). However, whether these substances can actually promote PMN emigration is unclear. Previous studies (6) indicate that cardiac interstitial mast cells degranulate after I/R, releasing chemotactic factors that can potentially attract PMN into the interstitium. Although well recognized as immune cells that contribute to the inflammatory response, mast cells constitute <0.1% of cells in the ventricular interstitium (25). One cell type that has been overlooked with respect to its potential to generate a chemotactic gradient is the cardiac myocyte, the predominant resident cell in the heart. Thus, in the present study, we have applied our in vitro model of the interstitial-vascular interface (3) to assess the ability of myocytes exposed to A/R to increase PMN transendothelial migration. Using this model, we show that cardiac myocytes challenged with A/R can indeed promote PMN transendothelial migration. This increased transendothelial migration can be attributed to the ability of A/R-conditioned myocytes to activate endothelial cells.

MATERIALS AND METHODS

Cardiac myocytes. Neonatal cardiac myocytes were isolated and cultured by using methods described previously (15) with some modifications. Briefly, newborn wild-type mice (C57BL6) were euthanized by cervical dislocation, and their hearts were removed aseptically and immersed in cold Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS). The tissue was minced and transferred into Ca^{2+}- and Mg^{2+}-free HBSS containing 160 U/ml of collagenase II (Worthington Biotech) and allowed to dissociate for 15 min at 37°C. This digestion procedure was repeated three more times, and the cells obtained were resuspended in medium 199 (M199). Because myocytes adhere less avidly to plastic than other cell types (e.g., fibroblasts), the cells were preplated in petri dishes for 1 h at room air with 5% CO_2 at 37°C and 95% humidity. The myocyte-rich supernatants were centrifuged for 5 min at 250 *g*. The pellets were resuspended in M199 supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin G, and 100 μg/ml streptomycin and seeded into fibronectin-coated 48-well tissue culture plates (Costar). After 72 h, the cardiac myocytes had formed a confluent monolayer consisting of 90–95% myocytes beating in synchrony and were used in experiments at this time.

Myocardial endothelial cells. Adult myocardial endothelial cells were isolated by using a modification of previously described methods (12, 26). Briefly, the ventricles were aseptically removed from heparinized (5,000 U/kg) and anesthetized (100 mg/kg of pentobarbital sodium) wild-type adult mice (C57/BL6) and transferred to ice-cold HBSS. After the ventricles were minced, the tissue was exposed to a digestion buffer containing 500 U/ml of collagenase II, 0.6 U/ml of dispase II (Boehringer-Mannheim), and 0.1% (wt/vol) bovine serum albumin (Sigma) in HBSS for 40 min at 37°C. The digested material was filtered through a 100-μm thick nylon mesh and washed twice by resuspension and centrifugation in fresh M199. The filtered cells were coincubated with lectin-coated (*Griffonia simplifolia*-1, Sigma) microbeads (model M-450, Dynal) in M199 supplemented with 1% FCS at room temperature. The microbeads with attached endothelial cells were captured by a Dynal magnet, seeded in a fibronectin-coated (Boehringer-Mannheim) T_{25} cell culture flask, and incubated in EBM-2 media supplemented with EGM-2 MV (Clonetic). The homogeneity of these ventricular endothelial cells was ≅85% (identified by DiI-Ac-LDL uptake). First- through third-passage cells were used for experiments when confluent.

For some experiments, ventricular endothelial cells were harvested as described above from manganese-superoxide dismutase (Mn-SOD)-overexpressing transgenic mice (10). For these experiments, the background strain (C57BL/6 × C3H hybrid) was used as a control.

Neutrophils. PMN were isolated from the marrow of hind leg bones of adult mice as previously described (19) with some modifications. Wild-type adult mice (C57BL6) were euthanized by cervical dislocation, the long bones of the hind legs were removed, and the ends were clipped and were then flushed with ice-cold Ca^{2+}- and Mg^{2+}-free HBSS. The obtained marrow was centrifuged (500 *g* at 4°C) for 5 min, and resuspended in 4 ml of HBSS. The suspension was subject to a Percoll (Sigma) step gradient. The Percoll gradient was centrifuged, and the cells were removed from the neutrophil-enriched fraction. This procedure yielded 5–6 million white blood cells, 95% of which were adult PMN, as identified by acetic acid crystal violet staining.

