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Neutrophil-Induced Myocyte Dysfunction: Role of the α_4-integrin

by

Betty Y. Poon

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ABSTRACT

The aim of this thesis was to examine the role of the α_4-integrin in cardiac myocyte dysfunction induced by emigrated neutrophils. Emigrated rat neutrophils express the α_4-integrin and use this ligand, in conjunction with β_2-integrin CD18 to adhere to isolated cardiac myocytes. We show that emigrated murine neutrophils also used both α_4- and β_2-integrins to adhere to myocytes, however immunosuppression of the α_4-integrin alone was able to prevent neutrophil-induced myocyte dysfunction, as measured by unloaded cell shortening. The myocyte injury was entirely dependent upon neutrophil-derived free radicals. Single cell imaging techniques showed that neutrophil-induced free radical generation in myocytes was coupled to the α_4-integrin. Myocytes were not protected by over-expression of endogenous superoxide dismutase, but were protected by exogenous superoxide dismutase added to the superfusate. Thus, emigrated neutrophils generate free radicals upon engagement of the α_4-integrin, and cause superoxide-dependent injury of the myocyte.

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LIST OF ABBREVIATIONS

Ab	antibody
BSA	bovine serum albumin
Ca^{2+}	calcium
CINC/gro	cytokine-induced neutrophil chemoattractant/gro
Cl^-	chloride
DCFH	6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate di(acetoxymethyl ester)
Fe	iron
fMLP	N-formyl-Met-Leu-Phe
H^+	hydrogen
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
I/R	ischemia-reperfusion
KO	knockout
LTB_4	leukotriene B_4
MPO	myeloperoxidase
Na^+	sodium
NO	nitric oxide
O_2	molecular oxygen
O_2^-	superoxide radical
OH^-	hydroxyl radical
$ONOO^-$	peroxynitrite
PAF	platelet activating factor
PBS	phosphate buffered saline
PECAM-1	platelet-endothelial cell adhesion molecule-1
PKC	protein kinase C

PMN	polymorphonuclear leukocyte
SH3	Src homology 3
SR	sarcoplasmic reticulum
SOD	superoxide dismutase
TNF-α	tumor necrosis factor-alpha
VCAM-1	vascular adhesion molecule-1
ZAP	zymosan-activated plasma

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

A heart attack, or myocardial infarction, is triggered by an interruption of the blood supply to tissue over a critical period, which can lead to tissue damage and irreversible cell death [1]. Upon reperfusion, and the restoration of oxygen, myocardial injury and inflammation is observed [2;3]. Since reperfusion is accompanied by a large influx of polymorphonuclear leukocytes (PMNs) [4-7], and depletion of PMNs from the circulation reduced myocardial injury after ischemia-reperfusion (I/R) [8-10], there is great interest in the PMN as a target for therapeutic intervention. It is thought that after PMNs infiltrate myocardium [11-14], they release cytotoxic factors like oxygen free radicals, proteases, and arachidonic acid metabolites [11;15;16]. Targeting these molecules can also reduce the extent of myocardial injury after I/R [17-20].

A large body of work has been dedicated to the analysis of PMN adhesion to vascular endothelium, and the ensuing PMN-induced endothelial injury [21-23]. Far less is known about the interaction between PMNs and parenchymal cells like cardiac myocytes. In the heart, a very important observation is that firm adhesion between cardiac myocytes and PMNs is absolutely required for the release of toxic mediators [24] and subsequent injury [25;26]. Detailed reports conclude that the engagement of the β_2-integrin CD18 is essential for PMNs to release cytotoxic molecules [24;27-29]. The tight seal between the PMN and myocyte may exclude plasma, which contains important anti-oxidants and anti-proteases [25;30]. When PMN adhesion is disrupted with anti-CD18 or anti-intercellular adhesion molecule-1 (ICAM-1) molecules, plasma-derived anti-oxidants and anti-proteases can prevent myocardial injury, highlighting the absolute requirement for PMN adhesion through CD18 in this pathology [25].

Although these seminal studies have convincingly demonstrated the essential role for adhesion between circulating PMNs and cardiac myocytes, the chosen experimental conditions differed from the physiological situation since PMNs must first emigrate out of the vasculature before they interact with cardiac myocytes. The emigration process is not trivial; emigrated PMNs have been shown to be far more responsive to inflammatory mediators [31; 32], and to express novel adhesion molecules, including α_4-integrin [33-35]. Indeed, the α_4-integrin has been shown to contribute significantly to emigrated PMN-myocyte interactions. Following emigration, targeting only CD18 with an anti-CD18 antibody (Ab) no longer inhibited adhesion [33]. Rather, both anti-CD18 and anti-α_4 Abs were required to prevent emigrated PMN-myocyte interactions in the rat model. This data has raised many new questions about the importance of the α_4-integrin as a mediator of emigrated PMN-dependent myocyte injury.

An activated PMN is able to produce a very high concentration of oxygen free radicals [11; 16], and this level increases upon adhesion. Indeed, human PMNs adherent to nylon fiber produced more superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) than the same cells in suspension [36]. Once adherent to cardiac myocytes, circulating PMNs were shown to generate CD18-dependent oxygen free radicals, a likely mechanism of PMN-dependent myocyte injury [25]. It is unknown whether emigrated PMNs will utilize this same mechanism to injure cardiac myocytes. The present study was designed to examine the mechanism of emigrated PMN-induced injury of cardiac myocytes through an α_4-integrin-controlled free radical pathway.

1.2 MECHANISMS OF MYOCARDIAL INJURY IN ISCHEMIA-REPERFUSION

Reperfusion of previously ischemic myocardium is crucial to patient recovery in the clinical setting. Reperfusion however, may also paradoxically exacerbate myocardial damage by causing morphological and metabolic de-arrangement and myocardial necrosis [37]. Although the pathogenesis of I/R has attracted great interest, a full understanding of the mechanism of myocardial injury in this pathology is incomplete.

Upon reperfusion of previously ischemic myocardium, there is a large influx of PMNs [4-7], and this PMN accumulation is associated with the areas of greatest injury in the heart [11; 38; 39]. Reduction of circulating PMN numbers with anti-PMN Abs [8], PMN depletion filters [40], or antimetabolites [14] all reduced infarct size in I/R challenged hearts. Limiting the recruitment of PMNs by immunosuppression of PMN adhesion molecules also limited myocardial injury [41-44]. Furthermore, experiments with complement depletion [45] and lypoxygenase inhibitors [46], aimed at reducing PMN chemotactic factors, limited infarct size. Increased levels of PMN-derived proteolytic enzymes, including elastases, β-glucosaminidases, β-glucuronidases, and myeloperoxidase (MPO), all of which break down the barrier function of the endothelium and lead to impaired myocyte function, have been measured in reperfused myocardium [47]. Inhibition of known PMN products, including oxygen free radicals, proteases, and arachidonic metabolites, also reduced the extent of myocardial injury [18; 19; 19; 48]. Although these data clearly show the PMN is a key player in myocardial injury in I/R, alternate pathways of injury have been proposed. PMN-independent mechanisms of injury include the study of the pH paradox

[49], reperfusion-induced calcium (Ca^{2+}) overload [50], and the generation of PMN-independent oxygen free radicals in the heart [51; 52].

An association between altered pH levels and contractile function has been well documented [53; 54]. In fact, a decrease of just 0.22 pH units caused a 50% decrease in contractile function of perfused rabbit hearts [49]. It is evident therefore, that maintenance of intracellular pH is crucial to proper cellular homeostasis. In the healthy myocardium, an optimal intracellular pH of 7.3 is maintained by the constant extrusion of protons by the sodium/hydrogen (Na^+/H^+) exchanger. It is well established that ischemia produces intracellular acidosis [55], and increased intracellular Na^+ [49; 56]. Theoretically, reperfusion at physiologic pH would lead to a pH gradient across the sarcolemma and activation of the Na^+/H^+ exchanger to restore intracellular pH levels [49; 57]. H^+ is moved out of the cell to increase intracellular alkalinity, while Na^+ is taken into the cell. An increase in intracellular Na^+ activates the Na^+/Ca^{2+} exchanger, causing a large influx of Ca^{2+} into the cell and subsequent cellular injury. Although theoretically viable, experiments that inhibited the Na^+/H^+ exchanger to limit the pH paradox have resulted in conflicting reports. Treatment of hearts at the time of reperfusion with amiloride, an inhibitor of the Na^+/H^+ exchanger [58; 59], showed either broad ranged protection and enhanced ventricular recovery [60], or no protection at all [61]. It is unclear, therefore if the pH paradox is the sole mechanism of cellular injury in myocardial infarction.

Cystolic Ca^{2+} overload also occurs as a result of impaired generation of ATP [62], as seen in ischemia where insufficient molecular oxygen (O_2) levels result in the depletion of ATP and the formation of ADP, AMP, adenosine, inosine and finally hypoxanthine [63]. The sarcoplasmic reticulum (SR) is therefore unable to take up Ca^{2+} at a normal rate,

resulting in abnormally high Ca^{2+} levels within the cell. Ca^{2+} overload activates phospholipases that can destroy the cell membrane, leading to Ca^{2+}-mediated arrhythmia and cell death [62]. Verapamil, a non-dihydropyridine type Ca^{2+} antagonist, showed reductions in mortality when administered 2-3days after the initial infarct [64], demonstrating a possible role for Ca^{2+} overload in late phase I/R. Despite encouraging data in animal studies, to date there is no convincing clinical data that humans benefit from the blockade of Ca^{2+} entry to the cell in the acute phase of myocardial infarction. In fact, major clinical trials using nifedipine, a dihydropyridine type Ca^{2+} antagonist, showed no significant benefits in post-myocardial infarct [65]. Although Ca^{2+} overload may contribute to myocardial tissue injury in I/R, there may be an alternative pathway of injury independent of, or working in concert with, the uncontrolled influx of Ca^{2+} to the cell.

The role of oxygen free radicals in myocardial I/R injury has been studied extensively. Free radicals have been shown to cause lipid peroxidation [66], a disruption of myocardial cell membranes [67], an imbalance in Ca^{2+} homeostasis [68; 69], and cardiac contractile dysfunction [70; 71]. In animal studies, the addition of free radical scavengers protected the heart from I/R-induced myocardial damage [72; 73]. In fact, the addition of exogenous free radical scavengers, superoxide dismutase (SOD) and catalase, protected isolated rat hearts from I/R-induced decrease in left ventricular pressure and increase in left ventricular end-diastolic pressure; and partially inhibited the I/R-induced disruption of SR Ca^{2+} uptake and extrusion from the cytosol [74]. During I/R, there are many potential sources of free radicals, including intracellular production from the mitochondria [67], conversion of xanthine oxidase to xanthine dehydrogenase [75], auto-oxidation of

catecholamines [76], and the arachidonic acid cascade [77]. It must be appreciated, however that the PMN is a major source of free radicals in the inflamed heart [11; 16].

Although the proposed PMN-independent mechanisms may contribute to the myocardial injury seen in I/R, it is clear that we cannot exclude the potential role of the PMN as a key player in the initiation and progression of these pathological pathways of injury.

1.3 PMN RECRUITMENT

In order to interact with cardiac myocytes, PMNs must first leave the vasculature to enter myocardial tissue. PMNs are recruited to areas of inflammation by a multi-step recruitment paradigm. Initially, circulating PMNs slow down by making temporary contacts with the endothelium, a process called tethering. After the initial contact, a succession of contacts is made and the PMN begins to roll along the vascular wall. The selectin family of adhesion molecules, including constitutively expressed L-selectin on the PMN, and inducible P- and E-selectin on the endothelium, mediates this process of tethering and rolling [78; 79].

To minimize steric interference, L-selectin is strategically located on the tips of microvilli projections and is shed following activation and subsequent emigration [80]. Possible ligands to L-selectin include P- and E-selectin on the endothelium [81]. P-selectin is preformed and stored in Weibel Palade bodies in endothelial cells and is rapidly mobilized to the endothelial cell surface in response to inflammatory mediators [4; 23]. P-selectin plays an important role in the early phase of inflammation since P-selectin levels are quickly decreased by 30-60min. P-selectin has been shown to bind primarily to P-

selectin glycoprotein ligand-1 on most leukocytes [82]. E-selectin is not preformed, but is synthesized in response to inflammatory cytokines [83; 84]. E-selectin may be more important in late stage inflammation since maximal synthesis levels require 4-6 hours. PSGL-1, E-selectin ligand-1, cutaneous lymphocyte antigen, and L-selectin are all possible ligands for E-selectin [81; 85; 86].

The next step in PMN recruitment to areas of inflammation is firm adhesion. Once the PMN has slowed down, it can then make more permanent interactions with the endothelium. The β_2-integrin (CD11/CD18) family of adhesion molecules is involved in cell-cell interactions and thus mediates firm adhesion of PMNs to endothelium [87]. The members of this adhesion molecule family include LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and p150/95 (CD11c/CD18) [88]. The β_2-integrins on PMNs adhere to ICAM-1 on cytokine stimulated endothelium [4], and on cells outside of the vasculature, including fibroblasts, dendritic cells, and epithelial cells [85]. β_2-integrins also have extravascular ligands, including matrix proteins and complement fragments [89], and these may become important once the PMN leaves the vasculature.

Once PMNs have firmly adhered to the endothelium, they undergo a shape change, which allows them to crawl between endothelial cells and move into tissue. This emigration process may be mediated by platelet-endothelial cell adhesion molecule-1 (PECAM-1), expressed along the border between endothelial cells and on PMNs [90]. A role for PECAM-1 in PMN transmigration across endothelium has been shown *in vitro* and is hypothesized to play a similar role *in vivo* [91; 92].

Following emigration, PMNs migrate along an increasing gradient of chemotactic agents, including cytokines (interleukin (IL)-1 and -8), complement cascade products

(C5a), bacterial products (N-formyl-Met-Leu-Phe (fMLP)), and products of phospholipid metabolism (platelet activating factor (PAF) and leukotriene B_4 (LTB_4)) [4]. PMNs are now localized within myocardial tissue and can interact with cardiac myocytes in pathophysiologies like I/R.

