Brief murine myocardial I/R induces chemokines in a TNF-α-independent manner: role of oxygen radicals

TARECK O. NOSSULI,1 NIKOLAOS G. FRANGOGIANNIS,1 PASCAL KNUEFERNAN,M2 VENKATESH LAKSHMINARAYANAN,1 OLIVER DEWALD,1 ALIDA J. EVANS,1 JACQUES PESCHON,3 DOUGLAS L. MANN,2 LLOYD H. MICHAEL,1 AND MARK L. ENTMAN1

1Section of Cardiovascular Sciences and Cardiology, Department of Medicine, the DeBakey Heart Center, Baylor College of Medicine, and the Methodist Hospital; 2Veterans Administration Medical Center, Winters Center for Heart Failure Research, Houston, Texas 77030; and 3Immunex Corporation, Seattle, Washington 98101

Received 9 February 2001; accepted in final form 22 August 2001.

Nossuli, Tareck O., Nikolaos G. Frangogiannis, Pascal Knueferrman, Venkatesh Lakshminarayanan, Oliver Dewald, Alida J. Evans, Jacques Peschon, Douglas L. Mann, Lloyd H. Michael, and Mark L. Entman. Brief murine myocardial I/R induces chemokines in a TNF-α-independent manner: role of oxygen radicals. Am J Physiol Heart Circ Physiol 281: H2549–H2558, 2001.—Early chemokine induction in the area at risk of an ischemic-reperfused (I/R) myocardium is first seen in the venular endothelium. Reperfusion is associated with several induction mechanisms including increased extracellular tumor necrosis factor (TNF)-α, reactive oxygen intermediate (ROI) species formation, and adhesion of leukocytes to the venular endothelium. To test the hypothesis that chemokine induction in cardiac venules can occur by ROIs in a TNF-α-independent manner, and in the absence of leukocyte accumulation, we utilized wild-type (WT) and TNF-α double-receptor knockout mice (DKO) in a closed-chest mouse model of myocardial ischemia (15 min) and reperfusion (3 h), in which there is no infarction. We demonstrate that a single brief period of I/R induces significant upregulation of the chemokines macrophage inflammatory protein (MIP)-1α, -1β, and -2 at both the mRNA and protein levels. This induction was independent of TNF-α, whereas levels of these chemokines were increased in both WT and DKO mice. Chemokine induction was seen predominantly in the endothelium of small veins and was accompanied by nuclear translocation of nuclear factor-κB and c-Jun (AP-1) in venular endothelium. Intravenous infusion of the oxygen radical scavenger N-2-mercaptopropionyl glycine (MPG) initiated 15 min before ischemia and maintained throughout reperfusion obviated chemokine induction, but MPG administration after reperfusion had begun had no effect. The results suggest that ROI generation in the reperfused myocardium rapidly induces C-C and C-X-C chemokines in the venular endothelium in the absence of infarction or irreversible cellular injury.

Address for reprint requests and other correspondence: M. L. Entman, Section of Cardiovascular Sciences, Baylor College of Medicine, One Baylor Plaza, M/S F-602, Houston, TX 77030 (E-mail: mentman@bcm.tmc.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
of age, 18.0–22.0 g body wt) were anesthetized by an intraperitoneal injection of pentobarbital sodium, and the extremities were taped to a lead II electrocardiogram (ECG) board to measure S-T elevation during the I/R protocol. The skin above the chest wall was then reopened. For animals randomized to the myocardial ischemia (15 min) and reperfusion (3 h) groups, the left jugular vein was cannulated to allow infusion of either normal saline (WT mice, n = 9; DKO mice, n = 7) or the oxygen radical scavenger N-2-mercapto-]propanol (MPG) (WT mice, n = 9; DKO mice, n = 7). The intravenous infusion was started 15 min before the initiation of ischemia, and continued throughout both I/R periods. In another group of mice, the intravenous infusion of MPG was started 5 min after reperfusion, and continued for the rest of the experimental protocol. The 8-0 suture, which had been previously exteriorized outside the chest wall and placed under the skin, was cleared of all debris from the skin and chest, and was carefully taped to heavy metal picks. Ligation of the LAD was accomplished by gently pulling the heavy metal picks apart until an S-T elevation appeared on the ECG. The ECG was continuously monitored to confirm that the entire ischemic interval to ensure persistent ischemia. At the end of ischemia, 3 h of reperfusion was accomplished by pushing the metal picks towards the animal and by cutting the suture close to the chest wall. Reperfusion was confirmed by resolution of the S-T elevation, which usually occurred very quickly. During this procedure, the animal was not reintubated and breathed room air under normal ventilation. At the end of the experiment (i.e., 3 h of reperfusion), the chest was opened and the heart was immediately excised, snap-frozen, and stored at −80°C until mRNA isolation. Sham animals (10-day sham group) were prepared identically without undergoing the I/R protocol (n = 9, WT mice; n = 6, DKO mice).