A/R protocol. The in vitro model of A/R used in the present study is identical to that described previously (2, 37). Briefly, confluent beating mouse cardiac myocyte monolayers were exposed to anoxia for 30 min in phenol red- and glucose-free Dulbecco's modified Eagle's medium and then reoxygenated (A/R). Thirty minutes after reoxygenation, the supernatants were removed and various assays performed. As a control, cardiac myocytes were treated identically except that they were exposed to normoxia instead of anoxia (normoxia-reoxygenation; N/R).

Assays. PMN transendothelial migration was assessed as previously described (3). Briefly, mouse heart endothelial cells were grown to confluence on 25 μg/ml fibronectin-coated Falcon cell culture inserts (3-μm diameter pores). ^{51}Cr-labeled mouse PMN in M199 were added to the apical aspect of the endothelial cell monolayer and coincubated for 90 min with supernatants from A/R- or N/R-conditioned myocytes in the basal compartment. The percentage of added neutrophils that migrated from the apical to the basal aspects of the insert membranes was quantified.

Oxidant production within the cardiac myocytes or endothelial cells was assessed by measuring the oxidation of intracellular dihydrorhodamine 123 (DHR 123; Molecular Probes), an oxidant-sensitive fluorochrome, as described previously (2). Briefly, the cells were treated with DHR 123 (5 μmol/l) for 1 h before A/R or N/R. After the A/R conditioning, the cells were lysed and analyzed for DHR 123 oxidation at excitation and emission wavelengths of 502 and 523 nm, respectively.

Total SOD activity was determined by using a Bioxytech SOD-525 kit (OXIS Health Products) as recommended by the manufacturer. Mn-SOD activity was determined by subtracting Cu/Zn-SOD activity obtained in chloroform-ethanol extracted samples (specifically inhibits Mn-SOD) from the total SOD activity.

Endothelial cell adhesion molecule expression was assessed as previously described (20) with modifications for mouse endothelial cells. Briefly, endothelial cells were fixed and treated with primary rat anti-mouse antibody directed

against E-selectin (10E9.6; BD Pharmingen) or ICAM-1 (BSA2; R&D Systems). The cells were subsequently reacted with biotinilated rabbit anti-rat secondary antibody and peroxidase-conjugated avidin (Rat-Extravidin, Sigma) with the use of 3′,3′,5′,5′-tetramethylbenzidine as the substrate.

Statistical analysis. All of the values are presented as means ± SE. Statistical analysis was performed with the use of ANOVA and Student's *t*-test with Bonferroni correction for multiple comparisons.

RESULTS

In previous studies on the effects of A/R on cardiac myocytes, the cells were exposed to anoxia for hours and cell death was used as the endpoint (30, 33, 34). In the present study, exposure of cardiac myocytes to A/R (30 min of anoxia) did not result in myocyte death, as judged by trypan blue exclusion. However, the myocytes incurred an oxidant stress as indicated by intracellular oxidation of DHR 123 (Fig. 1). Thus A/R in vitro mimics I/R in vivo where an oxidant stress is induced in the affected tissue.

To assess whether A/R-conditioned cardiac myocytes could produce chemotactic factors, supernatants were removed from the myocytes after A/R and evaluated for their ability to promote PMN transendothelial migration. As shown in Fig. 2, supernatants from A/R-conditioned myocytes increased PMN transendothelial migration compared with supernatants from the normoxic controls (cardiac myocytes exposed to N/R). These observations indicate that myocytes exposed to A/R produce stable, soluble factors that can promote PMN emigration. Both the PAF antagonist and catalase were effective in preventing the increase in PMN transendothelial migration induced by the supernatants from A/R-conditioned myocytes. Thus PAF and oxidants (most likely H_2O_2) appear to be produced by cardiac myocytes exposed to A/R and contribute to PMN transendothelial migration. Interestingly, both PAF and oxidants have also been implicated in the acute inflammatory response elicited by I/R in vivo (17).