1.4 PMN-INDUCED ENDOTHELIAL VS MYOCYTE DAMAGE

Although a role for the PMN in I/R-induced myocardial injury seems clear, it is unknown if the critical PMN-dependent injury is at the level of the endothelium or at the level of the myocyte. It is well appreciated that the I/R damages the microvasculature, and that the increased adhesiveness of PMNs to the endothelium contributes to the extent of the tissue injury [21; 93]. Indeed, adhesion of PMNs to the endothelium is a prerequisite to emigration, and endothelial injury may be so severe that the myocardium is irreversibly damaged and subsequent myocyte injury may play only a minimal role in decreased myocardial function.

PMNs can directly injure endothelial cells through proteases and oxygen free radicals [11; 15]. PMN-derived oxygen free radicals have been shown to cause disintegration of endothelial cell membranes, resulting in microvascular disorders arising from cell dysfunction, edema, and cell death [94]. Furthermore, oxygen free radicals stimulate PAF release from the endothelium, which further exacerbates the local PMN influx by an amplifying feedback loop [95]. PMN-derived elastase hydrolyzes a variety of biological substrates [96; 97], and its activity is increased in the blood of patients suffering from myocardial infarct [98]. Furthermore, endothelial cell monolayers exposed to anoxia induced elastase release from PMNs upon reperfusion [99]. These PMNs caused

endothelial cell detachment and resulted in a loss of cell-cell contact and exposure of the underlying matrix, which was ameliorated by the addition of elastase inhibitors. One consequence of endothelial detachment is the exposure of underlying smooth muscle to the direct vasoconstricting effects of platelet-derived factors [22].

The ability of the PMN to injure endothelium is well established, but whether this mechanism of myocardial injury in I/R is the dominant pathway is unclear. It is evident however, that patients may arrive at hospital after the myocardial infarct and thus PMNs have already been recruited and are in contact with cardiac myocytes. The adherence of PMNs to vascular endothelium occurs within 20mins post-reperfusion and these PMNs begin to emigrate as early as 1h post-reperfusion [100]. Many researchers have acknowledged this time frame and have chosen to study the interaction of PMNs and isolated cardiac myocytes and have shown that PMNs can indeed directly injure cardiac myocytes [25; 26]. PMNs treated with chemoattractant phorbol 12-myristate 13-acetate caused irregular contractions and subsequent contracture and blebbed formation in murine embryo ventricular myocytes [26]. Electron micrographs of these myocytes prior to contracture revealed swollen mitochondria, and ruptured plasma membranes and vacuoles.

PMN adhesion is critical to PMN-induced myocyte injury since supernatant from activated PMNs was unable to cause myocyte injury, and myocytes without adherent PMNs were not injured [25; 26; 101]. Researchers have, therefore begun to study the adhesion molecules involved in PMN-myocyte interactions and found that PMNs adhered to myocytes through a CD18-ICAM-1 mediated pathway [24; 101]. Furthermore, through fluorescence imaging, these investigators have shown that PMNs caused oxidant

generation in the myocyte and that this increase in oxidants was CD18-mediated [25]. Interestingly, this research group also found that one adherent PMN alone was able to cause myocyte injury, and that the magnitude of oxidant production was not increased when multiple PMNs were adherent to the myocyte.

Although these studies show the importance of PMN adhesion through a CD18-mediated pathway to myocyte injury, these experiments involved circulating PMNs isolated from whole blood. It is clear that PMNs must emigrate out of the vasculature before they can interact with cardiac myocytes. Emigrated rat and human PMNs have been shown to express the α_4-integrin, and this ligand is not expressed in the circulation [33-35]. Emigrated rat PMNs have also been shown to utilize this new adhesion molecule, in conjunction with CD18, to adhere to isolated ventricular myocytes [33]. It is unknown whether murine emigrated PMNs also express the α_4-integrin, and if this ligand plays a role in emigrated PMN-myocyte interactions in this model. Furthermore, the ability of emigrated PMNs to induce myocyte injury, and role of the α_4-integrin in mediating injury, requires further study.

1.5 PMN-DERIVED FREE RADICALS

PMNs are known to produce oxygen free radicals and their reactive oxygen intermediates have been shown to be toxic to many cell types [11; 16; 102]. Although cells have natural protective free radical scavenging systems, including SOD, catalase, and glutathione peroxidase, many ischemic diseases of the heart, bowel, liver, kidney, and brain have been linked to free radical damage caused by oxidative stress [63].

PMNs use the enzyme NADPH oxidase to mount a respiratory burst in response to an inflammatory condition [103]. NADPH oxidase transfers an electron from cytosolic NADPH across the plasma membrane to O_2. O_2^- is formed from the acceptance of one extra electron by O_2, and O_2^- or its secondary products can then be released and accumulate in the extracellular space. In the myocardium, when there is a sudden rise in intracellular Ca^{2+}, the "Ca^{2+} paradox" itself has also been shown to trigger the production of O_2^- [104]. In the vasculature, a major source of O_2^- is from the conversion of hypoxanthine to O_2^- by the enzyme xanthine oxidase found in endothelial cells.

In healthy cells, SOD catalyzes the dismutation of O_2^- to H_2O_2, which is then converted back to O_2 and water by catalase and glutathione peroxidase. In pathophysiological states like myocardial I/R, where free radical generation is increased, these scavenging systems may become overwhelmed and oxidant-induced injury may occur. Indeed, several studies with isolated heart preparations have reported a burst of oxygen free radicals generated following reperfusion [105-109]. Furthermore, free radical generation was measured up to 3h post-reperfusion using electron spin resonance and spin-trapping techniques, providing direct evidence for the production of free radicals in the setting of myocardial I/R [110].

O_2^- has been shown to react with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$). $ONOO^-$ is a stronger oxidizing agent than either O_2^- or NO alone, and quickly reacts with thiols, ascorbate, and lipids [68; 111]. Although $ONOO^-$ formation may be beneficial to some cells, there is also a vast amount of data showing $ONOO^-$-induced oxidative damage to biological tissues and subsequent pathogenic conditions [112; 113]. In the heart, $ONOO^-$ was shown to aggravate injury measured by depressed cardiac function

recovery, increased lactate dehydrogenase and creatine kinase release, and enlarged necrotic size [114; 115]. Researchers have confirmed $ONOO^-$ production upon reperfusion of previously ischemic myocardium [116-118]. The reaction rate for the formation of $ONOO^-$ is $6.7\pm0.9X10^9/M\cdot s$ [119], which is approximately six times faster than the scavenging rate of O_2^- by SOD [120; 121]. NO is the only known biological molecule produced in high enough concentrations in pathophysiological states to successfully out-compete SOD for O_2^- [112].

Normally, a biological system generating O_2^- will produce H_2O_2 by the dismutation reaction, unless SOD levels are depressed or if O_2^- is able to react immediately with another molecule like NO. In several experiments where exogenous free radical generating systems caused cell injury, H_2O_2 has been identified as the specific free radical causing injury since cells were protected with exogenous catalase and not SOD [122]. Furthermore, H_2O_2 not O_2^-, is able to cross biological membranes, allowing for intracellular cytotoxicity. In the heart, H_2O_2 caused contractile abnormalities and injury to cardiac myocytes which were linked to an H_2O_2-dependent increase in Ca^{2+} influx and subsequent Ca^{2+} overload [69]. H_2O_2 also damaged the SR, causing reductions in Ca^{2+} uptake and altered Ca^{2+} homeostasis [71]. Moreover, H_2O_2 has been shown to cause direct electrophysiological alterations to rat cardiac myocytes with a slowing of the inactivation of Na^+ channels and prolongation of the action potential [123].

Increasing attention has been focussed on the role of hypochlorous acid (HOCl) in tissue injury. Secretion of MPO into the phagocytic vacuole of the PMN can catalyze the oxidation of chloride (Cl^-) by H_2O_2 to yield HOCl. The local concentration of HOCl produced by activated PMNs is estimated to be 60-90μM [124; 125]. HOCl is highly reactive and, at concentrations as low as 10-20μM, can quickly oxidize many biological

molecules, causing cellular injury [126; 127]. HOCl is a weak acid at physiological pH, and especially under acidic conditions of I/R, remains mostly undissociated and permeable [128]. This may facilitate entry into the myocyte and allow HOCl to directly affect the myofilaments. Indeed, the addition of exogenous HOCl caused an increase in Ca^{2+} sensitivity, a decrease in maximal Ca^{2+} force, and an increase in the resting tension of skinned rat cardiac muscle [128]. Furthermore, HOCl has also been shown to mobilize intracellular zinc in cardiac myocytes [126], and free zinc has been shown to be a potent inhibitor of cardiac contractility [129]. Finally, HOCl from activated PMNs caused an 80-90% inhibition of Ca^{2+} uptake, indicating severe SR damage [71]. This inhibition was completely restored by the addition of L-Methionine, a known scavenger of HOCl [71; 130].

O_2^- can also, however enter the Fenton reaction in the presence of free iron (Fe) to produce the highly reactive hydroxyl radical (OH^-). The generation of OH^- by the Fenton reaction has been demonstrated in many *in vitro* studies [131-133], and many have proposed that it is the true agent behind the toxic effects attributed to O_2^- [131-134]. Formation of OH^- is controversial however, since it is unclear whether or not the body generates high enough free Fe levels to allow for this reaction to occur *in vivo*. Adult humans have 4g of Fe, with two-thirds present as hemoglobin and 10% found in myoglobin [135]. The remainder is present in intracellular storage proteins, ferritin, and hemosiderin found mainly in the liver, spleen, and bonemarrow [135]. Fe in the diet exists in the oxidized form Fe(III), and is generally tightly bound to transferrin, a carrier molecule glycoprotein with two binding sites for Fe(III) [135]. Under normal conditions, the transferrin present in the blood stream is only 30% loaded with Fe, so the amount of free Fe available in the blood plasma would be virtually zero [135; 136].

These reactive oxygen intermediates can affect a multitude of biological systems, including lipid peroxidation [66], modification of protein structure and function, and ultimately cell death [137]. The relationship between free radicals and the functional state of the myocardium has been studied extensively [25; 138-140]. Free radical generating systems administered exogenously have been shown to cause cardiac contractile dysfunction and electrophysiological abnormalities [141; 142]. These free radicals were also able to affect myocardial sarcolemmal membrane [143; 144], SR [145], and mitochondrial functions [146]. Furthermore, it has been shown that free radicals depress the sarcolemmal Ca^{2+}ATPase activity, resulting in reduced Ca^{2+} extrusion from the cytosol [144]. Free radicals also promote Ca^{2+} release from the SR and inhibit Ca^{2+} sequestration to the SR [68], leading to a disruption of Ca^{2+} homeostasis and subsequent Ca^{2+} overload.

1.6 STATEMENT OF HYPOTHESIS AND OBJECTIVES

Hypothesis 1: Emigrated murine PMNs use both β_2- and α_4-integrins to adhere to isolated cardiac myocytes.

Objectives:

1) To determine whether the process of emigration alters the mechanism by which murine PMNs adhere to cardiac myocytes.
2) To determine if murine PMNs express a new adhesion molecule profile following emigration.

Hypothesis 2: Emigrated PMNs cause injury to cardiac myocytes through the α_4-integrin.

Objectives:

1) To determine if emigrated PMNs cause myocyte dysfunction, and if so, whether adherence of the PMN to the myocyte is necessary for injury to ensue.
2) To determine whether injury is mediated through either CD18, α_4-integrin, or through both ligands.
3) To determine if circulating murine PMNs injure cardiac myocytes through the same mechanism as emigrated PMNs.

Hypothesis 3: PMN-induced myocyte injury is caused by the generation of free radicals.

Objectives:

1) To determine if PMNs require a respiratory burst to cause myocyte damage.

2) To visualize the production of free radicals upon PMN adhesion to the myocyte.
3) To determine whether free radical generation is mediated through either CD18 or α_4-integrin.
4) To determine if the specific free radical responsible for myocyte injury is O_2^-.

CHAPTER 2

METHODS AND MATERIALS

2.1 EXPERIMENTAL MODELS

2.1.1 PMN/Myocyte Adhesion Assay

To examine the adhesion of murine PMNs to isolated cardiac myocytes, an *in vitro* adhesion assay was employed [147]. Myocytes were coated onto a round glass coverslip and mounted onto the inside of one side of a metal chamber. A second clean coverslip was placed on top of the myocytes, separated by an O-ring gasket to form a chamber space of approximately 700-800μl. The other side of the metal chamber was then attached. PMN suspensions were injected into the chamber space between the coverslips via a syringe and 23G needle. The PMNs then settled by gravity onto the myocyte layer. Once the chamber was inverted, all nonadherent PMNs fell away from the myocyte layer, and those PMNs adherent to myocytes were counted with an inverted microscope.

Ventricular myocytes were isolated as previously described for rat ventricular myocytes [33] with minor modifications for murine cells. Briefly, six-week old male C57BL6 mice were anaesthetized and the hearts removed and placed into Tyrode's buffer (NaCl 140mM, KCl 5.4mM, Na_2HPO_4 1mM, HEPES 5mM, glucose 10mM, $MgCl_2$ 1mM, pH adjusted to 7.4 with NaOH) containing 1mM $CaCl_2$ at 4°C. Hearts were then cannulated via the aorta (within 3mins) for retrograde perfusion of the coronary arteries. Initially, the hearts were perfused with Tyrode's buffer containing 1mM $CaCl_2$ at 2ml/min for 5mins at 37°C and then with Tyrode's buffer containing no $CaCl_2$ at 2ml/min for 5mins. Perfusion was then switched to Tyrode's buffer containing 40μM $CaCl_2$, 20μg/ml collagenase, and 4μg/ml protease and perfusion continued at 2ml/min for 8mins. Digested hearts were then removed from the perfusion system and ventricles were

minced in Tyrode's buffer containing 1mM $CaCl_2$, 500μg/ml collagenase, 100μg/ml protease, and 2.5% bovine serum albumin (BSA). Ventricular tissue segments were then put into a shaking water bath for 10-20mins at 37°C to complete the dispersion and obtain a suspension of individual myocytes. Myocytes were then placed in a KB-type solution (K-glutamate 100mM, K-aspartate 10mM, KCl 25mM, KH_2PO_4 10mM, $MgSO_4$ 2mM, taurine 20mM, creatine 5mM, EGTA 0.5mM, glucose 20mM, HEPES 5mM, and BSA 1%, pH adjusted to 7.2 with KOH) at 4°C, and used within 5hrs.

