α_4-Integrin Mediates Neutrophil-induced Free Radical Injury to Cardiac Myocytes

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Abstract. Previous work has demonstrated that circulating neutrophils (polymorphonuclear leukocytes [PMNs]) adhere to cardiac myocytes via β_2-integrins and cause cellular injury via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme system. Since PMNs induced to leave the vasculature (emigrated PMNs) express the α_4-integrin, we asked whether (a) these PMNs also induce myocyte injury via NADPH oxidase; (b) β_2-integrins (CD18) still signal oxidant production, or if this process is now coupled to the α_4-integrin; and (c) dysfunction is superoxide dependent within the myocyte or at the myocyte–PMN interface. Emigrated PMNs exposed to cardiac myocytes quickly induced significant changes in myocyte function. Myocyte shortening was decreased by 30–50% and rates of contraction and relaxation were reduced by 30% within the first 10 min. Both α_4-integrin antibody (Ab)-treated PMNs and NADPH oxidase–deficient PMNs were unable to reduce myocyte shortening. An increased level of oxidative stress was detected in myocytes within 5 min of PMN adhesion. Addition of an anti–α_4-integrin Ab, but not an anti-CD18 Ab, pre-

vented oxidant production, suggesting that in emigrated PMNs the NADPH oxidase system is uncoupled from CD18 and can be activated via the α_4-integrin. Addition of exogenous superoxide dismutase (SOD) inhibited all parameters of dysfunction measured, whereas overexpression of intracellular SOD within the myocytes did not inhibit the oxidative stress or the myocyte dysfunction caused by the emigrated PMNs. These findings demonstrate that profound molecular changes occur within PMNs as they emigrate, such that CD18 and associated intracellular signaling pathways leading to oxidant production are uncoupled and newly expressed α_4-integrin functions as the ligand that signals oxidant production. The results also provide pathological relevance as the emigrated PMNs have the capacity to injure cardiac myocytes through the α_4-integrin–coupled NADPH oxidase pathway that can be inhibited by extracellular, but not intracellular SOD.

Key words: heart • neutrophils • integrins • free radicals • oxidant

Introduction

Neutrophils (polymorphonuclear leukocytes [PMNs][1]) infiltrate the postischemic myocardium and cause much of the myocardial dysfunction associated with this condition (Rossen et al., 1985; Dreyer et al., 1991; Hawkins et al., 1996). Therefore, much emphasis has been placed on preventing PMN recruitment in an attempt to minimize myo-

cardial injury. Reduction of circulating PMN numbers with anti-PMN antibodies (Abs; Romson et al., 1983) or PMN depletion filters (Engler et al., 1986) reduced infarct size in hearts challenged by ischemia-reperfusion. Reducing the recruitment of PMNs by immunosuppression of adhesion molecules also limited myocardial injury (Lefer et al., 1993, 1996; Tamiya et al., 1995; Zhao et al., 1997). Furthermore, experiments with complement depletion (Maroko et al., 1978) and lipoxygenase inhibitors (Shappell et al., 1990a), aimed at reducing PMN chemotactic factors, limited infarct size. Clearly, preventing the recruitment of PMNs into myocardial tissue reduces injury. However, once the PMNs leave the microvasculature, the molecular interactions are less well understood. Although antiadhesion therapy within the vasculature has received much at-

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[1]*Abbreviations used in this paper:* Ab, antibody; DCFH, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate di(acetoxymethyl ester); KO, knockout; NADPH, nicotinamide adenine dinucleotide phosphate; PMN, polymorphonuclear leukocyte; SOD, superoxide dismutase; WT, wild-type; ZAP, zymosan-activated plasma.

tention, patients with postischemic reperfusion already have PMN infiltration in the heart. This, coupled with the fact that emigrated PMNs in contact with cardiac myocytes are at least in part the injurious cells, emphasizes the importance of studying PMN events outside the vasculature. In fact, direct molecular contact between a PMN and myocyte is absolutely required before myocyte dysfunction can occur (Entman et al., 1990; Poon et al., 1999). Therefore, understanding the molecular detail of PMN–myocyte interactions may be key to effective therapeutic intervention for this common cardiovascular condition.

In 1995, it was demonstrated that human PMNs that transmigrated across endothelium expressed increased surface levels of α_4-integrin (Kubes et al., 1995). More recently, both rat (Reinhart et al., 1997) and murine (Poon et al., 1999) emigrated PMNs were also shown to express increased levels of α_4-integrin. Subsequent studies showed that the α_4-integrin was able to support adhesion of emigrated PMNs to cardiac myocytes, even after inhibition of the β_2-integrin, which is the dominant adhesion molecule on circulating PMNs (Reinhart et al., 1997). After adhering to the cardiac myocyte, emigrated PMNs reduced the contractile responses of the myocytes to electrical stimulation (Poon et al., 1999). Although in vivo inhibition of known PMN products, including oxygen free radicals, proteases, and arachidonic metabolites, reduces the extent of myocardial injury (Bednar et al., 1985; Simpson et al., 1987; Ma et al., 1992), to date the mechanisms by which emigrated PMNs impair myocyte function are unclear.

In seminal studies, Smith and colleagues showed that circulating PMNs adhered to cardiac myocytes via β_2-integrin (CD18) and then released oxidants which penetrated the myocyte, causing intracellular oxidative stress and myocyte death (Shappell et al., 1990b; Smith et al., 1991; Entman et al., 1992). The release of oxidants was entirely dependent on CD18, inasmuch as inhibition of this molecule with a monoclonal Ab prevented both the release of oxidants by PMNs and the subsequent myocyte death. However, based on the fact that emigrated PMNs use the α_4-integrin to bind to cardiac myocytes and these are in fact the cells (as opposed to circulating PMNs) that make contact with myocytes, we asked the following questions. (a) Are oxidants still the damaging molecules, even though emigrated PMNs are more apt to degranulate and release numerous proteins (Zimmerli et al., 1986)? (b) Does the β_2-integrin still signal oxidant production or is this very important function now replaced by the newly expressed α_4-integrin? (c) Is superoxide radical (O_2^-) responsible for the ensuing myocyte injury, and can O_2^- and the dysfunction be inhibited within the myocyte or at the PMN–myocyte interface? In this study, each of these issues was addressed in a murine model where emigrated PMNs were coincubated with isolated ventricular myocytes from transgenic and knockout (KO) mice.

Materials and Methods

All experimental protocols were reviewed and approved by the University of Calgary Animal Resource Center.