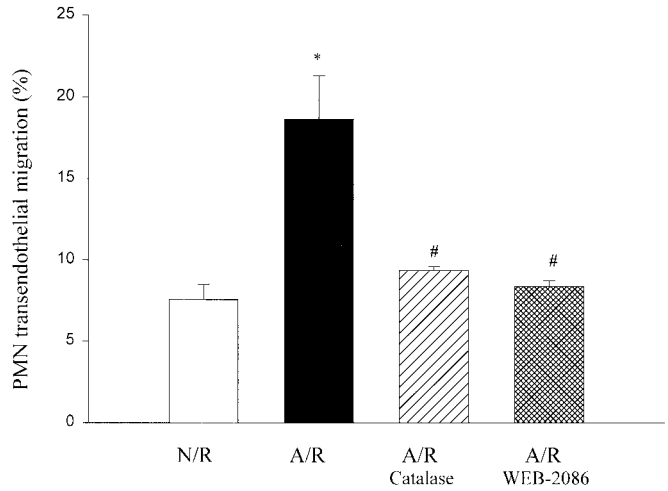

Fig. 2. Supernatants from A/R-conditioned myocytes promote polymorphonuclear (PMN) transendothelial migration: role of oxidants and platelet-activating factor (PAF). PMN were added to the apical surface of endothelial monolayers in inserts, and supernatants from A/R-conditioned myocytes were added to the basal compartment. At 90 min after coincubation, PMN transendothelial migration was assessed. PAF antagonist (WEB-2086; 50 μg/ml) or catalase (1,000 U/ml) was added along with the supernatants from A/R-conditioned myocytes. $n = 3$, in duplicate. *$P < 0.05$ compared with N/R; #$P < 0.05$ compared with A/R.

To assess whether substances produced by cardiac myocytes exposed to A/R could activate endothelial cells, supernatants obtained from A/R-conditioned myocytes were assessed for their ability to induce an oxidant stress in endothelial cells. As shown in Fig. 3, these supernatants increased oxidant stress in endo-

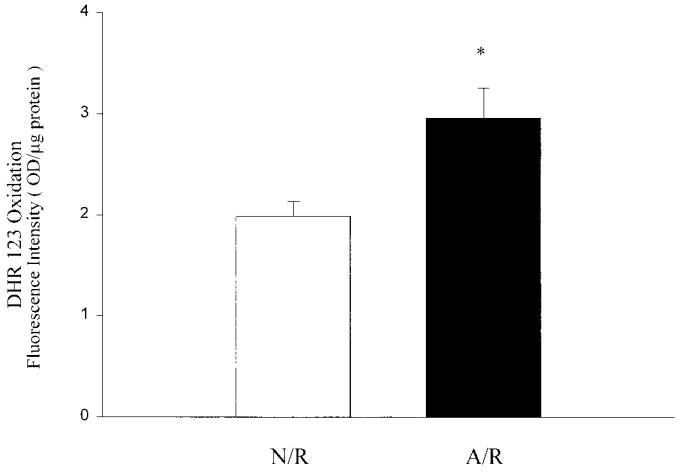

Fig. 1. Myocytes challenged with anoxia-reoxygenation (A/R) incur an oxidant stress. Myocytes were loaded with dihydrorhodamine (DHR) 123 (5 μmol/l) for 1 h before being exposed to normoxia-reoxygenation (N/R) or A/R. Supernatants were removed 30 min after N/R or A/R, and the myocytes were lysed {0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate} and assessed for DHR 123 oxidation. $n = 4$, in duplicate. OD, optical density. *$P < 0.01$ compared with N/R.