To obtain emigrated murine PMNs, six-week old male C57BL6 mice were injected intraperitoneally with 1% oyster glycogen in saline [148]. After 4h, mice were sacrificed and a peritoneal lavage performed with 3ml saline. Lavage fluid was placed on ice for 5mins then centrifuged at 1300rpm at 4°C for 6mins. Pellets were then resuspended in Tyrode's buffer with 1mM $CaCl_2$ at 4°C. This approach yielded a 99% pure population of emigrated PMNs as analyzed with Wright-Giemsa staining. In all experiments, PMNs were kept on ice and used within 2hrs of isolation.

Circulating murine leukocyte suspensions were isolated from whole blood by lysis of the red blood cells. Briefly, blood (800-900μl) was collected by cardiac puncture into a syringe with acid citrate dextrose (anticoagulant) (100μl), added to ddH_2O at 4°C, and gently mixed. KCl (0.6M), followed by phosphate buffered saline (PBS, NaCl 137mM, KCl 2.7mM, Na_2HPO_4 8.1mM, KH_2PO_4 1.47mM), was added and the sample centrifuged at 1300rpm at 4°C for 6mins. These steps were repeated to further isolate a pure circulating leukocyte population, and the pellet resuspended in Tyrode's buffer with 1mM $CaCl_2$ at 4°C. These leukocytes were initially exposed to myocytes and histological assessment revealed that all of the adherent cells were indeed PMNs.

Flow cytometry was used to measure the expression of CD11b, CD18, and α_4-integrins on circulating and emigrated PMNs. Circulating or emigrated murine PMNs ($1X10^6$ per tube) were stimulated with 1% zymosan-activated plasma (ZAP) (10mins at room temp) and then washed. Red blood cells were lysed and PMNs were fixed in 1% formalin (15mins at room temp) and then washed. Primary Abs were then added to stain for their respective adhesion molecules (CD11b, MK/170, 0.25μg per tube, Pharmingen; CD18, 2E6, 0.8μg per tube, Endogen; and α_4, R1-2, 1μg per tube, Pharmingen). After 30mins at room temperature, cells were washed and labeled with FITC-conjugated goat anti-rat IgG (Cedar Lanes Laboratories LTD) for CD11b and α_4, and FITC-conjugated goat anti-hamster IgG (Caltag Laboratories) for CD18. After 30mins at room temperature, cells were washed and fluorescence was measured on a FACScan flow cytometer (Becton Dickinson Immunocytochemistry Systems).

2.1.2 Unloaded Cell Shortening Assay

Isolated ventricular myocytes were allowed to adhere to a glass microscope stage for 5mins at room temperature [149]. Myocytes were then superfused at 1ml/min with normal Tyrode's buffer containing 1mM $CaCl_2$. Cells were field stimulated at 1Hz using a just threshold voltage level (Isolator II, Axon Instruments USA) to minimize production of free radicals due to hydrolysis. Unloaded cell shortening was recorded using an edge detection device (Solamere Technology Group) and the data acquired digitally at 10KHz sampling rate using customized software (Cellsoft V2.0, D. Bergman, University of Calgary, Canada). The number of PMNs adherent per myocyte and the time of onset of dysrythmia were recorded for each myocyte. For all experiments, cells were allowed to equilibrate while being electrically stimulated continuously for 15mins. To ensure that

myocytes exhibited normal contractile behavior and inotropic capacity before PMN treatment, the β-adrenergic agonist isoproterenol (0.1μM) was added and the resulting positive inotropic response to electrical stimulation was monitored. Myocytes exhibiting baseline shortening <5% of resting length, or those failing to respond to isoproterenol were excluded from the study. A positive response to isoproterenol included a 2-fold increase in extent of cell shortening, rate of contraction, and rate of relaxation from baseline.

After isoproterenol was washed out (10mins), baseline measurements were taken and then $1X10^6$ PMNs, pre-stimulated with 1% ZAP, were added to the superfusate. Myocyte contractility was then recorded continuously for 10mins. Isoproterenol was added again to the superfusate to reassess myocyte contractility. In all experiments, myocyte contractility was recorded to the completion of the protocol unless cell death occurred.

A cytochrome *c* reduction assay was utilized to measure the production of O_2^- from PMN suspensions [150]. Briefly, PMNs ($1X10^7$/ml) in PBS were added to PBS with $CaCl_2$ (1.19mM), $MgCl_2$ (0.54mM), and cytochrome *c* (1.5mM, Sigma) for a paired analysis. In one sample, SOD (from bovine erythrocytes, 264U/ml, Sigma) was added and both samples read at the same time in a spectrophotometer (U-2000 Spectrophotometer, Hitachi) at 550nm. Optical density differences between the two samples were recorded on an online chart recorder (Johns Scientific Inc). After 5mins of baseline measurements, 1% ZAP was added to both samples and optical density recorded for an additional 10mins.

2.1.3 Single Cell Imaging Assay

Isolated ventricular myocytes and emigrated PMNs were loaded with fluorescent probe, 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate di(acetoxymethyl ester) (DCFH, 1μM for myocytes and 10μM for PMNs, Molecular Probes) in Tyrode's buffer with probenecid 0.5mM (Sigma) at room temperature for 15mins. DCFH is oxidized to highly fluorescent 2',7'-dichlorofluorescein in the presence of free radicals. DCFH is not specific for any one oxidant, and thus can be used only as an overall indicator of oxidative stress within the cell [151-154]. Myocytes were allowed to adhere to a glass cover slip sealed by vacuum grease to the bottom of a plastic stage chamber, for 5mins at room temperature. The chamber was clipped into a fitted stage platform on an Axiovert-135 inverted microscope (Zeiss) equipped with an oil immersion FLUAR 100x/1.3 objective for single cell imaging [155]. A Delta-Ram High Speed Illuminator (Photon Technologies International), consisting of a 75Watt Xenon arc and a computer controlled random-access wavelength monochromator, provided excitation light. Wavelengths were further selected prior to cell illumination by a dichroic filter (Chroma Technology Corporation) mounted on a sliding apparatus under the objectives. ImageMaster v1.4 software (Photon Technologies International) allowed for 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 analysis. The cells were excited at 480nm and emission recorded at 510nm.

To ensure that morphologically viable myocytes were indeed healthy, single cell fluorescence intensities for each myocyte prior to exposure to PMNs were recorded for the first 5mins. Cells exhibiting a rise of greater than 10 raw intensity units were

assumed unhealthy and excluded from the study. Emigrated PMNs ($1X10^6$), pre-stimulated with 1% ZAP, were added to the myocytes and fluorescence intensities recorded every 10secs for 10mins. Phase contrast photos of the myocytes with adherent PMNs were recorded and stored digitally. At the end of each experiment, H_2O_2 (50mM, BDH) was added to the cells to confirm adequate loading of the cells with DCFH, and to demonstrate that all cells had the ability to fluoresce upon reaction with H_2O_2. Upon addition of the H_2O_2, all myocytes included in the study reached camera saturation intensity levels (255 raw intensity units).

2.2 EXPERIMENTAL PROTOCOLS

2.2.1 Emigrated Murine PMN Adhesion to Cardiac Myocytes via β_2- and α_4-integrins.

Round glass coverslips (25mm, Bellco Glass Inc) were pretreated with 1% filtered gelatin and incubated for 1hr at 37°C. The gelatin was then removed and a 1ml suspension of isolated cardiac myocytes in Tyrode's buffer ($1X10^4$/ml) was layered onto the coverslips, and incubated for an additional hour at 37°C. One coverslip with myocytes and one clean coverslip were then placed into the adherence chamber. Suspensions of either circulating murine leukocytes or isolated emigrated murine PMNs ($5X10^6$/ml) were pretreated with 1% ZAP, injected into the chamber space, and allowed to settle for 10mins. The chamber was then inverted and all nonadherent cells fell to the bottom of the chamber, leaving only cells adherent to the myocyte layer. The number of PMNs adherent per myocyte was counted at 200X magnification on an inverted microscope (Zeiss) (a minimum of 20 myocytes per coverslip was counted).

This adhesion assay was used to study the effect of known PMN stimulants, cytokines, and cell concentrations on PMN adhesion to myocytes to determine optimal conditions for adhesion. PMN stimulants tested included fMLP (5-20μM), PAF (50-1000ng/ml), cytokine-induced neutrophil chemoattractant/gro (CINC/gro, 5-20nM), LTB_4 (10^{-10}-10^{-8}M), KC (murine IL-8, 5-100nM), and ZAP (0.1-10%). Since 1% ZAP optimally increased PMN adherence and is present in pathophysiological states of I/R [156-158], this stimulant was used to activate PMNs in all subsequent experiments. Tumor necrosis factor-α (TNF-α, 100-500U/ml) treatment of the myocytes showed little difference in adhesion numbers as compared to untreated myocytes, therefore no myocyte pretreatment was used for subsequent experiments. Finally, various PMN concentrations ($1X10^6$-$5X10^6$/ml) were used in the assay and data showed optimal adhesion at the highest concentration tested. PMNs at $5X10^6$/ml were used for all adhesion studies, but for all other experiments (cell shortening and single cell imaging) the lower concentration ($1X10^6$/ml) was used to limit the number of mice required per experiment.

To examine the role of β_2- and α_4-integrins in PMN-myocyte interactions, functionally blocking Abs to CD18 (anti-CD18 Ab 2E6, Endogen) or to the α_4-integrin (anti-α_4 Ab R1-2, Pharmingen) were added alone, or in combination to the suspension of emigrated PMNs prior to injection into the adhesion chamber. Flow cytometry was used to determine the saturating dose of each Ab. Doses of the anti-CD18 and anti-α_4 Abs at 2-20μg/ml were tested. For the anti-CD18 Ab, 8μg/ml and for the anti-α_4 Ab, 10μg/ml showed maximal fluorescent staining and these doses were used for all experiments. For circulating leukocyte suspensions, addition of anti-CD18 Ab alone was sufficient to inhibit adhesion, therefore, both Abs were not added in combination.

2.2.2 PMN-Induced Myocyte Dysfunction via the α_4-integrin.

Following baseline cell shortening measurements and isoproterenol challenge, emigrated PMNs or circulating leukocytes ($1X10^6$) pretreated with 1% ZAP were added to the perfusion buffer. Unloaded cell shortening measurements, myocyte dysrythmia, and contracture were recorded, and the number of adherent PMNs noted. Experiments were conducted on myocytes alone, with emigrated PMNs or circulating leukocytes, and with anti-CD18 (2E6, 8μg/ml) or anti-α_4 Abs (R1-2, 10μg/ml). Data was subsequently analyzed for unloaded cell shortening, rate of contraction, and rate of relaxation for each myocyte at 5min intervals.

Isoproterenol was used to assess the contractile properties of all myocytes before PMN challenge. Myocytes were exposed to isoproterenol (0.1μM) for 5-20secs and the time to maximal response and the time required to return to pre-isoproterenol levels were recorded for each exposure time. For future experiments, all myocytes were exposed to isoproterenol for 10secs, with maximal response at 2mins, and returned to baseline cell shortening levels by 10mins.

2.2.3 Free Radical Generation in Cardiac Myocytes via the α_4-integrin.

Unloaded cell shortening was used to assess the role of PMN-derived free radicals on PMN-induced myocyte injury. Emigrated PMNs ($1X10^6$) were isolated from mice lacking the ability to generate free radicals (NADPH oxidase knock out (KO) mice) and added to myocytes from wild type (WT) mice (C57BL6). Cell shortening measurements were recorded for WT myocytes alone, and after the addition of either WT or NADPH oxidase deficient emigrated PMNs (pretreated with 1% ZAP).

As a control, the cytochrome *c* reduction assay was done comparing O_2^-

levels in PMNs from WT mice to those from NADPH oxidase deficient mice. As expected, results showed O_2^- levels below detection for the NADPH oxidase deficient PMNs, confirming that NADPH oxidase was indeed lacking in our transgenic mice.

The single cell imaging technique was used to visualize and measure the changes in oxidative stress in the myocyte upon adhesion of emigrated PMNs. Fluorescence measurements of WT myocytes alone, with WT emigrated PMNs ($1X10^6$) pretreated with 1% ZAP, and with anti-CD18 (2E6, 8μg/ml) or anti-α_4 Abs (R1-2, 10μg/ml) were recorded and images stored digitally. Images were analyzed for changes in fluorescence intensity in raw intensity units at baseline, 5 and 10mins.

To determine if the cause of myocyte injury was O_2^-, the unloaded cell shortening assay was used on myocytes from mice over-expressing endogenous Cu/Zn-SOD [159]. Myocytes isolated from these mice show a 10-fold increase in SOD expression [160] and as a result, should be able to scavenge intracellular O_2^-. Cell shortening was recorded for myocytes from WT (C57BL6) or SOD over-expressing mice in the presence and absence of WT emigrated PMNs ($1X10^6$). Additional cell shortening experiments were done with exogenous SOD (from bovine erythrocytes, 300U/ml, Sigma) to determine if extracellular O_2^- (O_2^- released by the PMN) was responsible for the PMN-induced myocyte injury.