Unloaded Cell Shortening Experiments

Unloaded cell shortening measurements were recorded with isolated murine ventricular myocytes and glycogen-elicited emigrated PMNs as described previously (Poon et al., 1999). In brief, myocytes were electrically stimulated at threshold voltage plus 10% and baseline unloaded cell shortening measured. Emigrated PMNs (10^6), prestimulated with 1% zymosan-activated plasma (ZAP), were then added and allowed to adhere to the myocytes, and cell shortening was measured for 10 min. An α_4-integrin Ab (R1-2, 10 μg/ml; BD PharMingen) was added to PMNs to ensure that impaired myocyte function was related to this integrin.

To evaluate the role of oxidants, emigrated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase–deficient PMNs (10^6) were isolated from mice with a nonfunctional allele for the gp91^{phox} subunit of the cytochrome b (NADPH oxidase KO mice). PMNs from these mice are completely unable to generate O_2^- or hydrogen peroxide (H_2O_2; Pollock et al., 1995). Myocyte cell shortening measurements were performed in the presence of either wild-type (WT) or NADPH oxidase–deficient emigrated PMNs (pretreated with 1% ZAP). In a second series of experiments, myocytes were isolated from transgenic mice overexpressing endogenous Cu/Zn-superoxide dismutase (SOD; Epstein et al., 1987). Myocytes isolated from these mice show a 10-fold increase in intracellular SOD expression (Wang et al., 1998). Cell shortening measurements were performed in myocytes from WT (C57BL6) or SOD overexpressing transgenic mice in the presence and absence of WT emigrated PMNs (10^6). Another series of cell shortening experiments was performed with exogenous SOD (from bovine erythrocytes, 300 U/ml; Sigma-Aldrich) to determine if extracellular SOD could alter significantly the emigrated PMN-induced myocyte injury. In each experiment the number of PMN–myocyte interactions was recorded. In no case was a myocyte recorded unless it had adherent PMNs throughout the experiment.

Cytochrome c Reduction Assay

A cytochrome c reduction assay was used to measure the production of O_2^- from suspensions of emigrated PMNs. In brief, PMNs (10^7/ml) were added to two cuvettes containing PBS with CaCl$_2$ (1.19 mM), MgCl$_2$ (0.54 mM), and cytochrome c (1.5 mM; Sigma-Aldrich). In one sample, SOD (from bovine erythrocytes, 264 U/ml; Sigma-Aldrich) was added and both samples were read at the same time in a spectrophotometer (model U-2000; Hitachi) at 550 nm. Optical density differences between the two samples were recorded on an online chart recorder (Johns Scientific, Inc.). After 5 min of baseline measurements, 1% ZAP was added to both samples and optical density was recorded for an additional 10 min.

Single Cell Imaging for Oxidant Production

Isolated ventricular myocytes and emigrated PMNs were loaded with fluorescent probe, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate di(acetoxymethyl ester) (DCFH, 1 μM for myocytes and 10 μM for PMNs; Molecular Probes) in Tyrode's buffer with probenecid (0.5 mM; Sigma-Aldrich) for 15 min at room temperature. DCFH is oxidized to highly fluorescent 2′,7′-dichlorofluorescein in the presence of free radicals. DCFH is not specific for any one oxidant and therefore can only be used as an overall indicator of oxidative stress within the cell (LeBel et al., 1992; Carter et al., 1994; Zhu et al., 1994; Murrant et al., 1999). Myocytes were allowed to adhere to a glass coverslip (which was sealed by vacuum grease to the bottom of a plastic stage chamber) for 5 min at room temperature. The chamber was clipped into a machined stage platform on an Axiovert-135 inverted microscope (ZEISS) equipped with an oil immersion FLUAR 100×/1.3 objective for single cell imaging (Murphy, 1999). A Delta-Ram high speed illuminator (Photon Technologies International), which consists of a 75-W Xenon arc and a computer-controlled random-access wavelength monochromator, provided excitation light. Wavelengths were further selected before cell illumination by a dichroic filter (Chroma Technology Corporation) mounted on a sliding apparatus under the objectives. The cells were excited at 480 nm and emission was recorded at 510 nm. ImageMaster v1.4 software (Photon Technologies International) allowed direct control of the camera, illumination, and data acquisition. Digital images of emissions from selected fields were saved to computer disk in sequential order for off-line analysis.

To ensure that the relaxed, evenly striated myocytes were indeed healthy, single cell fluorescence intensities were recorded every 10 s for 5 min. Cells exhibiting an increase in fluorescence of >10 raw intensity units were assumed to be unhealthy and were excluded from the study. Emigrated PMNs (10^6), prestimulated with 1% ZAP, were added to the myocytes and fluorescence intensities were recorded every 10 s for 10 min. Phase–contrast photos of the myocytes with adherent PMNs were recorded digitally. At the end of each experiment, H$_2$O$_2$ (50 μM; Fisher Scientific) was added to the cells to ensure adequate loading of the cells with DCFH. Upon addition of the H$_2$O$_2$, the fluorescent signal from all myocytes included in this study resulted in saturation of the sensor (255 raw intensity units).

Oxidative stress was first characterized in myocytes in the presence and absence of emigrated PMNs where both cell types were from WT animals. In addition, oxidative stress was measured in WT myocytes exposed to NADPH oxidase–deficient PMNs. WT myocytes and PMNs were also coincubated in the presence of exogenous SOD (300 U/ml) and myocytes from SOD overexpressing mice were exposed to WT PMNs. Finally, to further explore whether the oxidative stress detected within myocytes induced by emigrated PMNs could be modulated by adhesion molecules, WT myocytes and PMNs were coincubated with either an anti-CD18 Ab (2E6, 8 μg/ml; Endogen) or an anti-α_4-integrin Ab (R1-2, 10 μg/ml; BD PharMingen).

Integrin Expression on PMNs

Flow cytometry was used to measure the expression of CD11b/CD18 and α_4-integrin on circulating and emigrated PMNs. Circulating or emigrated murine PMNs were stimulated with ZAP (10 min at room temperature) and then washed. Red blood cells were lysed and PMNs were simultaneously fixed in 1% formalin (15 min at room temperature) and then washed. Primary Abs directed against their respective adhesion molecules (CD11b, MK/170, 0.25 μg per tube; α_4-integrin, R1-2, 1 μg per tube) were added. After 30 min at room temperature, cells were washed and labeled with FITC-conjugated goat anti–rat IgG (Cedar Lanes Laboratories, Ltd.) for α_4-integrin and FITC-conjugated goat anti–hamster IgG (Caltag Laboratories) for CD11b. After 30 min at room temperature, cells were washed and fluorescence was measured on a FACScan™ flow cytometer (Becton Dickinson). Appropriate controls (no primary, an isotype control, and no secondary Ab) were completed for each set of experiments.