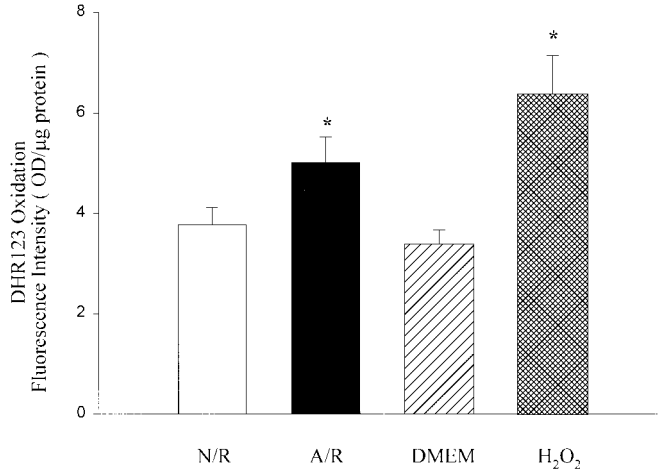

Fig. 3. Supernatants from A/R-conditioned myocytes induce an oxidant stress in endothelial cells. Endothelial cells were loaded with DHR 123 (5 μmol/l) 1 h before treatment with supernatants from myocytes exposed to either N/R or A/R. H_2O_2 (100 μM) dissolved in Dulbecco's modified Eagle's medium (DMEM) was used as a positive control. See Fig. 1 legend for details of the procedure for measuring DHR 123 oxidation. $n = 3$, in duplicate. *$P < 0.01$ compared with N/R or DMEM.

thelial cells as indicated by an increased intracellular oxidation of DHR 123.

To assess whether the induction of an oxidant stress in endothelial cells by supernatants from A/R-conditioned myocytes plays a role in the enhanced PMN transendothelial migration, endothelial cells derived from Mn-SOD-overexpressing mice were used. As shown in Fig. 4, *top*, ventricular endothelial cells from these mice had twice the Mn-SOD activity as their matched controls (C57BL/6 × C3H hybrid). The levels of Cu-Zn SOD were not increased (see Fig. 4, *bottom*). Supernatants from A/R-conditioned myocytes did not induce an oxidant stress in Mn-SOD-overexpressing endothelial cells (Fig. 5, *top*). Finally, the PMN transendothelial migration induced by supernatants obtained from A/R-conditioned myocytes was substantially reduced across monolayers of endothelial cells derived from the Mn-SOD-overexpressing mice compared with monolayers of endothelial cells derived from the background strain (see Fig. 5, *bottom*). Taken together, these findings indicate that endothelial cells