2.3 STATISTICS

All data are expressed as the arithmetic mean ± standard error of the mean. Data were compared between treatment groups using an analysis of variance of raw data with

the Dunnetts method for multiple comparisons to PMN only group, and the Student T-test within groups. Values of $P<0.05$ are considered statistically significant.

CHAPTER 3

EMIGRATED MURINE PMNs ADHERE TO CARDIAC MYOCYTES VIA β_2- and α_4-INTEGRINS

Hypothesis: Emigrated murine PMNs use both β_2- and α_4-integrins to adhere to isolated cardiac myocytes.

Objectives:

1) To determine whether the process of emigration alters the mechanism by which murine PMNs adhere to cardiac myocytes.
2) To determine if murine PMNs express a new adhesion molecule profile upon emigration.

3.1 RESULTS

ZAP and fMLP increase adhesion of emigrated murine PMNs to cardiac myocytes. To increase the number of adherent PMNs/myocyte, various PMN stimulants and concentrations were tested. Of those tested, fMLP and ZAP both showed consistent increases in adhesion over untreated cells. The addition of fMLP increased adhesion from 1.35±0.10 adherent PMNs/myocyte in untreated controls to 2.7±0.26 adherent PMNs at 10μM fMLP (Fig 3.1, N=11, $P<0.05$). The addition of ZAP also approximately doubled adhesion when administered at the 1% ZAP dose (Fig 3.2, N=4, $P<0.05$). fMLP is a bacterial product present in inflammatory states like sepsis. ZAP, however is present in pathophysiological states of I/R [156; 157] and is readily available and easy to use. Therefore, for subsequent experiments, 1% ZAP was used as a pretreatment for all PMNs.

PMN concentration affects adhesion of emigrated PMNs to cardiac myocytes. Preliminary studies showed that increasing the PMN concentration from $1X10^6$ to $5X10^6$/ml resulted in a greater than 2-fold increase in adhesion, therefore for optimal

adhesion, PMNs at a concentration of $5X10^6$/ml, pretreated with 1% ZAP, were used for all subsequent adhesion studies.

Cytokine pretreatment of isolated cardiac myocytes does not affect adhesion of emigrated PMNs. TNF-α pretreatment of the myocytes showed little difference in adhesion throughout all TNF-α doses (100-500U) as compared to untreated myocytes, consequently no myocyte pretreatment was used for future adhesion experiments.

Circulating murine PMNs adhere to isolated cardiac myocytes via CD18. Murine circulating PMNs, pretreated with 1% ZAP, avidly adhered to isolated cardiac myocytes (Figure 3.3). Addition of a functionally blocking Ab to CD18 (2E6, 8μg/ml) inhibited adhesion by 63% (N=4, $P<0.05$). Immunosuppression of the α_4-integrin with an anti-α_4 Ab (R1-2, 10μg/ml) however, did not inhibit adhesion (3.23±0.38 adherent PMNs/myocyte with anti-α_4 Ab, and 3.48±0.28 adherent PMNs/myocyte without anti-α_4 Ab, N=2). These data indicate that this adhesion pathway is CD18-, and not α_4-integrin-dependent.

Emigrated murine PMNs adhere to isolated cardiac myocytes via CD18 and α_4-integrin. As previously shown, ZAP (1%) increased emigrated PMN adhesion to cardiac myocytes (N=4, $P<0.05$, Figure 3.4). In contrast to circulating PMNs, addition of the anti-CD18 Ab (2E6, 8μg/ml) to emigrated PMNs did not affect adhesion to cardiac myocytes (N=4, P=NS from PMN only group). Addition of the anti-α_4 Ab (R1-2, 10μg/ml) also had no effect on adhesion (N=4, P=NS from PMN only group). Immunosuppression of both integrins with anti-CD18 and anti-α_4 Abs, however inhibited adhesion to pre-ZAP levels (N=4, $P<0.05$ compared to 1% ZAP group).

Murine PMNs express CD11b, CD18, and α_4-integrin upon emigration.

Flow cytometry fluorescence data for CD11b, CD18, and α_4-integrin on circulating and emigrated murine PMNs is summarized on Table 3.1. Mean fluorescence increased upon emigration and subsequent stimulation with ZAP for all adhesion molecules. Mean fluorescence increased from 74.6±4.3 to 311.61±3.57 for CD11b, from 89.36±25.6 to 277.34±26.15 for CD18, and from 2.31±0.06 to 17.96±1.50 for the α_4 integrin (N=4, $P<0.05$ for all adhesion molecules).

3.2 DISCUSSION

Human and rat PMNs have been shown to express new adhesion molecule profiles upon emigration [33-35], and rat PMNs utilize the newly expressed α_4-integrin, in conjunction with CD18 to adhere to parenchymal cells like cardiac myocytes [33]. The purpose of the present study was to determine if this same paradigm exists in the murine system. Indeed, our data showed that adhesion profiles of murine PMNs changed after emigration. We found that circulating murine PMNs adhered to cardiac myocytes via CD18, confirming previous results showing the importance of CD18 in circulating PMN-myocyte interaction [25]. Application of PMN stimulant, ZAP increased adhesion of emigrated murine PMNs by 2-fold. Anti-CD18 Ab however, was unable to inhibit adhesion of these PMNs. Flow cytometry confirmed the expression of the α_4-integrin upon emigration, and co-administration of anti-CD18 and anti-α_4 Abs inhibited adhesion of ZAP-pretreated emigrated PMNs to untreated control levels.

Interestingly, the addition of PMN stimulants increased adhesion of PMNs to cardiac myocytes by 2-fold. In the rat system, α_4-integrin expression in untreated

emigrated PMNs increased 5-fold after re-stimulation with fMLP [33]. It is conceivable that the increased level of α_4-integrin expression after stimulation allowed for greater ligand-ligand interactions between the α_4-integrin on PMNs with the corresponding ligand on the myocytes. The addition of PMN stimulants may have also re-mobilized some of the α_4-integrin that was expressed after emigration. Furthermore, it has been shown that both CD18 [161; 162] and the α_4-integrin [163; 164] can exist in high and low affinity states and further stimulation is required for their activation. This parallels the pathophysiological condition where PMNs are exposed to increasing gradients of stimulants as they migrate out of the vasculature to areas of inflammation. Newly mobilized Mac-1 (CD11b/CD18) required increased levels of stimulation to participate in PMN adhesion [165], and the same may be true for the α_4-integrin.

It is possible that the expression of the α_4-integrin on emigrated PMNs, and its role in adhesion to parenchymal cells, is a result of the re-internalization or shedding of β_2-integrins. Indeed, it has been proposed that Mac-1 (CD11b/CD18) was shed upon emigration of human PMNs [166]. Our flow cytometry data, showing increased expression of both CD11b and CD18 after emigration, do not support this view. Furthermore, the fact that both CD18 and α_4-integrins were necessary for adhesion to myocytes further supported our data suggesting that CD18 is indeed expressed on, and plays a role in adhesion of, emigrated PMNs.

The α_4-integrin, a 150-kD 999 amino acid protein subunit [167], can associate with either the β_1 (VLA-4) or β_7 (LPAM-1) subunit. VLA-4 can adhere to vascular adhesion molecule-1 (VCAM-1) [168] or to the cell attachment domain (CS-1) in an alternatively spliced region of fibronectin [169]. LPAM-1 can adhere to mucosal addressin cell adhesion

molecule, VCAM-1, and fibronectin [170-172]. It should be noted that the present study does not address whether the α_4-integrin was bound to the β_1 or β_7 subunit. Since LPAM-1 is predominately expressed on lymphocytes and not PMNs [172; 173], and β_1 is expressed on human and rat PMNs [174-176], it is reasonable to suggest that the β_1 subunit may have played a more dominant role in our study. Moreover, the present data does not elucidate which ligand the α_4-integrin adhered to on the myocyte. It has been suggested that in emigrated PMN-myocyte interactions in the rat system, the α_4-integrin adhered to the myocyte via fibronectin [33]. It is possible that this same ligand is used in the murine system.

3.3 LIMITATIONS

Ideally, we should have used pure populations of isolated murine circulating PMNs instead of isolating circulating leukocytes for these experiments. Unfortunately, all protocols attempted resulted in very few PMNs isolated per mouse. These protocols would have required at least 8-10 mice (8-10ml of blood) per assay and we performed 3 assays per day, resulting in a total of 25-31 mice per experiment day (24-30 for the PMNs and 1 for myocytes). The isolation of circulating leukocytes was ethically necessary to keep the number of mice used per experiment to a minimum (6 mice per experiment day for 3 assays). Staining of the slides after the adhesion assay however, revealed that those cells adherent to the myocytes were indeed PMNs and not other leukocytes.

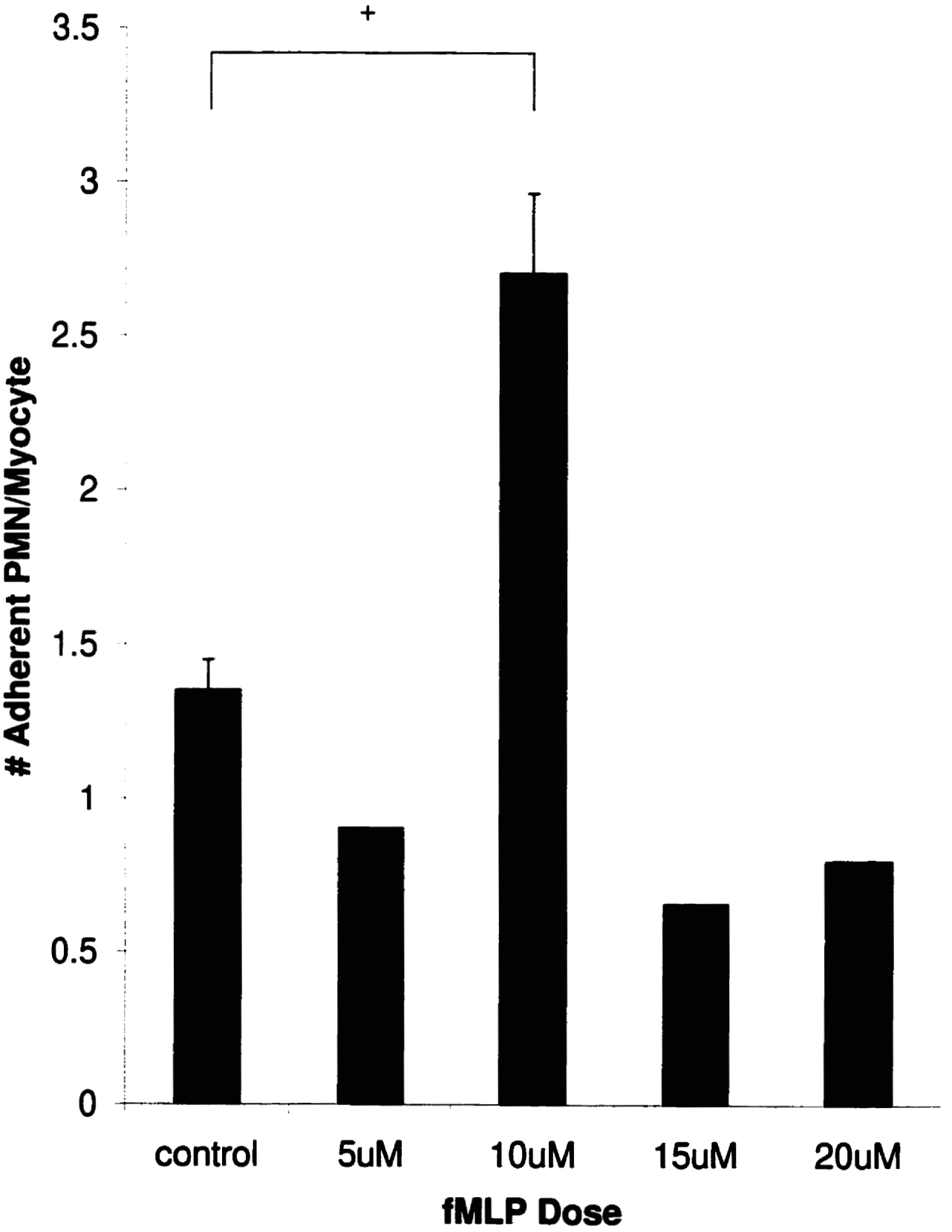

Figure 3.1 Adhesion assay dose response of fMLP pretreated emigrated PMNs to cardiac myocytes. '+' $P<0.05$