Additional mice (n = 9 for both groups) were utilized as a control naïve heart group, to assess constitutive levels of MIP-1α, MIP-1β, and MIP-2. These mice were anesthetized with pentobarbital sodium, as was done for all other mice. With no prior instrumentation, these hearts were immediately excised, snap-frozen, and stored at −80°C until mRNA isolation, as described below.

Measurement of S-T elevation. The S-T elevation was measured in microvolts from baseline to the top of the T wave for each animal. Because there is no consistently distinguishable S-T segment in mice due to their extremely high heart rates, the S-T elevation was measured to the top of the T wave as a consistent approximation of the S-T segment. This was done at the end of 15 min of ischemia for the animals undergoing the I/R protocol. The S-T segment elevation was not significantly different among any groups of mice undergoing the I/R protocol.

MPG. MPG (Sigma; St. Louis, MO) is a low molecular weight synthetic analogue of glutathione that is freely diffusible across cell membranes (41), and has been found to scavenge oxygen-derived free radicals such as hydroxyl (49) and hydrogen peroxide (8, 21). The MPG solution was prepared fresh every morning before use as previously described (17). MPG, which is highly soluble in aqueous solutions, was dissolved in normal saline. Because an aqueous solution of MPG is highly acidic (pH ~2.0), the pH was adjusted to 7.4 by the addition of sodium hydroxide (0.1 N). Because MPG has a half-life in plasma of ~15 min (41), it had to be continuously infused intravenously throughout the entire experimental protocol, starting either 15 min before ischemia, or 5 min after reperfusion.

mRNA isolation. All solutions for RNA analysis were treated with 0.1% diethylpyrocarbonate and sterilized or prepared in diethylpyrocarbonate-treated water. Glassware
CHEMOKINES IN MURINE I/R

was baked at 240°C for 5 h to remove trace RNases. Total RNA was isolated from whole heart according to the acid guanidium thiocyanate-phenol-chloroform extraction method developed by Chomczynski and Sacchi (9). Briefly, whole hearts were homogenized in RNA STAT-60 solution (Tel-Test; Friendswood, TX). For RNA extraction, 0.2 volumes of R-chloroform were then added per volume homogenate. This mixture was incubated on ice for 15 min, and then spun at 12,000 g for 15 min at 4°C. The supernatant was transferred to another tube, and an equal volume of isopropanol was added for RNA precipitation overnight at 4°C. The tubes were then spun at 12,000 g for 15 min at 4°C, and the supernate was then decanted. The pellet was washed twice with 75% ethanol, briefly dried, and dissolved in 1% diethylpyrocarbonate-treated water. Quantification and purity of RNA was assessed by A260/A280 ultraviolet absorption, and RNA samples with ratios >1.9 were utilized for further analysis.