Immunogold electron microscopy was also performed on emigrated PMNs stimulated with 1% ZAP and labeled with either the rat anti–mouse α_4-integrin Ab (R1-2, 10 μg/ml) or isotype-matched control Ab (rat IgG2b, 10 μg/ml) for 10 min at room temperature. After Ab labeling, PMNs were fixed in PBS containing 0.05% glutaraldehyde for 10 min at room temperature, blocked in PBS containing 5% fetal calf serum (PBS-FCS) for 30 min, and then labeled with secondary goat anti–rat IgG conju-

gated to 12 nm colloidal gold (1:5 dilution in PBS-FCS; Jackson ImmunoResearch Laboratories) for 60 min at room temperature. The cells were then transferred to sodium cacodylate (0.05 M) buffer, refixed in 1% glutaraldehyde for 60 min, and postfixed in sodium cacodylate containing 1% tannic acid (5 min) just before osmium postfixation. This was followed by en bloc uranyl acetate staining (60 min), dehydration through ethanol, and embedding in LX112 resin (Polysciences, Inc.). Ultrathin sections (80 nm) were stained with uranyl acetate and lead citrate and viewed on a JEOL 200 CX electron microscope.

To quantitate α_4-integrin labeling, leukocyte profiles were viewed at 200,000× magnification and the numbers of gold particles associated with the cell surface were counted. Cells examined included PMNs ($n = 20$ for α_4-integrin and $n = 10$ for control IgG2b), monocytes/macrophages ($n = 8$ for α_4-integrin and $n = 9$ for control IgG2b), and lymphocytes ($n = 4$ for α_4-integrin and $n = 3$ for control IgG2b). Gold particle counts were expressed as number of gold particles per cell profile.

Statistical Analysis

All data are expressed as the arithmetic mean ± SEM. Data were compared between treatment groups using an ANOVA of raw data with the Dunnett method for multiple comparisons between groups. Values of $P < 0.05$ are considered statistically significant.

Results

Emigrated PMNs Decrease Cell Shortening via α_4-Integrin

Fig. 1 A shows the pattern of shortening of a myocyte electrically stimulated at 1 Hz and at a strength 10% above threshold voltage. The cell shortens 14 μm, or 12% of its
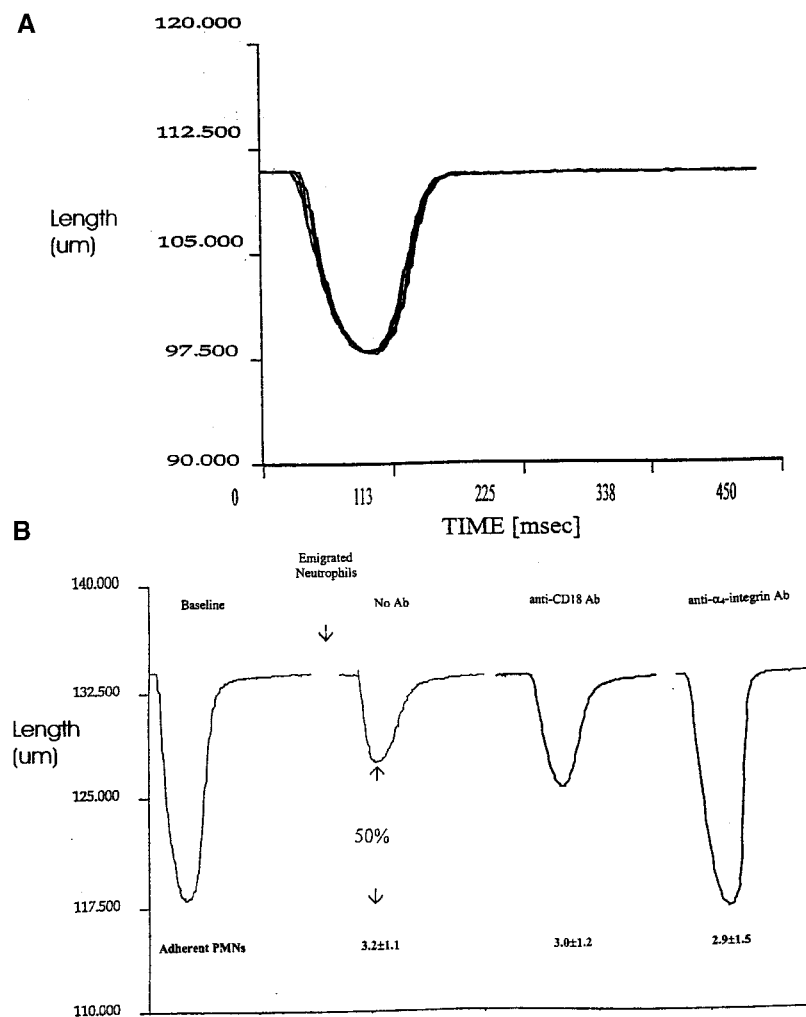

Figure 1. (A) Representative unloaded cell shortening traces from a murine ventricular myocyte which was paced at threshold voltage +10%. (B) Recordings of unloaded cell shortening of a myocyte at baseline and in the presence of emigrated PMNs with and without anti-CD18 (2E6, 8 μg/ml) or anti–α_4-integrin Ab (R1-2, 10 μg/ml) at 5 min.
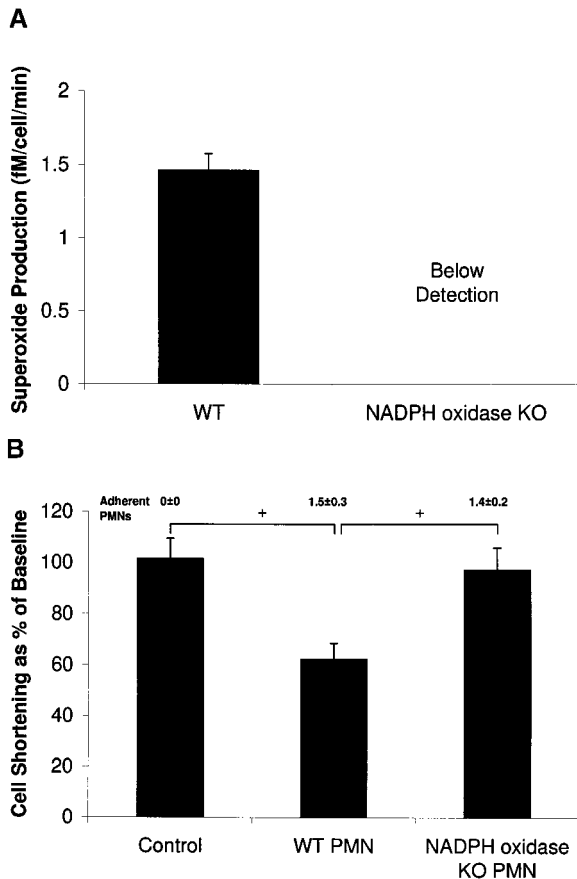
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Figure 2. (A) Superoxide production in PMNs from WT ($n = 4$) and NADPH oxidase KO mice ($n = 2$). (B) Cumulative unloaded cell shortening in control (no PMNs, $n = 6$; WT PMNs, $n = 7$; and NADPH oxidase KO PMNs, $n = 6$) at 5 min. $+$, $P < 0.05$ between indicated groups.

resting cell length, and then relaxes in the expected, reproducible pattern. The responses over a 10-min period of field stimulation remain completely unchanged (multiple overlapping traces), suggesting no decline in function with time. Fig. 1 B shows a representative record of cell shortening, where the addition of emigrated PMNs reduced cell shortening by 50% and this decrease was not prevented by an anti-CD18 Ab, but was completely inhibited in the presence of the anti-α_4-integrin Ab.