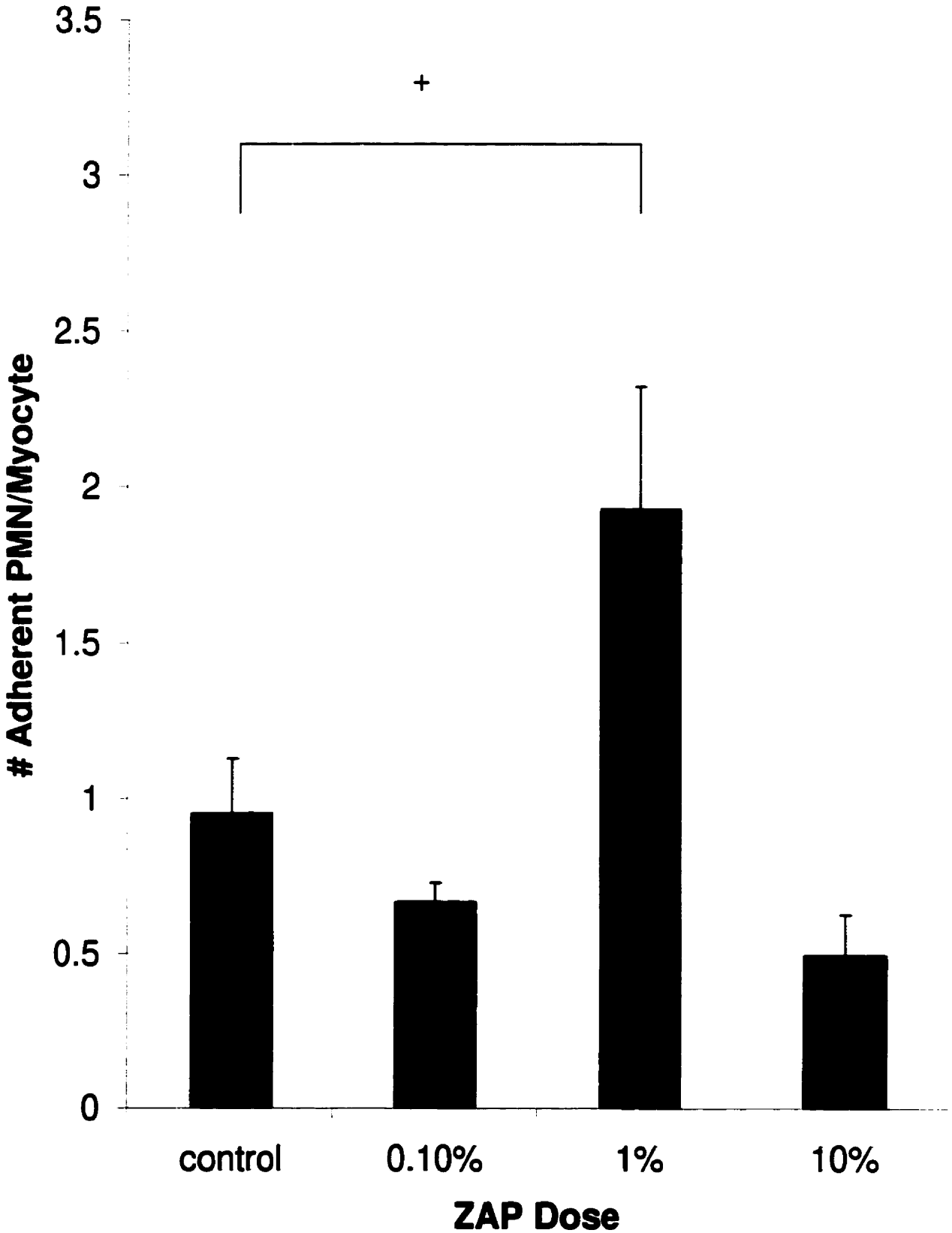

Figure 3.2 Adhesion assay dose response for ZAP pretreated emigrated PMNs to cardiac myocytes. '+' $P<0.05$

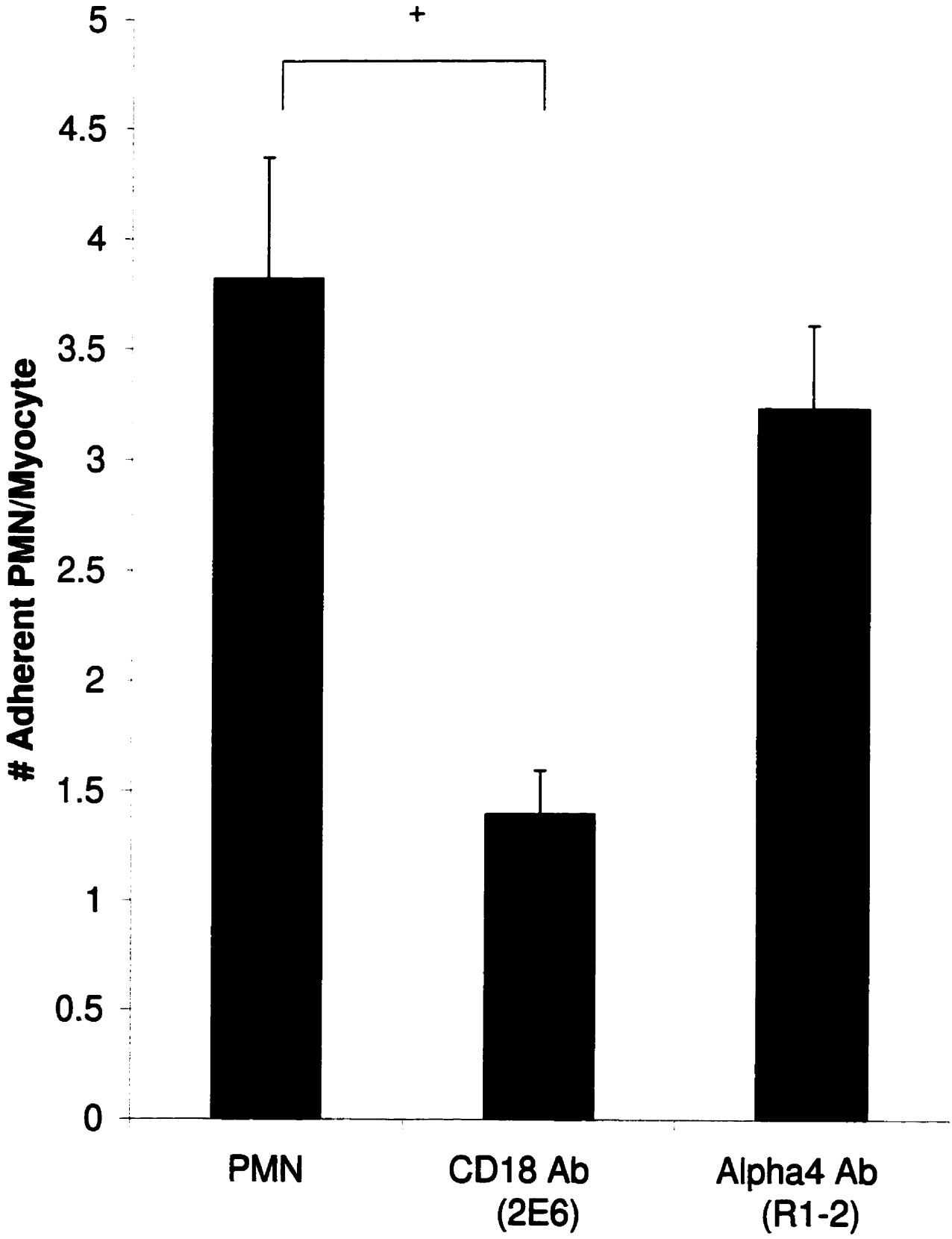

Figure 3.3 Adhesion assay of circulating PMNs to cardiac myocytes in PMN (PMNs only) N=4, CD18 Ab (PMNs + anti-CD18 Ab 2E6, 8ug/ml) N=4, and Alpha4 Ab (PMNs + anti-alpha4 Ab R1-2, 10ug/ml) N=2. '+' $P<0.05$

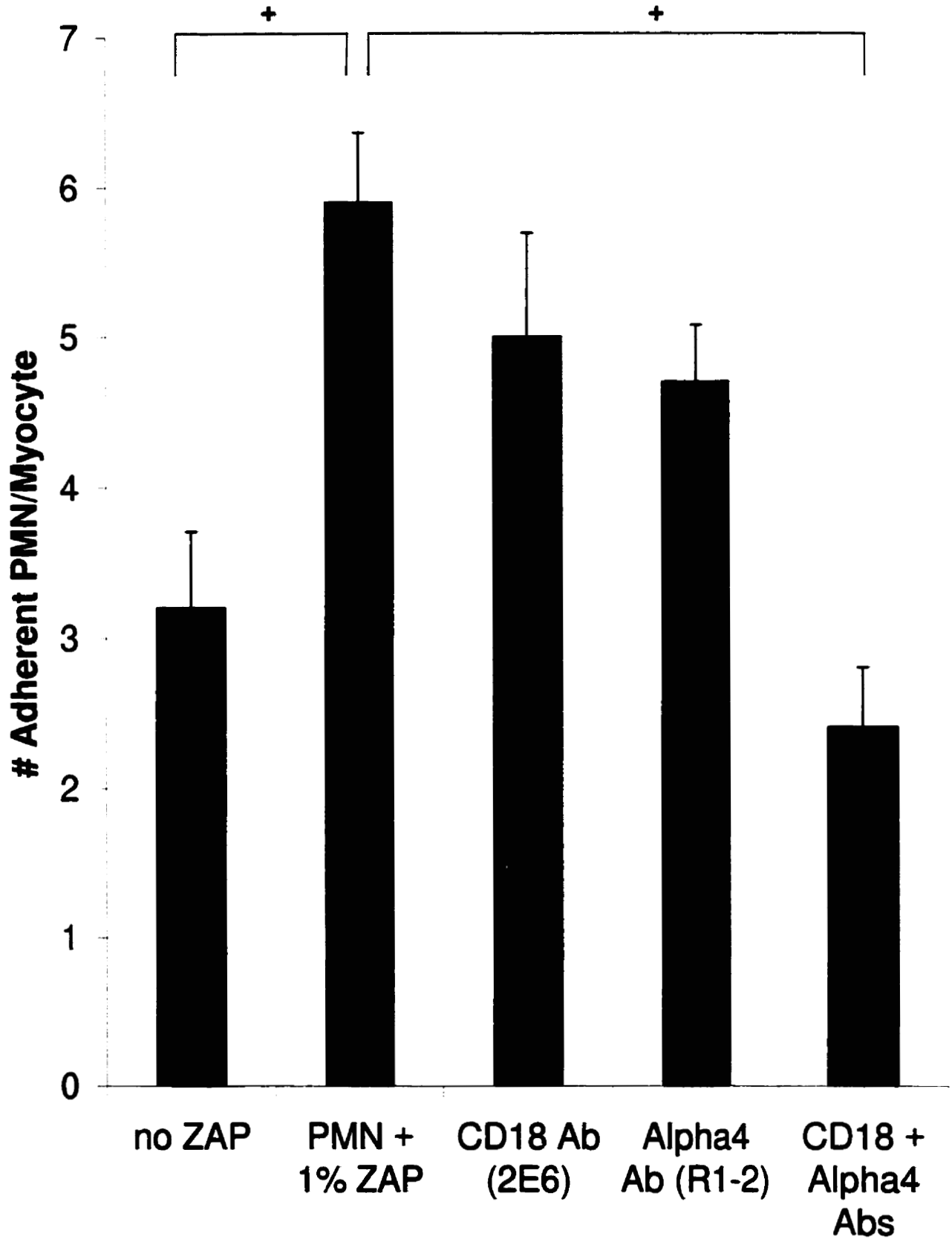

Figure 3.4 Adhesion assay of emigrated PMNs to cardiac myocytes in no ZAP (N=4), PMN + 1% ZAP (N=4), CD18 Ab (PMNs + anti-CD18 Ab 2E6, 8ug/ml) N=4, Alpha4 Ab (PMNs + anti-alpha4 Ab R1-2, 10ug/ml) N=4, and CD18 + Alpha4 Abs (N=4). '+' $P<0.05$

Mean Fluorescence

Adhesion Molecule	*Circulating PMNs*	*Emigrated PMNs*
CD11b	74.6 ±4.30	311.61 ±3.57 +
CD18	89.36 ±25.60	277.34 ±26.15 +
Alpha4	2.31 ±.060	17.96 ±1.50 +

*Mean fluorescence measurements for CD11b, CD18, and Alpha4 in circulating and emigrated murine PMNs (N=4). '+' $p<0.05$ relative to circulating PMNs.

CHAPTER 4

PMN-INDUCED MYOCYTE DYSFUNCTION VIA THE α_4-INTEGRIN

Hypothesis: Emigrated PMNs cause injury to cardiac myocytes through the α_4-integrin.

Objectives:

1) To determine if emigrated PMNs cause myocyte dysfunction, and if so, if adherence of the PMN to the myocyte is necessary for the injury to ensue.
2) To determine whether injury is mediated through either CD18, α_4-integrin, or through both ligands.
3) To determine if circulating murine PMNs injure cardiac myocytes through the same adhesive mechanism as emigrated PMNs.

4.1 RESULTS

Emigrated Murine PMNs Can Injure Cardiac Myocytes.

Figure 4.1 demonstrates a representative pattern of unloaded cell shortening observed during the entire 10min protocol in myocytes that were not exposed to PMNs. These cells were electrically stimulated at 1 Hz, and as expected, the unloaded cell shortening at the beginning and end of each experimental protocol remained unchanged. When the myocyte was exposed to isoproterenol, it showed the characteristic positive inotropic responses to β-adrenergic stimulation; 1) marked increase in the extent of cell shortening 2) faster rate of contraction and 3) increased rate of relaxation. These responses were the same at the beginning and end of each experiment. In the next series of experiments, the cells were again first exposed to isoproterenol, and then emigrated PMNs were added and allowed to adhere to the myocytes.

Figure 4.2 demonstrates a representative recording of cell shortening from this experiment: following administration of emigrated PMNs, the unloaded cell shortening

decreased by approximately 50% (from 10% to 5% cell shortening) within 5mins. This represents a very profound alteration in myocyte function that was also observed after 10mins of PMN exposure.

Adhesion of Emigrated PMNs to Myocytes is Required for the Ensuing Injury. Analysis of the data within each group by considering only those myocytes which had adherent PMNs, compared with those without adherent PMNs, demonstrated the importance of adherence via the α_4-integrin (Figure 4.3). In the group that received PMNs only (no Ab), only 1 myocyte out of 9 experiments had no adherent PMNs, and this cell did not show any change in unloaded cell shortening (Figure 4.3A, left panel). This confirms previous studies suggesting the absolute requirement of adherence in PMN-mediated myocyte dysfunction [25]. Of the remaining cells, 5 myocytes survived to the end of the experiment and these had adherent PMNs ranging from 1 to 8 per myocyte. A negative inotropic effect was measured in all of these cells at 5mins and all but one cell at 10mins (Figure 4.3A, right panel). It is noteworthy that there was no correlation between the number of adherent PMNs and the amount of cellular dysfunction since a single PMN was apparently able to induce similar amounts of myocyte dysfunction as 8 PMNs. Finally, three myocytes in this group went into contracture and died within 5mins of PMN exposure (Table 4.1) and they had 1, 2 and 4 adherent PMNs, further emphasizing the ability of as few as one adherent PMN to induce myocyte dysfunction.