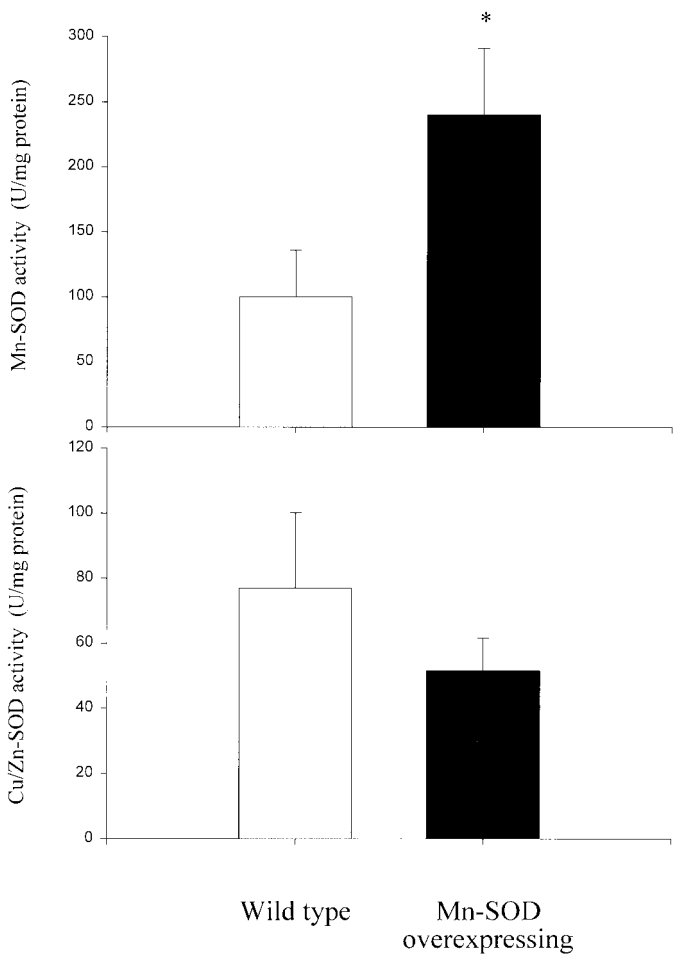

Fig. 4. Mn superoxide dismutase (SOD; *top*) and Cu-Zn SOD (*bottom*) activity in endothelial cells derived from hearts of Mn-SOD-overexpressing mice. Total SOD activity was measured, and Mn-SOD was determined by subtracting Cu/Zn SOD activity measured in chloroform-ethanol extracted samples. $n = 3$. $^*P < 0.05$ compared with endothelial cells obtained from the background strain (C57BL/6 × C3H hybrid).

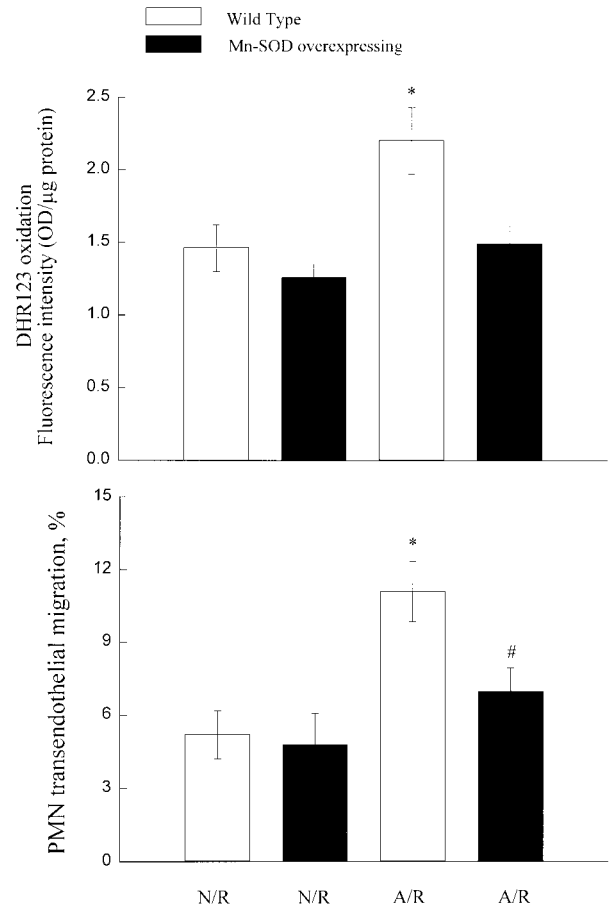

Fig. 5. Endothelial cell oxidant stress (*top*) and PMN transendothelial migration (*bottom*) induced by supernatants from A/R-conditioned myocytes was diminished when endothelial cells derived from hearts of Mn-SOD, overexpressing mice were used. Oxidant stress was measured as described in Fig. 1 and PMN transendothelial migration was measured as described in Fig. 2. $n = 3$ in duplicate. $^*P < 0.05$ compared with N/R for wild-type and Mn-SOD-overexpressing mice, respectively. $\#P < 0.05$ compared with A/R in wild-type mice.

play an active role in the PMN transendothelial migration elicited by supernatants derived from A/R-conditioned myocytes and that the generation of an oxidant stress within the endothelial cells is a pivotal event.