The number of adherent PMNs to myocytes is also provided in Fig. 1 B. Approximately three PMNs adhered per myocyte. Addition of either an anti-CD18 Ab alone or an anti-α_4-integrin Ab alone did not reduce the number of adherent myocytes. Tandem administration prevents PMN–myocyte interactions (data not shown). Although the function of a myocyte was not studied unless at least one adherent PMN was present, it is important to note that cellular dysfunction was very similar regardless of the number of PMNs that adhered. A single PMN could induce the same amount of myocyte dysfunction as multiple PMNs.

Emigrated PMNs Induce Ventricular Myocyte Dysfunction via Reactive Oxidants

Emigrated PMNs from WT mice generated O_2^- levels of 1.46 ± 0.12 fM/cell/min ($n = 4$). This level is approximately half of the levels produced by maximally stimulated human
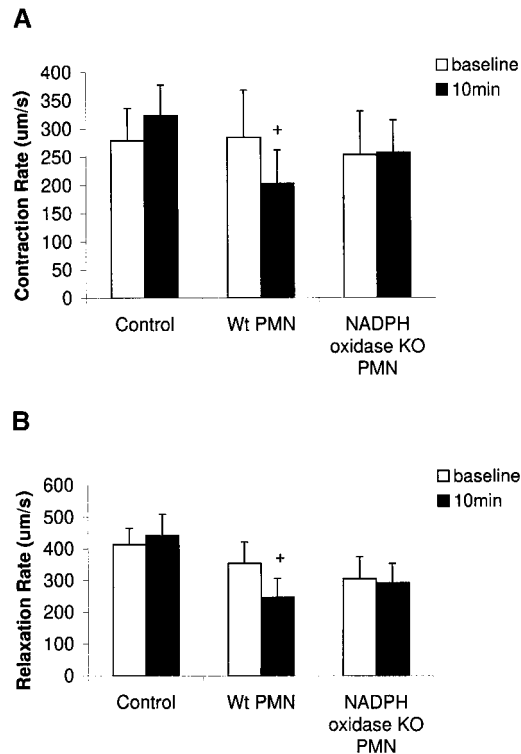
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B

Figure 3. Change in rates of (A) contraction ($+dP/dt$) and (B) relaxation ($-dP/dt$) in myocytes under baseline conditions after 10 min in control (no PMNs, $n = 6$; WT PMNs, $n = 7$; and NADPH oxidase KO PMNs, $n = 6$). $+$, $P < 0.05$ relative to respective baseline.

circulating PMNs (West et al., 1983). Emigrated PMNs from NADPH oxidase–deficient mice did not produce free radicals. Results showed O_2^- levels below the detectable limits of the assay in these cells ($n = 2$) (Fig. 2 A). Cumulative cell shortening data are shown in Fig. 2 B. Unloaded cell shortening remains at baseline at 5 min in controls ($n = 6$). The addition of emigrated PMNs to the myocyte caused ~40% reduction in cell shortening within 5 min ($n = 7$, $P < 0.05$). This result was observed regardless of whether one or many PMNs adhered to the myocyte. In contrast, PMNs from NADPH oxidase–deficient mice did not alter cell shortening patterns ($96.8 \pm 8.9\%$ of baseline, $n = 6$, $P < 0.05$ compared with PMN-only group). There was no difference in adhesion efficiency to cardiac myocytes between WT and NADPH oxidase–deficient PMNs, as shown in the inset. Leaving the NADPH oxidase–deficient PMNs bound to the myocytes for even 10 min still revealed no impairment (data not shown).

Data summarizing the maximal rates of contraction and relaxation of myocytes within these groups are shown in Fig. 3. The maximal rate of contraction and relaxation remained constant in the control group throughout the entire 10-min experimental period ($n = 6$, $P = $ NS). The addition of WT emigrated PMNs decreased both contraction and relaxation rates by ~30% from baseline at 10 min ($n = 6$, $P < 0.05$). It is noteworthy that changes in the rate of contraction and relaxation did not occur at 5 min of PMN exposure, the time at which a decrease in cell shortening was observed. PMNs from NADPH oxidase–deficient mice did not alter either contraction or relaxation rates in these myocytes. Late in most experiments, after exposure
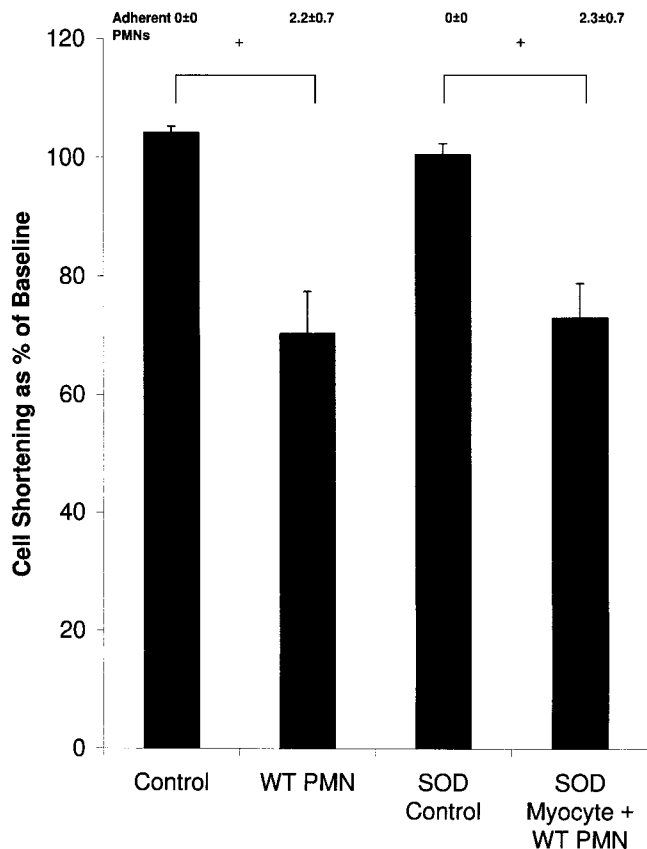
Figure 4. Unloaded cell shortening of control (WT myocyte only, $n = 4$), WT PMNs (WT Myocyte + WT PMN, $n = 4$), SOD control (SOD myocyte only, $n = 4$), and SOD Myocyte + WT PMNs ($n = 5$) at 5 min. +, $P < 0.05$ between indicated groups.
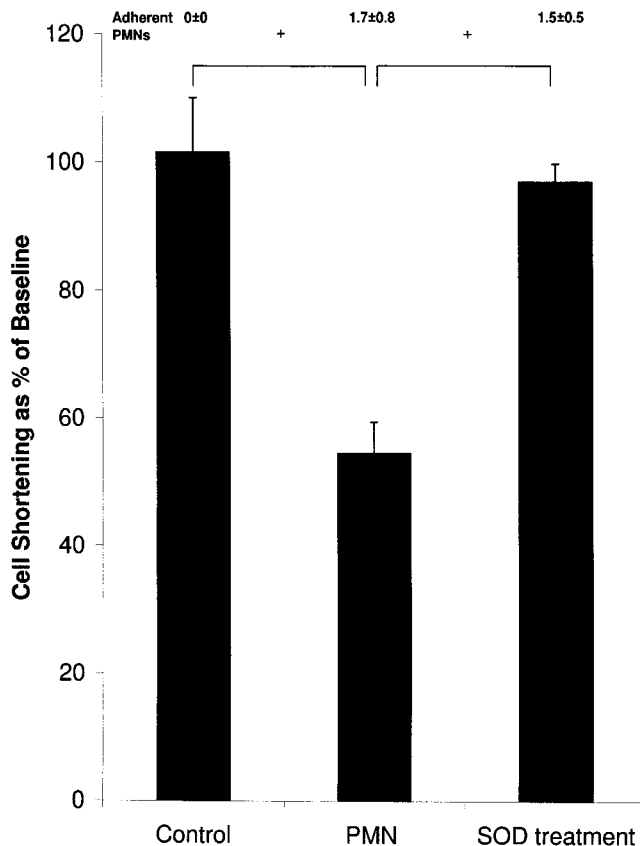