Ribonuclease protection assay and quantitation. The expression levels of MIP-1α, MIP-1β, MIP-2 mRNA, interferon-inducible protein (IP)-10 and monocyte chemoattractant protein-1 (MCP-1) were determined using a ribonuclease protection assay (RPA). A commercially available kit (Riboquant, Pharmingen; San Diego, CA) and antisense RNA probe were utilized (mck-5 probe, Pharmingen) according to the manufacturer’s protocol as previously described (34). Phosphorimaging of the RPA gels was performed with a Storm 860 phosphorimagerg (Molecular Dynamics). Signals were quantified using ImageQuaNT software and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Immunohistochemistry. For immunohistochemistry, mouse hearts from the control naive group, 10-day sham group, and the I/R groups (with and without MPG infusion) were isolated from both WT and DKO mice. After the experimental protocol, hearts were excised and immediately fixed in zinc formalin buffer (Anatech; Battle Creek, MI), dehydrated by incubation in increasing concentrations of ethanol with a standard protocol, and then embedded in paraffin; 4-μm sections were obtained with microtomy. For immunostaining, the slides were rehydrated and incubated with 3% hydrogen peroxide. After being rinsed in phosphate-buffered saline, sections were blocked from nonspecific antibody staining with 10% goat serum in phosphate-buffered saline. Immunostaining with antibodies to MIP-1α, MIP-1β, MIP-2, the p65 subunit of NF-κB, c-Fos, and c-Jun (all from Santa Cruz Biotechnology; Santa Cruz, CA), was performed with the Elite kit (Vector; Burlingame, CA), which has a peroxidase-based detection system. The color reaction was developed with a diaminobenzidine kit (Vector) as a substrate. For positive control of MIP-1α, MIP-1β, and MIP-2 staining, mouse spleen was utilized from an LPS-stimulated mouse according to the method of Rovai et al. (38). Endothelial cell labeling was performed using lectin histochemistry with Griffonia simplicifolia-I (Vector) as previously described (1). Sections from the spleen of a normal mouse were utilized as negative controls for chemokine staining.

Statistical analysis. All values are means ± SE. Statistical significance among control naive heart, 10-day sham, and the I/R groups (with and without MPG infusion) for MIP-1α, MIP-1β, and MIP-2 mRNA were analyzed by two-tailed analysis of variance with Bonferroni correction. Results were considered significant at \( P < 0.05 \).

RESULTS

I/R induced chemokine mRNA expression in WT mice. To determine chemokine expression in myocardial tissue, mRNA from whole hearts of WT mice was isolated in the control naive group (i.e., no instrumentation), 10-day sham group, and the 15-min ischemia and 3-h reperfusion groups (i.e., with and without MPG infusion). Figure 1A illustrates a representative autoradiograph of mRNA from each group of mice obtained by RPA of MIP-1α, MIP-1β, and MIP-2. The densitometric analysis of each mRNA normalized to GAPDH is shown in Fig. 1, B–D.

Figure 1B demonstrates that 15 min of ischemia and 3 h of reperfusion caused a marked upregulation of MIP-1α mRNA in the myocardium compared with both control (i.e., noninstrumented) and sham-operated groups (\( P < 0.001 \)). In addition, infusion of the oxygen radical scavenger MPG starting 15 min before ischemia and continuing throughout reperfusion caused a significant decrease in MIP-1α mRNA levels vs. I/R (\( P < 0.001 \)).

Figure 1C shows that 15 min of ischemia and 3 h of reperfusion caused a pronounced upregulation of MIP-1β mRNA compared with the control (i.e., noninstrumented) group (\( P < 0.001 \)), but not compared with the sham-operated group (not significant). As with MIP-1α, the MPG infusion caused a decrease of MIP-1β mRNA that came back down to control (i.e., noninstrumented levels; \( P < 0.05 \) vs. I/R).

Figure 1D demonstrates that 15 min of ischemia and 3 h of reperfusion significantly increased MIP-2 mRNA levels compared with both control (\( P < 0.001 \)) and sham-operated (\( P < 0.01 \)) groups. Similar to MIP-1α, infusion of the oxygen radical scavenger MPG caused a decrease of MIP-2 mRNA, which came back down to control levels (NS vs. control, and \( P < 0.05 \) vs. I/R).

The experiments with MPG described in Fig. 1 involved pretreatment of the animals with the ROI scavenger. We evaluated the efficacy of MPG in subsequent experiments by starting the MPG infusion 5 min after reperfusion. In contrast with pretreatment, MPG infusion initiated after reperfusion caused levels of MIP-1α, MIP-1β, and MIP-2 that were similar to those observed in the untreated I/R groups (data not shown, NS vs. I/R). Taken together, these data demonstrate that brief periods of ischemia cause a significant induction of chemokine mRNA, which require only short exposure to ROIs. Finally, permanent occlusion of 15 min or 3 h of ischemia did not induce measurable MIP-1α, MIP-1β or MIP-2.