Finally, studies were undertaken to determine if supernatants from A/R-conditioned myocytes could induce a proadhesive phenotype in endothelial cells, which would prolong the endothelial cell contribution to the inflammatory response. To this end, the surface levels of E-selectin and ICAM-1 were measured 4 h after exposure of the wild-type endothelial cells to supernatants from A/R-conditioned myocytes. As shown in Fig. 6, both E-selectin and ICAM-1 were increased on the surface of endothelial cells after exposure to supernatants from A/R-conditioned myocytes. These findings indicate that substances produced by myocytes exposed to A/R can activate the endothelium in a manner that results in a proadhesive phenotype for as long as 4 h after the A/R challenge.

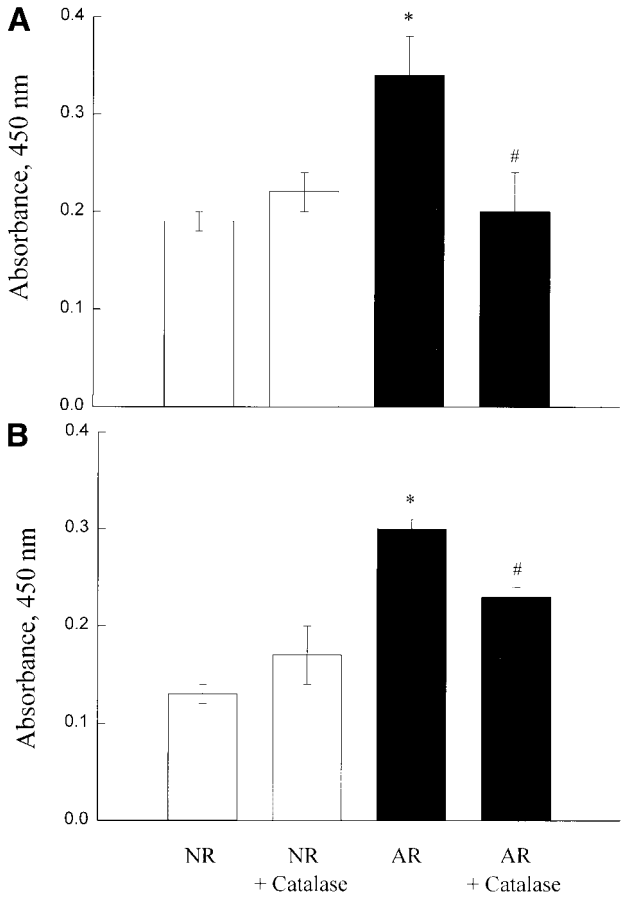

Fig. 6. Endothelial cell surface levels of E-selectin (*A*) and ICAM-1 (*B*) are increased 4 h after exposure of endothelial cells to supernatants from A/R-conditioned myocytes. E-selectin and ICAM-1 surface levels were assessed as described in MATERIALS AND METHODS. $n = 3$ in duplicate. *$P < 0.05$ compared with N/R; #$P < 0.05$ compared with A/R.

DISCUSSION

Reperfusion of the ischemic myocardium induces a local inflammatory response in the tissue supplied by the affected blood vessels (5, 14). The resultant PMN-endothelial cell adhesive interactions (adhesion and emigration) are believed to play a pivotal role in the myocardial dysfunction induced by I/R. This contention is based on in vivo observations indicating that I/R-induced myocardial dysfunction is ameliorated in animals that *1*) have been rendered neutropenic (5), *2*) have had either endothelial or neutrophil adhesion molecules neutralized by antibodies (9, 18, 21, 28, 32), or *3*) have been rendered devoid of endothelial or neutrophil adhesion molecules by genetic manipulation (22, 23).

In vitro approaches have been used to dissect out the molecular determinants of neutrophil-endothelial cell adhesive interactions. In general, these studies have employed models in which cultured endothelial cells are exposed to anoxia (hypoxia) and reoxygenated (A/R) and subsequently interacted with PMN (11, 17). With the use of these in vitro approaches, a great deal of useful information has been gleaned regarding the molecular determinants of A/R-induced PMN adhesion to endothelial cells. However, one issue that has not been addressed in vitro is the source of the chemotactic gradient that promotes PMN transendothelial migration. In the present study, we used Transwell inserts to mimic the interstitial-vascular interface (3) and assessed the contribution of myocytes to the development of a chemotactic gradient. By using this approach, we provide evidence that cardiac myocytes can be activated by A/R and that they produce substances that promote PMN transendothelial migration (see Figs. 1 and 2). To our knowledge, this is the first report to indicate that cardiac myocytes may play a significant role in the PMN emigration after I/R of the heart.