Figure 5. Unloaded cell shortening for control (no PMNs, $n = 4$), PMNs (PMN only, $n = 4$), and SOD treatment (PMN + SOD 300 U/ml, $n = 3$) at 5 min. +, $P < 0.05$ between indicated groups.

to PMNs, myocytes also became dysrythmic. Specifically, control myocytes alone showed no myocyte dysrythmia ($n = 6$), but the addition of WT PMNs caused five of the seven myocytes recorded to become dysrythmic. When PMNs from NADPH oxidase–deficient mice were added to the myocytes, only one of the six myocytes in this group showed any dysrythmia.

SOD Inhibits Dysfunction at the PMN–Myocyte Interface

First, we examined whether intracellular SOD could prevent the myocyte dysfunction. Patterns of unloaded cell shortening in ventricular myocytes isolated from SOD overexpressing mice are shown in Fig. 4. There was no difference in maximal cell shortening between WT and SOD over-expressing myocytes under baseline conditions ($n = 4$, $P = $ NS). The addition of WT emigrated PMNs caused ∼30–40% reduction in cell shortening at 5 min in both of these myocyte groups. Prolonged exposure of both WT and SOD myocytes to PMNs for 10 min revealed the same pattern of dysfunction (data not shown). The addition of WT PMNs caused ∼50% reduction in contraction and relaxation rates in both WT and SOD overexpressing myocytes (data not shown). One important difference between WT and SOD overexpressing myocytes was a lesser propensity for dysrythmia in the latter. Specifically, although there was no dysrythmia recorded for WT or SOD myocytes alone, the addition of WT PMNs caused dysrythmia

in three of the four WT myocytes, but only one of the five SOD myocytes. This could not be explained by differences in the number of adherent PMNs. As shown in Fig. 4, the same number of PMNs adhered to myocytes regardless of the magnitude of intracellular SOD.

Next, we examined whether exogenous application of SOD could affect unloaded cell shortening (Fig. 5). Control myocytes (no PMNs) maintained cell shortening at baseline levels after 5 min ($n = 4$). The addition of PMNs caused a 45% reduction in cell shortening in the absence of SOD ($n = 4$, $P < 0.05$). The addition of exogenous SOD protected the myocytes from injury (cell shortening at 97 ± 3% of baseline at 5 min, $n = 3$, $P < 0.05$). Addition of exogenous SOD to the emigrated PMNs did not affect their adhesion efficiency to cardiac myocytes, as shown in the inset. A similar pattern of cell shortening was observed at 10 min of PMN exposure (data not shown).

Control myocytes maintained both contraction and relaxation rates at baseline levels throughout the 10-min experimental period (data not shown). The addition of PMNs alone caused a 40% reduction in contraction rate and a 32% reduction in relaxation rate at 10 min. Exogenous SOD prevented all PMN-induced decreases in contraction and relaxation rates. There was no dysrythmia recorded for myocytes alone (baseline conditions). The addition of PMNs caused dysrythmia in all of the myocytes studied. When exogenous SOD was present, none of the myocytes recorded showed dysrythmia.
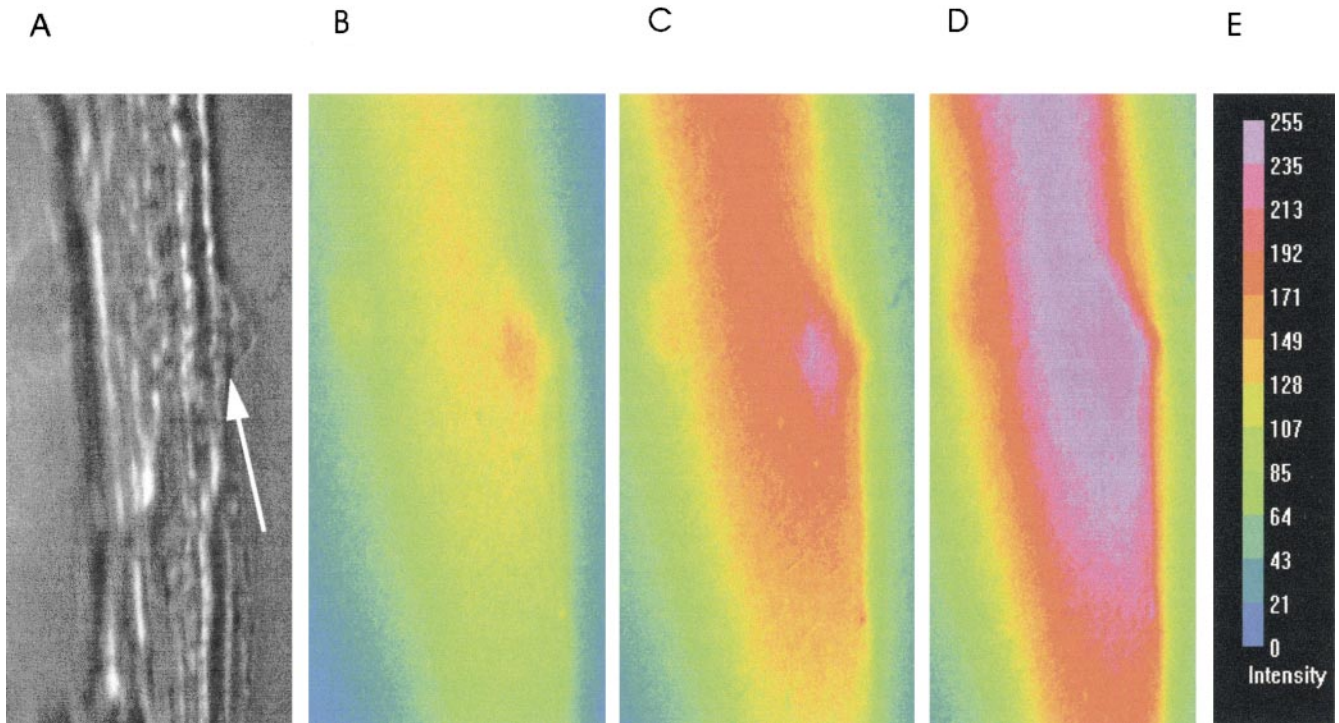
Figure 6. Representative fluorescence images of an adherent WT PMNs (arrow) to a WT myocyte. (A) Phase–contrast photo, (B) immediately after adhesion of PMNs to the myocyte, (C) after 5 min of adhesion, (D) after 10 min of adhesion, and (E) color bar.