In the anti-CD18 group (Figure 4.3B, left panel), 2 myocytes did not have any adherent PMNs and they showed no significant decrease in unloaded cell shortening. One of these cells showed a 17.6% decrease from baseline at 5mins, but this cell

completely recovered by 10mins. However, in 6 of 7 cells that had adherent PMNs, there was a decrease in unloaded cell shortening despite the presence of anti-CD18 Ab. These findings suggest, for the first time, that immunoneutralization of CD18 is not sufficient to completely prevent myocyte dysfunction in the presence of emigrated PMNs. Finally, 2 of the myocytes in this group, which had 1 and 3 adherent PMNs, went into contracture within the first 5mins and died (Table 4.1).

Importantly, in the anti-α_4 Ab group, 6 of the 8 myocytes had no adherent PMNs, and the majority of these myocytes showed no significant change in contractile activity (unloaded cell shortening at 5mins), although 2 of these cells showed a decline at 10mins. In this group it was very difficult to find any myocytes that supported PMN adhesion. In the two myocytes that did have adherent PMNs (1 and 4 PMNs), there was a 19% decrease in unloaded cell shortening in the former at 5mins but this cell completely recovered by 10mins. In the myocyte with 4 adherent PMNs, there was no impairment in unloaded cell shortening at either time point. In the group receiving anti-α_4 Ab, no myocytes went into contracture or failed to respond to the stimulus during the experiment (Table 4.1).

Emigrated Murine PMNs injure cardiac myocytes via the α_4-integrin.

Cumulative unloaded cell shortening data (data as a percentage of resting cell length) are shown in Figure 4.4. Control unloaded cell shortening in myocytes (not exposed to PMNs) was 10.06±1.16% (N=10). When PMNs were added to the myocytes, a reduction of approximately 50% in unloaded cell shortening was observed (N=9, $P<0.05$). Addition of anti-CD18 Ab did not protect the myocyte from the negative inotropic effect of emigrated PMNs (N=9, P=NS compared to PMN only group). The

anti-α_4 Ab, however, greatly reduced PMN-induced impairment of cell shortening at 5mins (9.42±0.94%, N=8, $P<0.05$). A similar pattern of results was observed at 10mins (Figure 4.5).

Myocyte dysfunction for all groups is summarized in Table 4.1. In the absence of PMNs, all myocytes remained viable for the entire experimental protocol and none had any signs of dysrythmia or contractile dysfunction. When the emigrated PMNs were added, 6 myocytes survived the protocol, but 4 of these exhibited dysrythmia. This abnormal activity included contractions independent of electrical stimulation, or a lack of, or delayed response to, electrical stimulation. This phenomenon was also noted in 3 of 7 myocytes in the group exposed to PMNs in the presence of anti-CD18 Ab. In contrast, none of the myocytes exposed to PMNs in the presence of anti-α_4 Ab behaved in this fashion.

Rates of contraction and relaxation for all groups are summarized in Figure 4.6. The maximal rate of contraction and relaxation did not change from baseline in the absence of PMNs (control group, N=10, P=NS). The addition of emigrated PMNs, however, significantly reduced both contraction and relaxation rates by 40% from baseline at 10mins (N=9, $P<0.05$). The addition of either anti-CD18 or anti-α_4 Ab protected the myocytes from this PMN-induced decrease in contraction and relaxation. The fact that rates of contraction and relaxation were not reduced with adherent PMNs in the group that received Abs, suggests that the reduction in contraction and relaxation rates observed in the PMN only group was not a simple physical impedance of myocytes to contract due to attached PMNs.

Circulating Murine PMNs Injure Cardiac Myocytes via CD18.

Further experiments with circulating cells showed these cells could reduce myocyte cell shortening by 35% from baseline at 5 and 10mins (N=2) (myocytes with 1 or 2 adherent PMNs) (Figure 4.7). Furthermore, the addition of anti-CD18 Ab protected the myocyte (N=2), primarily through inhibition of PMN adhesion (all experiments in the presence of anti-CD18 Ab showed myocytes with no adherent PMNs). Rates of contraction and relaxation for circulating PMNs are shown in Figure 4.8.

4.2 DISCUSSION

Previous work from our laboratory has shown that both CD18 and α_4-integrin were essential for emigrated PMN adherence to rat ventricular myocytes [33], and we have now shown the same adhesion profile in the murine myocardium. The present results extend this work and, for the first time, suggest that engagement of the α_4-integrin is critical for the ensuing myocyte damage. In our study, the anti-CD18 Ab was able to protect the myocyte from damage to mechanisms controlling contraction and relaxation rates, but was not able to protect against decreased cell shortening, myocyte dysrythmia, or contracture. This suggests that α_4-integrin, not CD18, is the dominate molecule in PMN-induced myocyte dysfunction. These observations complement and significantly extend previous studies wherein pretreatment with anti-CD18 Ab prevented PMN recruitment into tissues [4; 177; 178]. Clinically, patients arrive at hospital after, not before, an infarct at which point PMNs have already infiltrated the myocardium. Our data suggest that one could therapeutically target the emigrated PMN to prevent ongoing myocardial injury. Perhaps, both CD18 and α_4-integrin pathways need to be inhibited to completely

prevent PMN-dependent injury in these pathophysiological states wherein the endothelium is injured by circulating PMNs and myocytes are injured by emigrated PMNs.

Previous reports have described the ability of integrins to receive signals from outside the cell that can, in turn, signal the release of cytotoxic mediators from within the cell [179]. Indeed, engagement of CD18 on PMNs leads to reorganization of the cytoskeleton, oscillating cystolic free Ca^{2+} levels, shape change, and subsequent secretion of granule proteins and oxidants [180; 181]. This type of outside-in signaling can also be mediated by the α_4-integrin. Signal transduction through the α_4-integrin activates protein tyrosine kinase activity in T cells [182], and engagement of this fibronectin receptor induces gene expression of enzymes, including collagenase and metalloproteinase stromelysin in fibroblasts [183]. The second messenger pathways regulated by the α_4-integrin in PMNs have yet to be explored. Since both adhesion pathways are involved in emigrated PMN adhesion to myocytes, one might expect that inhibition of either CD18 or α_4-integrin would lead to protection. Our study would suggest that adherence of emigrated PMNs only minimally activated a CD18-dependent pathway of injury.

CD18 is upregulated on the PMN in response to stimulants in the vasculature, which allows for firm adhesion to the endothelium and subsequent emigration [184]. Since the CD18 integrin has already been exposed to a stimulus prior to emigration, it has already engaged its ligand. It is conceivable that CD18 can no longer respond to a stimulus after emigration and therefore is much less effective in initiating the release of specific cytotoxins from the PMN. In this study, and in previous studies, the α_4-integrin is expressed at only very low levels on circulating PMNs, and this expression level is

increased following emigration and stimulation [33; 185]. Thereafter, α_4-integrin is ready to engage its receptor and signaling via this ligand may be possible. The binding of the α_4-integrin to its ligand on the myocyte may cause a release of proteases and oxidants from the PMN, which can directly degrade the extracellular matrix. This may lead to changes in membrane potential or integrity, thereby affecting the availability of cystolic Ca^{2+} and thus decrease the magnitude of cell shortening.

It is intriguing that the PMNs appear to injure myocytes in a time and site specific manner. This is evidenced by the fact that global dysfunction did not occur in individual myocytes at the same time periods. Although we observed a very profound decrease in cell shortening at 5mins after PMN exposure with no Ab, we did not see a change in rate of contraction or relaxation until 10mins. These results suggest that the emigrated PMN was able to reduce cell shortening, before impacting upon contraction or relaxation mechanisms. It is well appreciated that the degree of contraction, the rate of contraction, and the rate of relaxation are all mediated by different ionic events. This raises the possibility that the myriad of molecules released by the PMN impacts on ion channels through phosphorylation of proteins with differing degrees of efficiency. Furthermore, it is possible that the PMN-induced damage is initially restricted to the sarcolemma, affecting L-type Ca^{2+} channels, which trigger contraction by initiating a much larger Ca^{2+} release from the SR. PMNs may subsequently cause membrane depolarization, which could further reduce Ca^{2+} influx via L-type Ca^{2+} channels. At later times, intracellular organelles essential for excitation-contraction coupling and Ca^{2+} homeostasis may be compromised. A decrease in Ca^{2+}-induced Ca^{2+} release from the SR

results in a decreased rate of contraction. Moreover, the Ca^{2+} pump in the SR and the rate of relaxation may also be significantly affected.

Our results demonstrate that unlike circulating PMNs, emigrated PMNs use α_4-integrin to mediate the myocyte damage induced by PMNs. To date most studies have focussed on the mechanisms by which PMNs adhere to the endothelium and infiltrate the myocardium, with the goal of targeting this mechanism to reduce the injury associated with pathophysiological conditions like myocardial infarction. The time window of opportunity to intervene in the recruitment process may be so brief however, that therapy may only work prophylactically (i.e. patients already have PMNs in the myocardium upon arrival at hospital). Our results provide a novel basis for therapeutic intervention on the PMN that one may target even after this leukocyte has reached the myocardium. This approach has the potential to reduce or prevent myocyte dysfunction without affecting PMN function in the circulation.

4.3 LIMITATIONS

The adhesion chamber data from the previous chapter was obtained using a static assay. PMNs were added to the chambers and allowed to settle by gravity onto the myocytes. In contrast, the cell shortening assay is not a static model. The PMNs were superfused over the myocytes and then subsequently washed away with buffer. Furthermore, we used $5X10^6$ PMN/ml in the adhesion assay, and only $1X10^6$ PMNs/ml in the cell shortening assay to limit the number of mice used per study. This difference in PMN concentration explains why more PMNs adhered per myocyte in the adhesion assay compared to the cell shortening assay.

Moreover, it was very difficult to find PMNs adherent to the myocytes in the cell shortening assay when anti-α_4 Ab was present. Data in the previous chapter clearly showed that both anti-CD18 and -α_4 Abs were required to inhibit adhesion in the static model. In the presence of anti-α_4 Ab, PMNs adhere via a CD18-ICAM-1 pathway. It has been shown that the adhesion of leukocytes to VCAM-1 coated cover slips increased at lower shear force (minimal adhesion at 20dynes/cm^2 and maximal adhesion at 5dynes/cm^2) [186]. It is not possible to calculate shear forces in our cell shortening model, but it is apparent that there is more shear stress in the cell shortening model where myocytes are subjected to buffer flow rates of 1ml/min as compared to the static state in the adhesion chambers. It is possible that this same effect is seen with CD18 and ICAM-1.

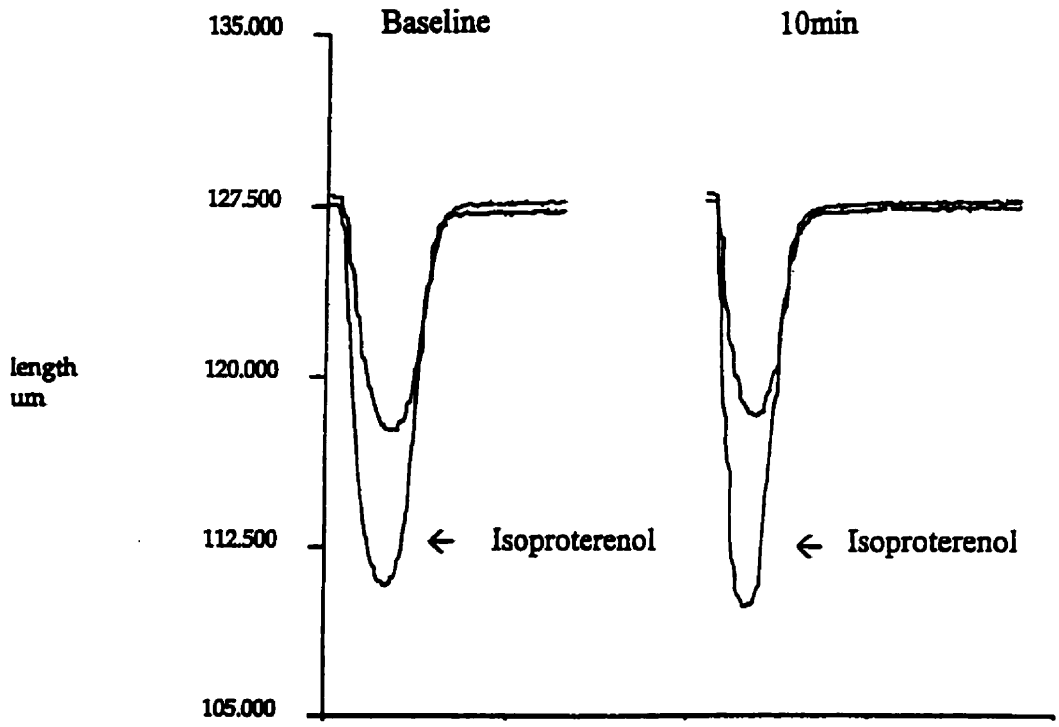

Figure 4.1 Representative cell shortening trace of a control cardiac myocyte before and after isoproterenol challenge.