I/R does not induce IP-10 or MCP-1. In contrast with MIP-1α, MIP-1β, and MIP-2 induction, there was no detectable induction of IP-10 or MCP-1 after 15 min of ischemia and 3 h of reperfusion in any of the experimental groups (data not shown).

Localization of chemokine protein in I/R. We then determined the cellular source of the chemokine upregulation observed in the various groups of WT mice. Figure 2, A–C, demonstrates immunolocalization of MIP-1α, MIP-1β, and MIP-2 protein to myocardial microvascular endothelial cells, identified by Griffonia simplicifolia-I lectin histochemistry (Fig. 2D), in animals subjected to 15 min of ischemia and 3 h of reperfusion. Staining for these chemokines was much reduced in sham-operated mice and in MPG-treated...
Control murine hearts showed minimal chemokine immunoreactivity.

**I/R induced chemokine mRNA expression in TNF-α DKO mice.** To determine chemokine expression in myocardial tissue, mRNA from whole hearts of TNF-α DKO mice was isolated in the control group (i.e., no instrumentation), 10-day sham group, and the 15 min of ischemia and 3 h of reperfusion groups (i.e., with and without MPG infusion). Figure 3A illustrates a representative autoradiograph of mRNA from each group of mice obtained by RPA of MIP-1α, MIP-1β, and MIP-2. The densitometric analysis of each mRNA normalized to GAPDH is shown in Fig. 3, B–D.

Figure 3B demonstrates that 15 min of ischemia and 3 h of reperfusion upregulated MIP-1α mRNA compared with control (P < 0.05) and sham-operated (P < 0.05) groups. MPG infusion initiated 15 min before ischemia, and, continuing throughout reperfusion, markedly reduced MIP-1α mRNA levels (P < 0.05 vs. I/R) to control values (NS).

Figure 3C shows marked upregulation of MIP-1β mRNA in the 15 min of ischemia and 3 h of reperfusion group compared with control (i.e., noninstrumented, P < 0.001), but not compared with the sham-operated group (NS). Similarly to MIP-1α, MPG infusion initiated 15 min before ischemia, and, continuing through-
out reperfusion blunted MIP-1β mRNA levels ($P < 0.01$ vs. I/R) to control values (NS).

Figure 3D indicates that 15 min of ischemia and 3 h of reperfusion caused a significant increase of MIP-2 mRNA compared with both control ($P < 0.01$) and sham-operated groups ($P < 0.05$). As was the case for both MIP-1α and MIP-1β infusion of MPG caused a marked downregulation of MIP-2 mRNA, which was not different than that of control (NS).

As with WT mice, when the MPG infusion was started 5 min after reperfusion, thus not quenching ROI production on reperfusion, the levels of MIP-1α, MIP-1β, and MIP-2 were comparable to those observed in the I/R groups (data not shown, NS). Taken to-
together, these data demonstrate that brief periods of ischemia, which induce neither irreversible cellular injury nor leukocyte infiltration, cause a marked increase in chemokine levels that are mediated by a short burst of oxygen radical production and are TNF-α independent.

**Localization of chemokine protein in DKO mice with I/R.** We then determined the cellular source of the chemokine upregulation observed in the groups of DKO mice. MIP-1α, MIP-1β, and MIP-2 protein were localized in venular endothelial cells in the mouse myocardium of the groups subjected to 15 min of ischemia and 3 h of reperfusion (data not shown), identical to the staining found in WT mice (Fig. 2, A-C). Similar to WT mice, staining for these chemokines was much reduced in sham-operated and MPG-treated mice. Control hearts showed minimal chemokine staining.