NADPH Oxidase Is Coupled to the α_4-Integrin

Fig. 6 consists of a phase–contrast image of a WT PMN adhering to a myocyte, where both cells are loaded with DCFH as an indicator of overall oxidative stress within the cells. Fluorescent images at baseline, 5 min, and 10 min of PMN exposure and a transmission image of the cells are shown. Fluorescence in the ventricular myocyte is first detected at the point of PMN attachment. With time, it then spreads throughout the myocyte. Fig. 7 A shows fluorescence intensity changes at 5 min after PMN exposure. Control myocytes showed a minimal increase in fluorescence (4.4 ± 0.7 raw intensity units above baseline, $n = 5$). In contrast, the addition of WT PMNs caused a fivefold increase in fluorescence intensity over myocyte-only controls ($n = 4$, $P < 0.05$). However, PMNs deficient in NADPH oxidase did not increase fluorescence levels above controls ($n = 5$, $P = $ NS from control). Similar patterns were also seen at 10 min (data not shown).

In the presence of exogenous SOD, WT emigrated PMNs could not induce an increase in fluorescence (Fig. 7 B). Indeed, fluorescence levels of myocytes increased from 4.8 ± 0.5 in controls ($n = 4$) to 36.0 ± 3.6 in the presence of PMNs at 5 min ($n = 4$, $P < 0.05$). The addition of exogenous SOD completely inhibited this PMN-induced increase in fluorescence ($n = 4$, $P < 0.05$ compared with PMN-only group). Similar patterns were also seen at 10 min (data not shown). In complete contrast, 10-fold higher levels of intracellular SOD within SOD transgenic myocytes did not reduce the PMN-induced increase in fluorescence (Fig. 7 B).

Fluorescence intensity changes of myocytes in the presence or absence of Abs are shown in Fig. 8. Myocyte controls show minimal increase in fluorescence (5.2 ± 1.8 raw intensity units above baseline, $n = 5$). The addition of em-

igrated PMNs caused fluorescence levels to increase to 31.7 ± 9.1 intensity units above baseline ($n = 6$, $P < 0.05$). Addition of an anti-CD18 Ab still resulted in at least a threefold increase in fluorescence over controls ($n = 6$, $P = $ NS compared with PMN-only group). However, addition of an anti–α_4-integrin Ab reduced fluorescence back to control levels (5.3 ± 2.0 units above baseline, $n = 4$, $P < 0.05$ compared with PMN-only group). Similar patterns were also seen at 10 min (data not shown).

Circulating PMNs were able to cause dysfunction once bound to myocytes, but both the PMN adhesion and therefore, not surprisingly, the dysfunction were completely inhibited by CD18 Ab (data not shown).

Emigrated PMNs Express α_4-Integrin

Fig. 9 summarizes the degree of α_4-integrin expression on circulating versus emigrated PMNs. The data reveal a significant rightward shift of α_4-integrin expression after emigration. Fig. 9 also shows CD11b expression on the surface of emigrated PMNs and demonstrates high levels of fluorescence. Although a direct quantitative comparison cannot be made between the two adhesion molecules (due to potentially different affinities of the Abs), the data do suggest a much larger amount of surface CD11b than α_4-integrin.

To further examine the amount of α_4-integrin on the surface of PMNs, immunogold staining was performed and the results show that 0.8 ± 0.2 gold particles/cell profile (80 nm thick) were consistently found on PMNs, whereas monocytes had approximately five times as much α_4-integrin (4.3 ± 0.9 gold particles/cell profile) and lymphocytes had approximately six times as much α_4-integrin (5.3 ± 2.6 gold particles/cell profile). This clearly demonstrates that lymphocytes and monocytes do express significantly more α_4-
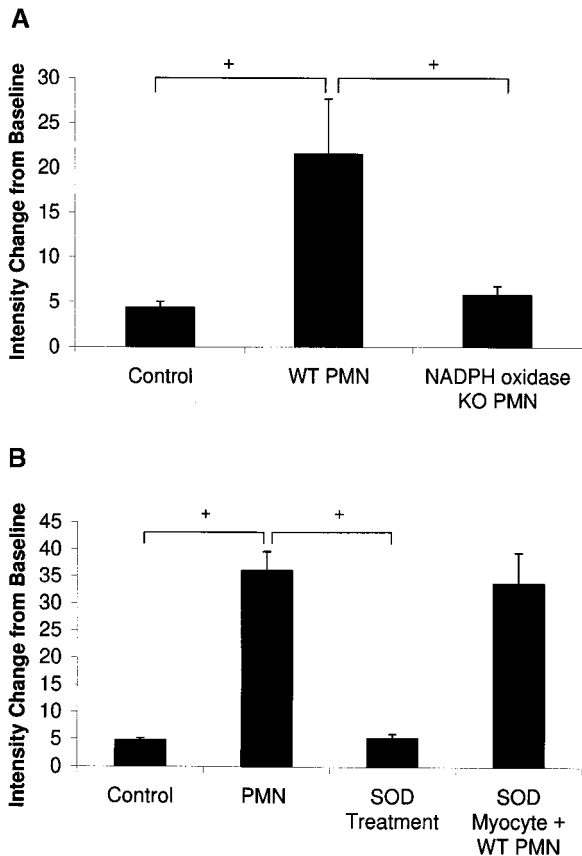
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Figure 7. (A) Change in fluorescence intensity from baseline for control (no PMNs, $n = 5$), WT PMNs ($n = 4$), and NADPH oxidase KO PMNs ($n = 5$) at 5 min. +, $P < 0.05$ between indicated groups. (B) Change in fluorescence intensity from baseline for control (no PMNs, $n = 4$), PMNs (PMN only, $n = 4$), SOD treatment (PMN + SOD 300 U/ml; $n = 4$), and SOD Myocyte + WT PMNs ($n = 4$) at 5 min. +, $P < 0.05$ between indicated groups.