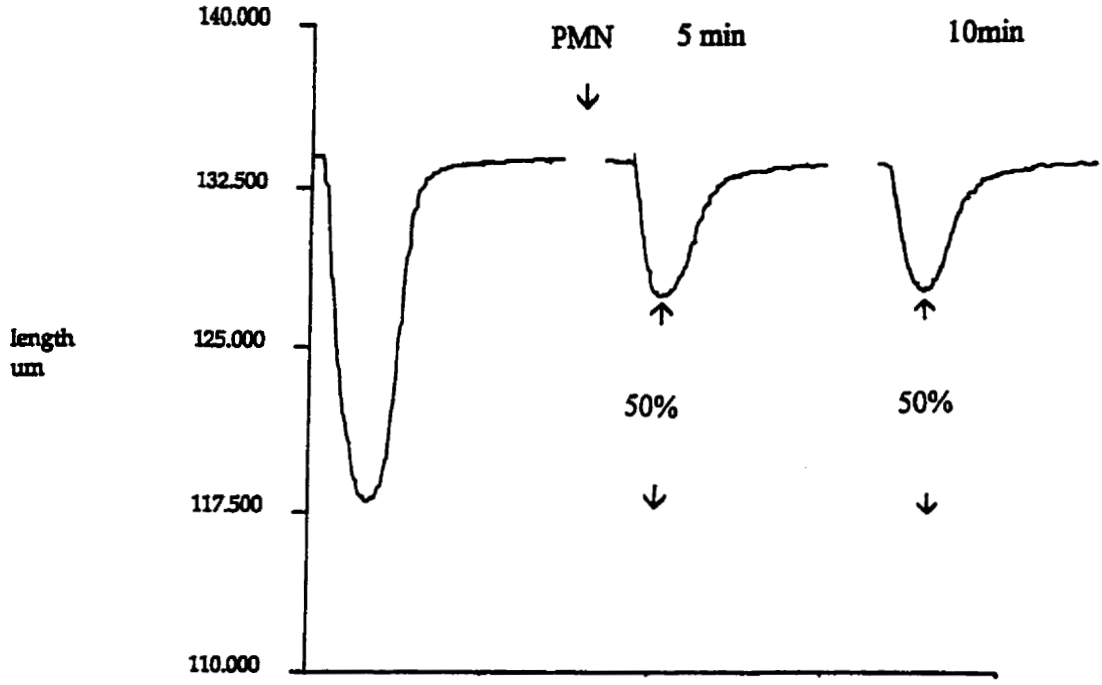

Figure 4.2 Representative cell shortening trace of a cardiac myocyte + emigrated PMN at 5 and 10mins.

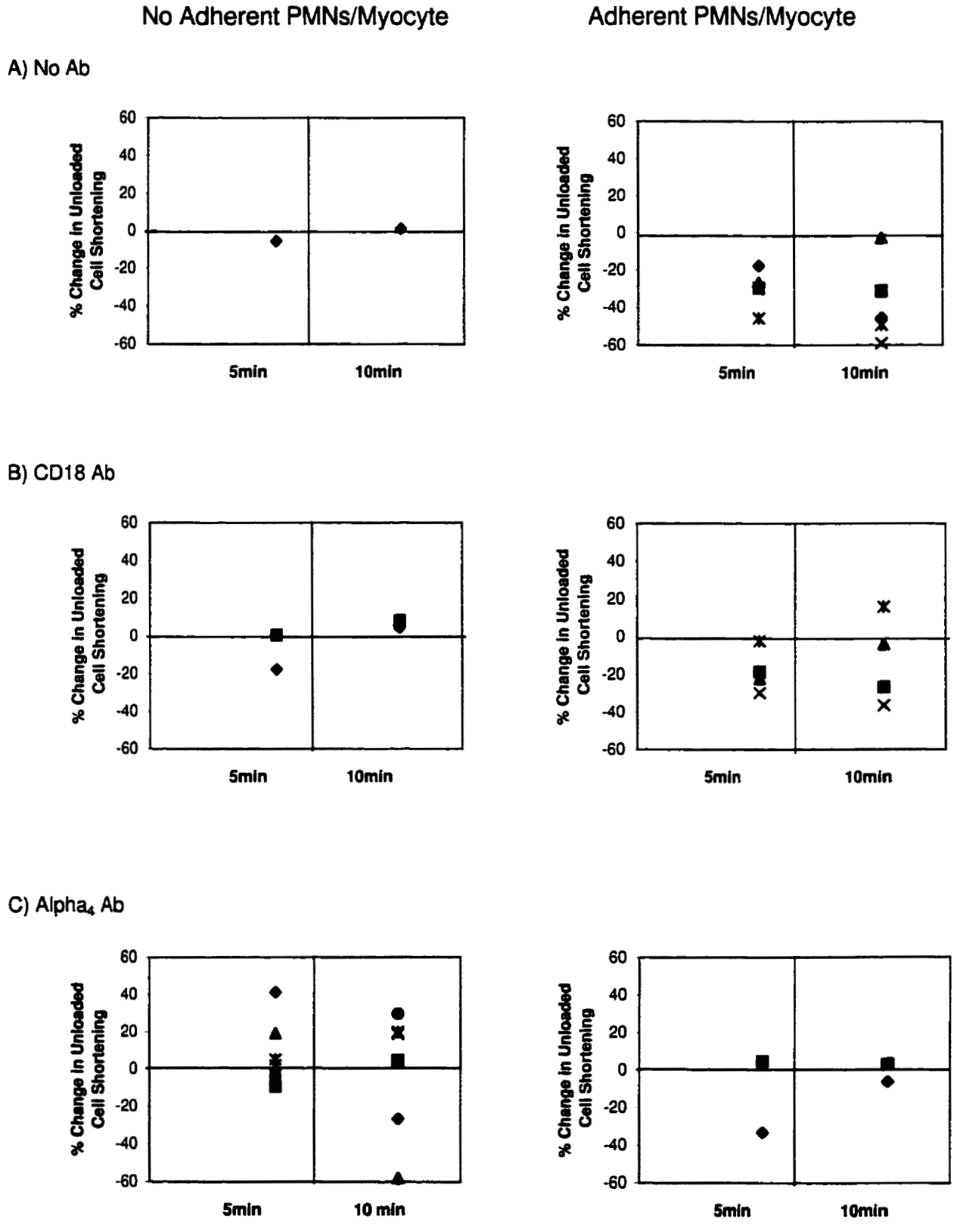

Figure 4.3 Unloaded cell shortening expressed in terms of whether or not there were adherent PMNs for A) PMNs only, B) CD18 Ab (PMNs + anti-CD18 Ab 2E6, 8ug/ml), and C) Alpha4 Ab (PMNs + anti-alpha4 Ab R1-2, 10ug/ml)

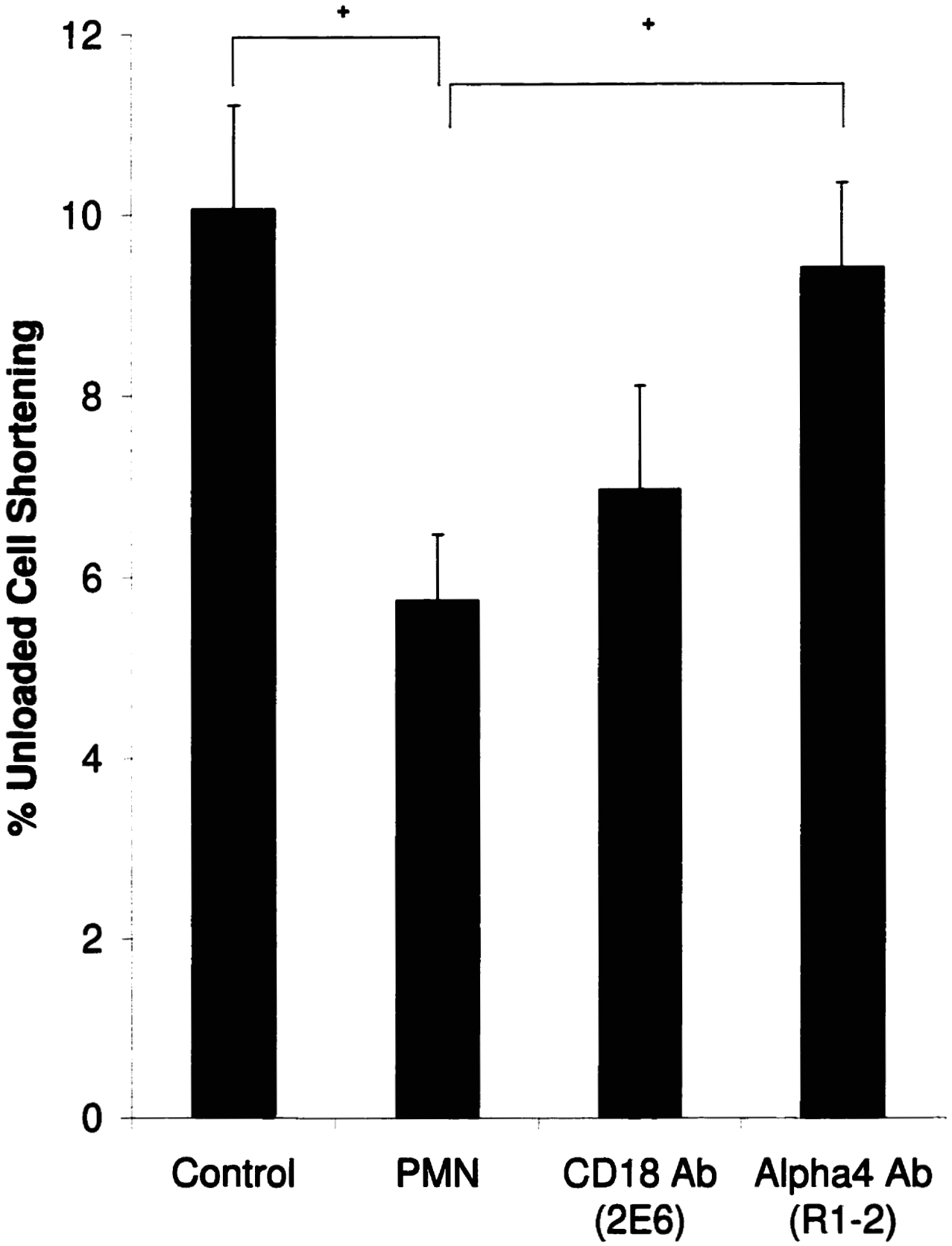

Figure 4.4 Unloaded cell shortening in control (no PMNs) N=10, PMN (PMN only) N=9, CD18 Ab (PMN + anti-CD18 Ab 2E6, 8ug/ml) N=9, and Alpha4 Ab (PMN + anti-alpha4 Ab R1-2, 10ug/ml) N=8 at 5mins. '+' $P<0.05$

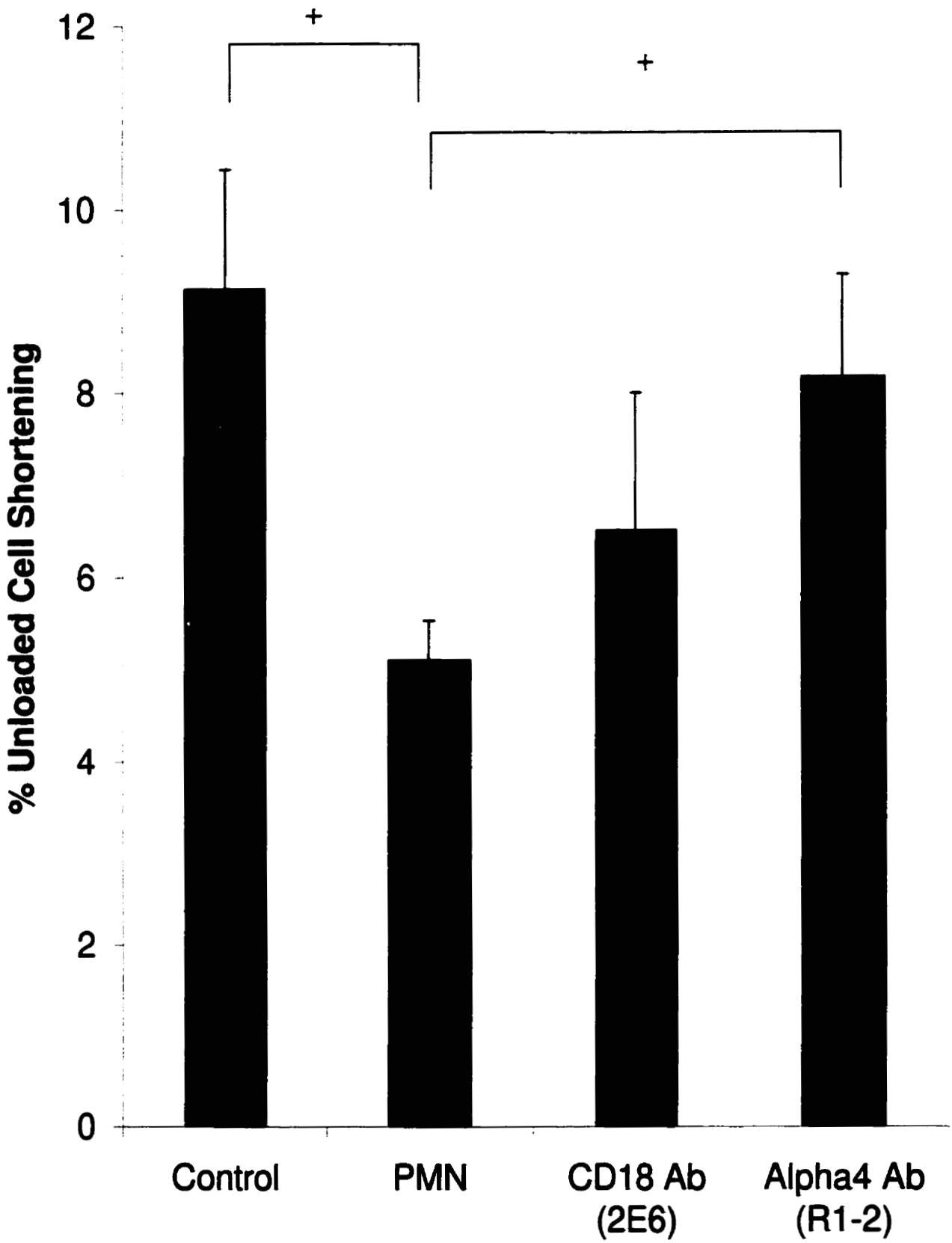

Figure 4.5 Unloaded cell shortening in Control (no PMN) N=10, PMN (PMN only) N=9, CD18 Ab (PMN + anti-CD18 Ab 2E6, 8ug/ml) N=9, and Alpha4 Ab (PMN + anti-aplha4 Ab R1-2, 10ug/ml) N=8 at 10mins. '+' "P<0.05