**Mechanism of oxygen radical-induced chemokine expression.** It is well known that nuclear translocation (activation) of the transcription factors NF-κB and AP-1 (i.e., c-Fos and c-Jun) are primary mechanisms for oxygen radical mediated upregulation of inflammatory mediators, and that many chemokines contain NF-κB and AP-1 sites in their promoters. Therefore,
we sought to determine the mechanism of TNF-α-independent chemokine production induced by oxygen radicals by staining sections of mouse myocardium with antibodies directed against NF-κB, c-Fos, and c-Jun. In WT mice, 15 min of ischemia and 3 h of reperfusion caused a marked nuclear translocation of NF-κB and c-Jun (Fig. 4, A and B), proteins in the same venular endothelial cells that demonstrated chemokine expression (Fig. 2). A similar pattern of staining was observed in DKO mice that underwent the I/R protocol (data not shown). NF-κB and c-Jun staining was virtually absent in WT mice receiving MPG infusion initiated 15 min before ischemia (Fig. 4, D and E). MPG infusion in DKO mice that were subjected to the I/R protocol also showed virtual absence of NF-κB, and c-Jun staining (data not shown). C-Fos was not stained in any of the groups. These data suggest that oxygen radicals modulate chemokine induction via NF-κB and AP-1 sensitive mechanisms.

**DISCUSSION**

In our previous work (22, 23) using a reperfused infarcted model in the dog, we demonstrated early induction of MCP-1 and IL-8 on reperfusion and that this induction occurred almost exclusively in the small venules of the myocardium. The observation that these chemokines could be induced in venular endothelium by TNF-α led us to hypothesize that TNF-α was responsible for this induction (22, 23). Subsequently, we noted that TNF-α activity in early reperfusion arose from preformed TNF-α in cardiac mast cells (12). This observation led us to question the role of TNF-α in chemokine induction because it was difficult to explain the very localized chemokine induction when the TNF-α release was quite general (present in cardiac extracellular fluid). The literature demonstrating a role for reactive oxygen in induction of chemokine synthesis (35, 42, 43, 46) led us to consider the alter-
native hypothesis that reactive oxygen might be responsible for this localized induction.

Previous work (4) in the area of chemokine induction had been done in an infarct model where leukocyte adhesion and transendothelial migration to the venules was occurring during early reperfusion and being driven, at least in part, by generation of C5a. Because leukocyte adhesion in early reperfusion occurs at the cardiac venular level, signals from these adherent leukocytes to the endothelium were also considered as possible candidates of endothelial gene induction. Another potential confounding problem in delineating the role of reactive oxygen was the fact that reactive oxygen scavengers may disrupt TNF-α signal transduction (7). We elected, therefore, to take advantage of the fact that short periods of coronary occlusion, followed by reperfusion, result in reactive oxygen formation (2, 5, 15), but in the absence of infarction. A significant ROI burst occurs during the first 5 min of reperfusion (2, 5, 27, 47, 48). In addition, substantial literature (17, 33, 41, 45) exists suggesting that free-radical scavengers can obviate the functional effects of reactive oxygen generated by short periods of ischemia, followed by reperfusion. These investigators demonstrated a significant reduction of infarct size when the animals were subjected to long periods of ischemia and marked improvement of myocardial function after a brief period of ischemia concomitant with a blunting of the ROI induction during I/R. Thus we elected to examine the role of a single short period of occlusion (i.e., 15 min), followed by reperfusion on chemokine induction and its potential transcriptional mechanisms in WT mice. To assess the role of reactive oxygen, we investigated the effect of the free-radical scavenger MPG that has been used in numerous experiments with I/R (6, 33, 45). In addition, to obviate the difficulty with regard to the potential effects of free-radical scavengers on TNF-α signal transduction, we repeated the experiments in a mouse model with genetic deletion of p55 and p75. We have utilized this model in the past to demonstrate the role of TNF-α as an anti-apoptotic molecule in similar experiments (24).

To examine the role of ROIs in chemokine induction, we utilized our previously described model of I/R in the mouse with a shorter occlusion time (i.e., 15 min) (30, 31). We soon found that the experiments had to be modified because of the high levels of chemokine induction in the sham animals. Therefore, we (34) developed a technique by which the animal could recover from surgical trauma resulting from the initial instrumentation, and I/R protocols could be carried out at later periods of time. In our study describing this model (34), we point out that failure to use this chronic model results not only in higher sham levels of cytokines compared with naïve hearts, but also in exaggerated responses of cytokine upregulation after an I/R insult. On a pragmatic basis, we chose 10 days of recovery as an ultimate period of time to reduce our shams to an acceptable level of chemokine expression.