Figure 8. Change in fluorescence intensity from baseline for control (no PMNs, $n = 5$), PMNs (PMN only, $n = 6$), CD18 Ab (PMN + anti-CD18 Ab 2E6, 8 μg/ml, $n = 6$), and Alpha4 Ab (PMN + anti-α_4 Ab R1-2, 10 μg/ml, $n = 4$) at 5 min. +, $P < 0.05$ between indicated groups.

integrin than PMNs. Nevertheless, in the nine PMNs examined with an isotype-matched control Ab, we observed no gold particles bound to the surface. In fact, in the 22 cells examined only 1 cell (a macrophage) had nonspecific binding (1 gold particle), suggesting that there is significant α_4-integrin on PMNs. The presence of this molecule on the PMN surface is consistent with the view that this molecule could mediate signaling of oxidant production.

Discussion

Previous work from our laboratory has shown that emigrated murine PMNs express the α_4-integrin and use this ligand to mediate PMN-induced myocyte damage (Poon et al., 1999). The present results extend these findings by showing, for the first time, that emigrated PMNs impair the function (cell shortening) of cardiac myocytes through an α_4-integrin–coupled free radical pathway. Adherent emigrated PMNs, isolated from NADPH oxidase–deficient mice, had no affect on the contractile responses of the myocytes. Furthermore, the number of myocytes that displayed signs of dysrythmia was dramatically reduced when the PMNs could not generate free radicals. Although all mammalian cells produce some antioxidants, the PMN has the capacity to overwhelm this antioxidant
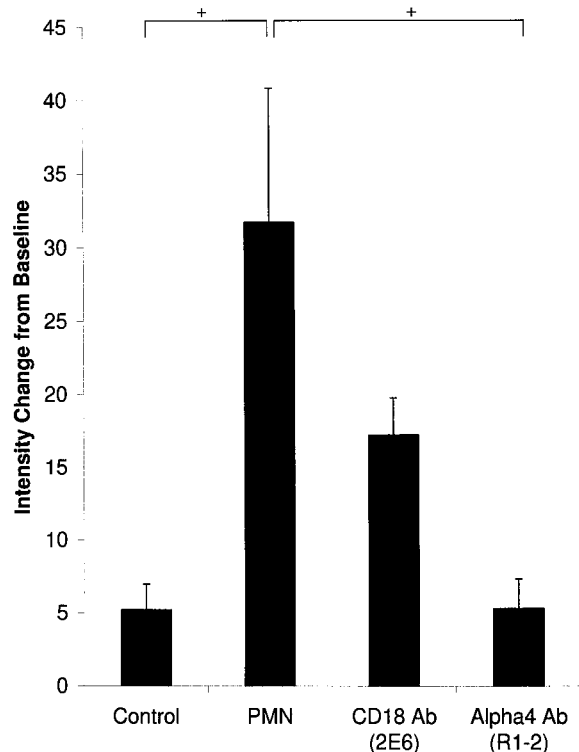
"shield" and quickly induce severe cellular injury. In this study, we have found that overexpressing intracellular SOD in the myocyte was not sufficient to protect the cell from PMN-induced reductions in cell shortening, contraction, or relaxation rate. However, addition of extracellular SOD entirely prevented these negative inotropic effects.

Our work demonstrates that the α_4-integrin is coupled to free radical production by emigrated PMNs. The addition of an anti–α_4-integrin Ab completely inhibited oxidant production (Fig. 8). That is, oxidant levels were at control levels (myocyte only) even though PMNs continued to adhere to the myocyte via CD18. Previous work had suggested that a circulating PMN binds to endothelium via CD18 and this event induces oxidant production (Smith et al., 1991). Our previous work showed that immunosuppression of the α_4-integrin, but not CD18, inhibited all parameters of emigrated PMN-induced myocyte dysfunction (Poon et al., 1999) and, in this study, inhibited oxidative stress. Taken together, these data suggest that after the PMN emigrates out of the vasculature, the CD18-NADPH oxidase pathway becomes uncoupled and thereafter oxidant production requires the α_4-integrin. These results provide the mechanistic evidence to suggest that α_4-integrin on PMNs couples to NADPH oxidase to induce myocyte dysfunction.

It is well documented that integrins are involved in cell signaling events. Current models postulate that when ligands are engaged, kinases bound to the cytoplasmic tail of integrins can initiate signaling cascades within the cell (Yamada and Miyamoto, 1995). Signaling through the α_4-
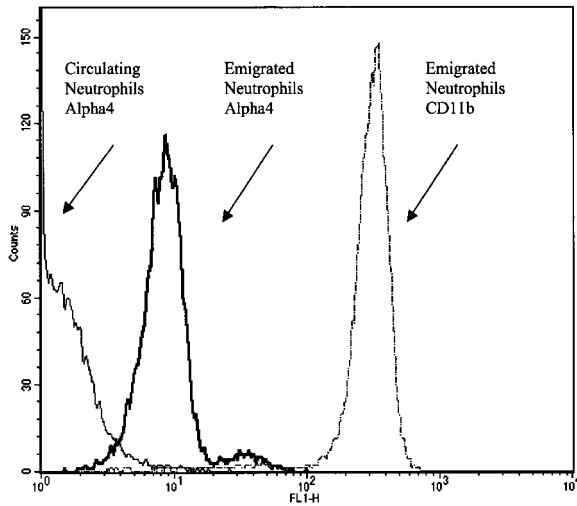
Figure 9. Mean fluorescence intensity of α_4-integrin on the surface of circulating and emigrated PMNs. Nonspecific binding was essentially zero. For comparison we also provide mean fluorescence data for the CD11b integrin subunit. Each experiment was run at least four times.

integrin resulted in protein kinase C activation in murine T cells (Tchilian et al., 1997), an event known to phosphorylate a key protein (p47^{phox}) in the NADPH oxidase complex. Indeed, p47^{phox} was phosphorylated by purified protein kinase C in a cell-free system (Kramer et al., 1988; Majumdar et al., 1991; Pilloud-Dagher et al., 1992). p47^{phox} is a cytosolic protein with a Src homology 3 domain structural motif involved in specific molecular interactions during signal transduction (de Mendez et al., 1997). p47^{phox} is critical for oxidase activation in intact cells and the Src homology 3 domain has been implicated in the assembly and maintenance of the NADPH oxidase activity in PMNs (de Mendez et al., 1996). Accordingly, it is possible that the adhesion of emigrated PMNs to cardiac myocytes via the α_4-integrin caused protein kinase C activation within the PMN and subsequent p47^{phox} phosphorylation, and that the multicomponent NADPH oxidase within the PMN can then assemble, resulting in free radical production. Previously, the α_4-integrin was thought to be expressed only on nonneutrophilic leukocytes and since NADPH oxidase is primarily associated with PMNs, the novel observation that emigrated PMNs also express the α_4-integrin led, in this study, to the demonstration that signaling through the α_4-integrin induces oxidant production in these cells.