A variety of inflammatory mediators (or chemotactic factors) have been implicated as playing a role in I/R-induced myocardial inflammation, including tumor necrosis factor-α, PAF, interleukin (IL)-8, IL-6, complement, histamine, and leukotrienes (5, 14). These mediators have been implicated on the basis of their detection in coronary sinus or cardiac lymph after I/R. One approach that has been particularly insightful is the analysis of cardiac lymph obtained after I/R (4, 38). The significance of this approach is that the interstitium should be the source of the chemotactic gradient directing PMN out of the vasculature and into the interstitial/parenchymal compartment. Thus cardiac lymph allows for sampling of the interstitial fluid for the presence of relevant chemotactic factors. In the present study, we used a similar approach to identify the chemotactic substances produced by A/R-conditioned cardiac myocytes. We exposed cardiac myocytes to A/R and assessed the ability of supernatants (analogous to lymph in vivo) obtained from these A/R-conditioned myocytes to promote PMN transendothelial migration. By using our model of the vascular-interstitial interface, we were able to directly demonstrate that supernatants from A/R-conditioned myocytes promote PMN transendothelial migration. Previous studies (13, 35) indicate that myocytes exposed to hypoxia/reoxygenation can generate proinflammatory mediators, such as PAF and IL-6. In the present study, we were able to completely block the supernatant-induced PMN migration with catalase or a PAF antagonist. Thus our studies indicate a role for stable oxidants and PAF in the PMN transendothelial migration elicited by A/R-conditioned myocytes.

Previous studies have shown that both H_2O_2 and PAF can promote adhesion of PMN to endothelial cells (16). Furthermore, PAF introduced to the basal aspect of endothelial cell monolayers can promote PMN migration from the apical to the basal aspect of these monolayers (3). Thus it is tempting to conclude that PAF and H_2O_2 are the activating/chemotactic factors responsible for the PMN transendothelial migration induced by supernatants from A/R-conditioned myocytes. However, there may be one caveat in this line of reasoning. The earlier studies were performed with the use of purified reagents in the absence of other cells or material derived from other cells. In contrast, in the

present study, supernatants obtained from A/R-conditioned myocytes were used to promote PMN migration. Thus it may be possible that in our system PAF and H_2O_2 may simply be promoting PMN adhesion to endothelial cells (a prerequisite to PMN transendothelial migration) and that some other factors produced by myocytes may be generating the actual chemotactic gradient. The identity of this factor, if it exists, is unknown.

Another issue that we addressed in the present study was whether A/R-conditioned myocytes could be promoting PMN transendothelial migration by activating the adjacent endothelium. As shown in Fig. 3, supernatants from A/R-conditioned myocytes increased endothelial cell oxidant stress. To assess whether the generation of an oxidant stress within endothelial cells was a prerequisite for PMN migration, we used endothelial cells from Mn-SOD-overexpressing mice. Supernatants from A/R-conditioned myocytes did not induce an oxidant stress in endothelial cells overexpressing Mn-SOD (see Fig. 5). Furthermore, PMN transendothelial migration induced by supernatants from A/R-conditioned myocytes was dramatically reduced across monolayers of Mn-SOD-overexpressing endothelial cells (see Fig. 5). Taken together, these findings indicate that induction of an oxidant stress in endothelial cells is an important component of the PMN transendothelial migration induced by supernatants from A/R-conditioned myocytes.