The present study demonstrates induction of MIP-1α, MIP-1β, and MIP-2 utilizing RPA to assess mRNA levels (Figs. 1, A–D, and 3, A–D), and immunocytochemistry to demonstrate the presence of the protein confined to cardiac venules (Fig. 2). The chemokine induction correlated with histological demonstration of nuclear translocation of NFkB, c-Fos, and c-Jun in cardiac venules (Fig. 4). Induction of all three of these chemokines (i.e., MIP-1α, MIP-1β, and MIP-2), was suppressed by MPG infusion when it was begun before reperfusion and continued throughout the 3-h reperfusion time. Because there was some residual chemokine expression may be because MPG does not efficiently scavenge superoxide radicals. However, when MPG was initiated 5 min after reperfusion, it did not suppress any of these signals (NS vs. I/R). Myers et al. (33) have shown that MPG administration initiated after reperfusion resulted in loss of functional improvement of the myocardium after a single brief ischemic episode. This suggests that a short burst of reactive oxygen occurring immediately on reperfusion is necessary and sufficient to induce transcription of these three genes. Similar experiments were done in the TNF-α DKO mice with identical results. There was no statistical difference between the WT and DKO animals. It is to be noted that the MIP-1β induction consistently observed histologically was attended in RPA studies by a trend toward increased mRNA concentration, which was not statistically different than the sham-operated animals. However, MPG markedly suppressed the MIP-1β mRNA and protein expression. We presume that the failure to find statistical significance relates to a greater variability in the level of MIP-1β in sham-operated animals. The balance of the data appears to support induction of MIP-1β.

In our infarction studies in dogs (22, 23), we found that the chemokine induction was primarily in the endothelial cells of the venules, and this also appeared to be so in this noninfarction short occlusion model in mice. One of the potential mechanisms proposed for venular induction was cytokine or ROI secretion from leukocytes adhering to these venules after infarction (10). This noninfarction model suggests that venular chemokine induction is not dependent on leukocyte products because the absence of infarction obviates significant leukocyte infiltration into the reperfused myocardium (10). Interestingly, MCP-1 and IP-10 do not appear to be induced after a single brief ischemic insult in mice, whereas other C-C and C-X-C chemokines are upregulated. The reason for the difference in induction of these chemokines, despite containing similar NF-κB and AP-1 sites in their promoter (39, 51) is not exposed by the present data. On balance, these experiments support a critical role for reactive oxygen in induction of chemokines in the small venules even in the absence of infarction. These venules are strategically placed with regard to two known actions of the chemokines: 1) the postcapillary venular network is the site of transendothelial migration in the heart (16), and 2) angiogenesis begins in the venular endothelium (11).

Perhaps more intriguing is the potential role of chemokines as angiogenic agents under circumstances of
transient ischemia without tissue infarction. IL-8 has been demonstrated to be an angiogenic agent (20) and endothelial cells contain a CCR-2 receptor (32). Ito and co-workers (18) suggested that MCP-1 can also function in the induction of collateral circulation to ischemic skeletal muscle. Furthermore, Salcedo et al. (40) recently demonstrated that endothelial cells do indeed contain a CCR-2 receptor, which may be pertinent to its potential angiogenic role. A study by Weir and co-workers (50), which utilized a model of repeated short occlusions of dog coronary arteries over a 21-day period, demonstrates a significant increase in collateral circulation in a model in which no infarction occurred. It is interesting to speculate that ROIs may be one of the mediators of the formation of collateral circulation in a compromised vascular bed by virtue of their induction of chemokines in the postcapillary venules.

We thank Sharon Malinowski and Concepcion Mata for editorial assistance with the manuscript.

This work was supported by National Heart, Lung, and Blood Institute Grant HL-42550, the Medallion Foundation, and a grant from the Methodist Hospital Foundation (to N. G. Frangogiannis). T. O. Nossuli is a postdoctoral fellow supported by National Heart, Lung, and Blood Institute Grant T32 HL-07747-06.

REFERENCES


13. Frangogiannis NG, Mendoza LH, Lindsey ML, Ballantyne CM, Michael LH, Smith CW, and Entman ML. IL-10 is induced in the reperfused myocardium and may modulate the reaction to injury. *J Immunol* 165: 2798–2808, 2000.