Although to date, the α_4-integrin has been thought of as primarily an adhesion molecule, our data suggest that it also functions as an important signaling molecule. In fact, the amount of α_4-integrin found on the surface of PMNs is significantly less than that found on monocytes and lymphocytes, where the α_4-integrin is thought to be the dominant adhesion molecule. Indeed, dual addition of both anti-CD18 and anti–α_4-integrin Ab was required to inhibit emigrated PMN adhesion to myocytes (Reinhart et al., 1997). Although the low level of α_4-integrin raises some concern about the feasibility of this molecule to function as an adhesion molecule, it is conceivable that engagement of α_4-integrin may either rapidly mobilize or cluster more α_4-integrin on the PMN surface. Indeed, Alon and colleagues

have very recently documented subsecond induction of α_4-integrin on the leukocyte membrane (Grabovaky et al., 2000). With this type of flexibility to express α_4-integrin, it is also possible that in the absence of ligand (as would be the situation in our flow cytometry and immunogold binding assays) α_4-integrin may be rapidly reinternalized, shed, or simply not upregulated to the same degree as in the presence of myocyte ligands. Finally, it is possible that engagement of α_4-integrin signals other adhesive pathways to be induced. Indeed, α_4-integrin engagement by vascular cell adhesion molecule 1 enhances CD18-dependent adhesion (Chan et al., 2000). Whether other adhesion pathways are also upregulated remains unknown.

Our purpose was not to identify the molecular mechanisms by which the PMN-derived oxidants injure myocytes. Nevertheless, the results provide some meaningful insights in this regard. In the mouse, as in humans, contractility is regulated by numerous mechanisms within the myocyte (Opie, 1992). Phasic contraction is predominately controlled by the magnitude of calcium (Ca^{2+}) flux through dihydropyridine-sensitive L-type Ca^{2+} channels on the sarcolemma, and the much larger subsequent Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (Katz, 1992b). The rate of contraction (cell shortening) is controlled through the magnitude of cytoplasmic Ca^{2+} available for contraction and the rate of recycling of the actin-myosin cross-bridges (Katz, 1992a). However, the rate of relaxation is dependent on the rate of Ca^{2+} reuptake through sarco(endo)plasmic reticulum Ca^{2+}-ATPase2 (SERCA2) on the sarcoplasmic reticulum (Katz, 1992b). Since our data show that cell shortening is reduced before any significant changes in rate of contraction or relaxation (Figs. 2 and 3), much of the initial oxidant-induced injury appears to be targeting the myocyte at the plasma membrane surface. It is possible that the oxidants are affecting the fast inward current (Na^+ channel) or the slow inward current (Ca^{2+} channel), which depolarize the myocyte upon electrical stimulation. Since the intracellular level of both of these ions can alter mechanical activity and $[Ca^{2+}]_i$, an alteration in either ion channel could affect the magnitude of Ca^{2+} available for Ca^{2+}-induced Ca^{2+} release, or result in Ca^{2+} overload of the cell. The fact that myocytes are still able to contract and relax, albeit at a slower rate in the presence of emigrated PMNs, implies that the cardiac cell is able to maintain its intracellular milieu at sufficient levels to avoid contracture. This argues against massive nonspecific cell injury and suggests a more subtle alteration in the control of the contractile machinery of the myocyte.

Activated PMNs can produce several different O_2^--derived species, including H_2O_2, hypochlorous acid, peroxynitrite, or hydroxyl radical (Halliwell and Gutteridge, 1984, 1986; Liu et al., 1997). At present, there is no fluorescent probe sufficiently specific for the detection of a single oxidant species. Indeed, although some investigators have used DCFH (the probe used in this study) as an H_2O_2 detection system (Carter et al., 1994), others have demonstrated that DCFH reacts with other oxygen species, including nitric oxide and hydroxyl radical (LeBel et al., 1992; Zhu et al., 1994; Murrant et al., 1999). We would predict from our measurement of O_2^- production that a single emigrated PMN might optimally release nanomolar levels

of oxidant into a myocyte, assuming a cellular volume of 1 μl. Since H_2O_2 oxidizes DCFH between 5 and 500 μM (LeBel et al., 1992), or at least 100-fold higher than the amount of H_2O_2 produced by PMNs in our system, it is likely that DCFH was detecting some other oxidant. Since SOD scavenges O_2^- but does not affect H_2O_2 levels, yet SOD prevents oxidation of DCFH in our system, it is likely that we were not detecting H_2O_2 and that H_2O_2 was not the toxic molecule. It is noteworthy that extracellular SOD could inhibit both the oxidative stress and myocardial dysfunction, whereas overexpression of SOD within the myocyte, even at 10-fold supranormal levels, did not inhibit either the oxidative stress or myocardial dysfunction. This would be consistent with the very restricted diffusion capacity of O_2^- and supports the view that if the toxic oxidant had penetrated the cell, its phenotype has altered such that it is no longer inhibitable by intracellular SOD. For example, it is plausible that a derivative of O_2^-, such as the combination of O_2^- and nitric oxide, caused the negative inotropic responses in the myocytes seen after exposure to emigrated PMNs. Indeed, preliminary data using inducible nitric oxide synthase–deficient mice have suggested a role for nitric oxide in our model (Poon, B.Y., and P. Kubes, preliminary observations).

In conclusion, our results reveal that profound molecular changes occur within PMNs as they emigrate, such that CD18 and associated intracellular signaling pathways leading to oxidant production are uncoupled and α_4-integrin functions as the ligand that predominantly signals oxidant production. This may be an important brake preventing PMNs from producing oxidants until the α_4-integrin detection system is engaged. In fact, in a normal inflammatory process the uncoupling of CD18 with the NADPH oxidase system may in part explain why oxidant release is limited from PMNs until after the cell reaches the extracellular space.

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