Not only does activation of the endothelial cells by supernatants from A/R-conditioned myocytes contribute to the early initiation of PMN transendothelial migration, it may also contribute to a more prolonged inflammatory response. In the present study, we show that at 4 h after exposure of endothelial cells to supernatants from A/R-conditioned myocytes, the endothelial surface levels of E-selectin and ICAM-1 are increased. These observations indicate that endothelial cells develop a sustained proadhesive phenotype in response to substances generated by myocytes exposed to A/R. Taken together, our findings suggest that in vivo cardiac myocytes may contribute to the inflammatory response in the heart after I/R in two distinct phases. In an early phase (90 min or less), the myocytes produce substances that activate the endothelium and promote PMN emigration into the myocardial interstitium. The activated endothelial cells also set in motion a series of intracellular events that result in the induction of ICAM-1 and E-selectin expression over the next several hours. This proadhesive phenotype could contribute to a more prolonged inflammatory response.

The mechanisms involved in the generation of this proadhesive phenotype (increased expression of ICAM-1 and E-selectin) were not addressed in the present study. However, previous literature on this issue offers a viable explanation. Both E-selectin and ICAM-1 production by and expression on endothelial cells is regulated by NF-κB, which transactivates the genes encoding for E-selectin and ICAM-1 (1). Intracellular oxidants and PAF are common activators of NF-κB, promoting its translocation to the nucleus of endothelial cells (1, 27, 36). In accord with this, our findings indicate that catalase could largely prevent the increase in endothelial cell E-selectin and ICAM-1 surface levels induced by supernatants from A/R conditioned myocytes (Fig. 6). Thus, in the present study, the supernatants from A/R-conditioned myocytes could have activated endothelial cell NF-κB, resulting in an increased expression of both E-selectin and ICAM-1 on the endothelial cell surface.

Previous studies (2, 11) provided evidence that direct exposure of endothelial cell monolayers to A/R can induce an oxidant stress within the cells and increase surface levels of ICAM-1 and E-selectin. The magnitude of these endothelial cell responses was comparable with that reported in the present study by using supernatants from A/R-conditioned myocytes. Thus the relative contribution of myocytes to endothelial cell activation and the I/R-induced myocardial inflammation in vivo is unclear. One compounding variable limiting the application of in vitro observations to the situation in vivo is the Krogh model of P_{O_2} gradients from capillary to tissue. On the basis of the Krogh model, one would predict that during ischemia, anoxia would be induced earlier and be of longer duration in myocytes than in endothelial cells, because myocytes are further removed from the source of oxygen (blood capillaries). Conversely, on the basis of the same model, one would predict that reoxygenation during reperfusion would occur sooner in the endothelial cells than in the myocytes. The relative contribution of these two events to the overall response in vivo is unpredictable. Because endothelial cell activation in response to oxidants (e.g., H_2O_2) is dose dependent (7, 31), myocytes have the potential to contribute to the activation of endothelial cells during I/R. However, whether myocytes initiate the endothelial cell activation or amplify the endothelial cell activation is unclear. Irrespective of the role of the myocyte in endothelial cell activation, a chemotactic gradient must be generated within the interstitium to attract PMN into the tissue. Here, we provide evidence that cardiac myocytes have the potential to generate this chemotactic gradient.

In summary, we show that myocytes exposed to A/R can create an extracellular milieu, which promotes PMN transendothelial migration. This is accomplished by the production and release of oxidants and PAF by A/R-challenged myocytes. This chemotactic milieu can be generated within 30 min of reoxygenation and could conceivably contribute to the early inflammatory response in the myocardium after I/R in vivo. We further show that the PMN transendothelial migration in this situation is largely dependent on endothelial cell activation, specifically the generation of an intracellular oxidant stress within the endothelial cells. Finally, we show that endothelial cell activation results in the expression of a proadhesive phenotype (increased E-

selectin and ICAM-1 expression), which could prolong the inflammatory response. On the basis of these observations, it would seem prudent that any evaluation of the mechanisms involved in the PMN infiltration of the myocardium after I/R should take into account the potential contribution of cardiac myocytes to this phenomenon.

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