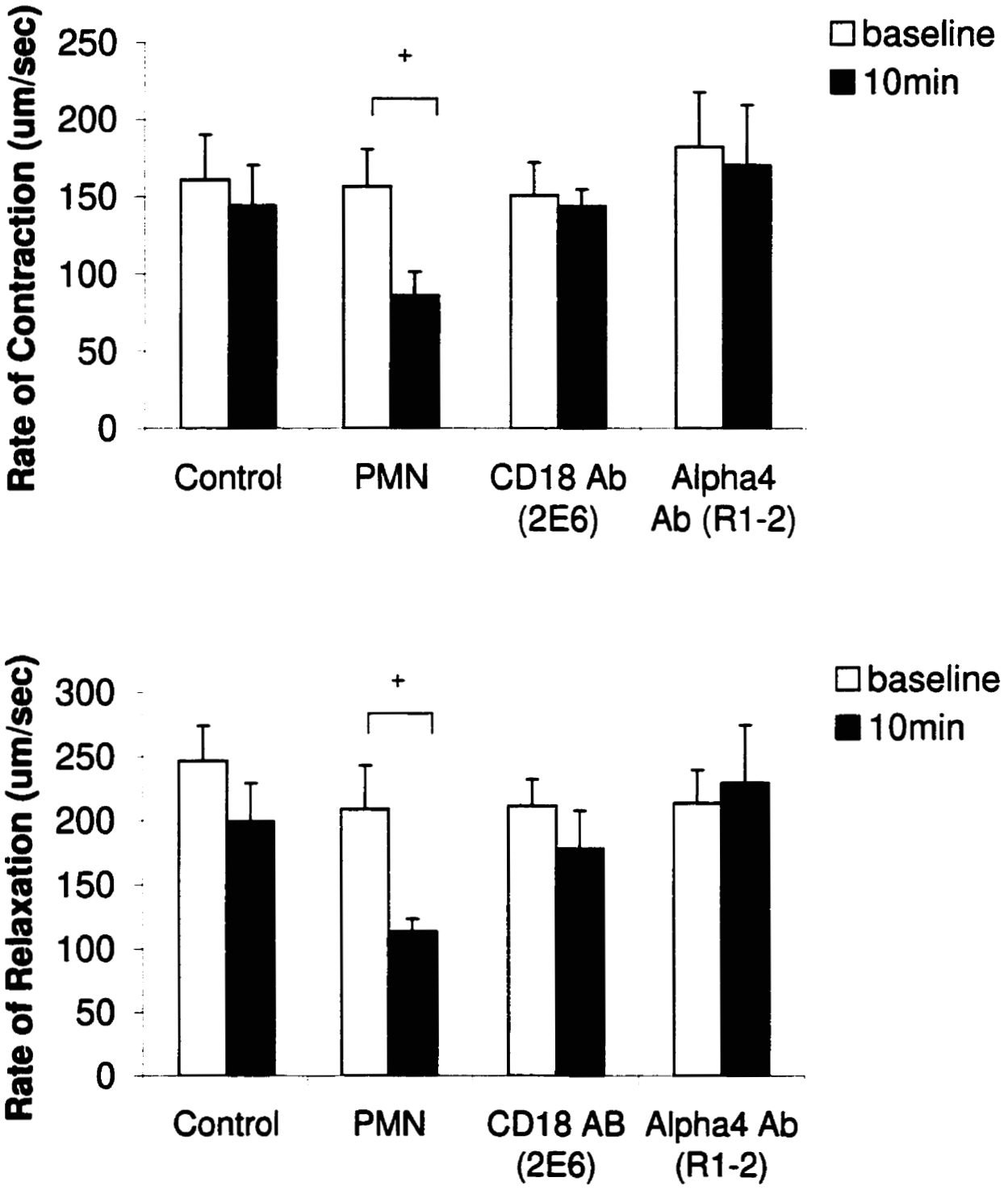

Figure 4.6 Change in rate of contraction and relaxation in Control (no PMNs) N=10, PMN (PMN only) N=9, CD18 Ab (PMNs + anti-CD18 AB 2E6, 8ug/ml) N=9, and Alpha4 Ab (PMNs + anti-alpha4 Ab R1-2, 10ug/ml) N=8. '+' $P<0.05$

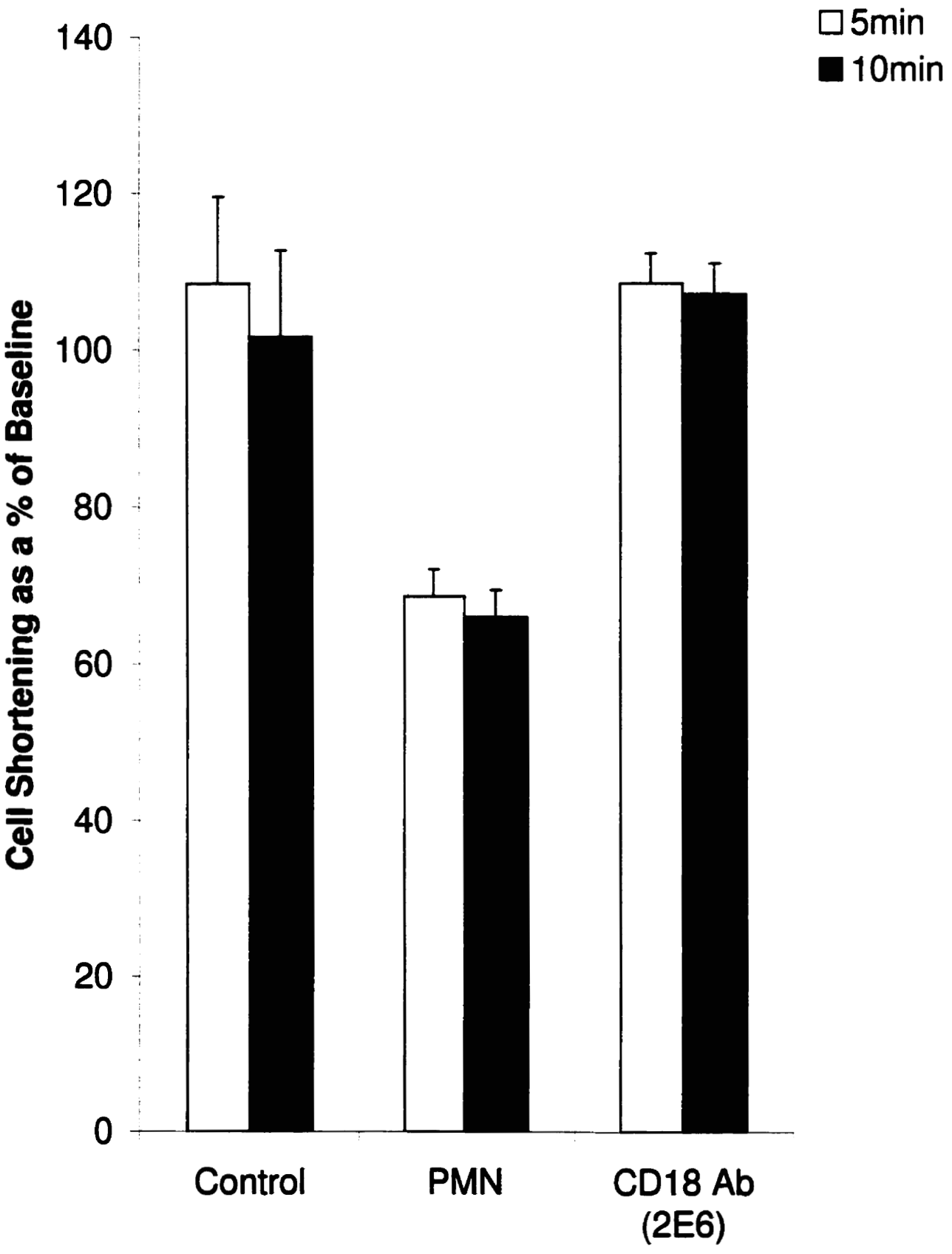

Figure 4.7 Unloaded cell shortening with circulating PMNs in Control (no PMNs) N=2, PMN (PMN only) N=2, and CD18 Ab (PMNs + anti-CD18 Ab 2E6, 8ug./ml) N=2. Each experiment was completed on 2 seperate days

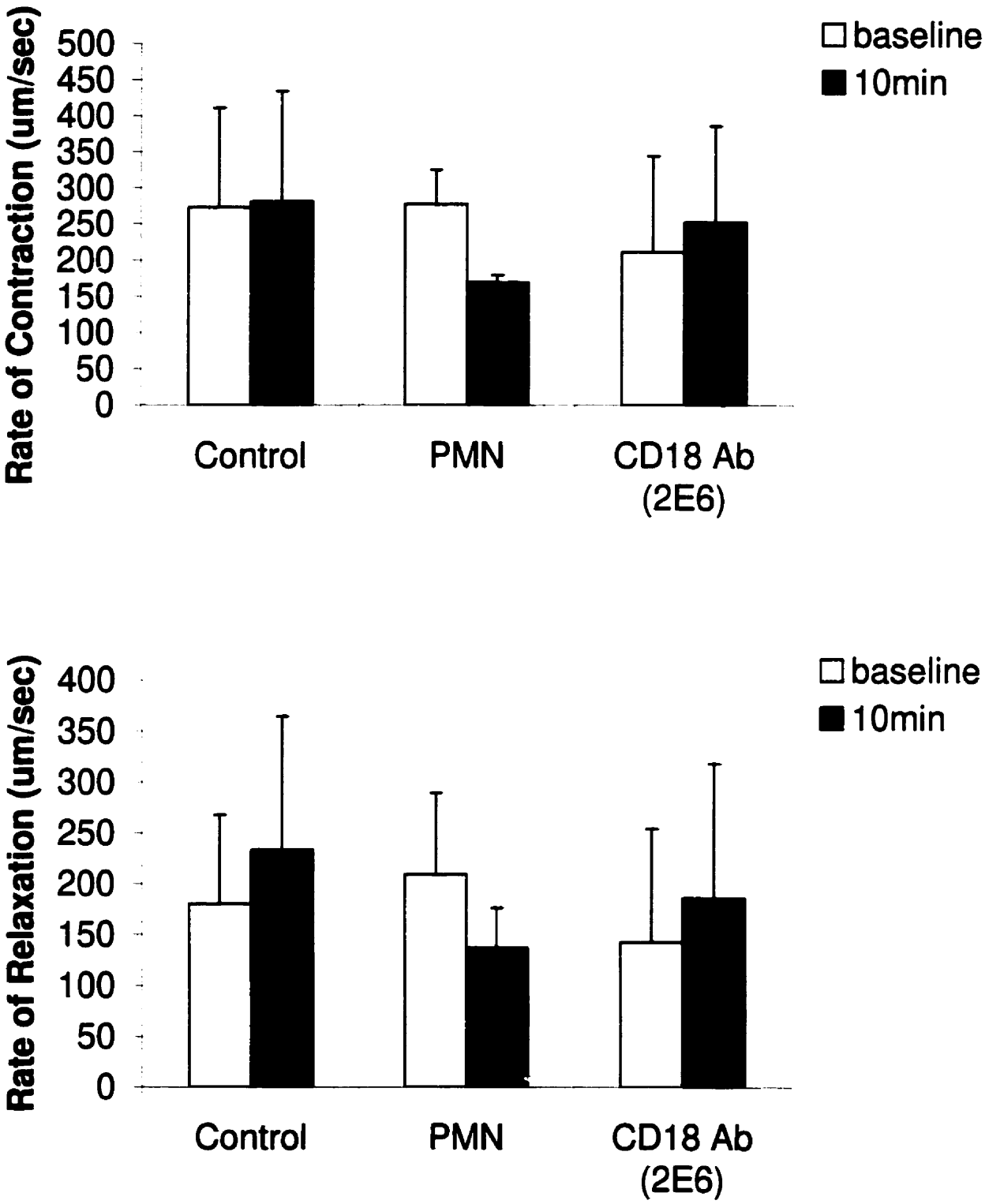

Figure 4.8 Change in rates of contraction and relaxation with circulating PMNs in Control (no PMNs) N=2, PMN (PMN only) N=2, and CD18 Ab (PMN + anti-CD18 Ab 2E6, 8ug/ml) N=2. Each experiment was completed on 2 seperate days

Group	Baseline (N)	Contracture (N)	Dysrythmia (N)	% Dysfunction
Control	10	0	0	0%
PMN	9	3	4	78%
CD18 Ab (2E6)	9	2	3	56%
Alpha4 Ab (R1-2)	8	0	0	0%

*Functional observations of myocytes in Control (no pPMNs), PMN (PMN only), CD18 Ab (PMNs + anti-CD18 Ab 2E6, 8ug/mL), and Alpha4 (PMNs + anti-alpha4 Ab R1-2, 10ug/mL). Those cells that appear in the Dysrythmia column are not the same cells as those that appear in the Contracture column.

CHAPTER 5

α_4-INTEGRIN MODULATES FREE RADICAL INJURY TO CARDIAC MYOCYTES

Hypothesis: PMN-induced myocyte injury is caused by the generation of free radicals.

Objectives:

1) To determine if PMNs require a respiratory burst to cause myocyte damage.
2) To visualize the production of free radicals upon PMN adhesion to the myocyte.
3) To determine whether free radical generation is mediated through either CD18 or α_4-integrin.
4) To determine if the specific free radical responsible for myocyte injury is $O_2^{\cdot-}$.

5.1 RESULTS

PMN-Derived Free Radical Injury to Cardiac Myocytes.

Figure 5.1 shows unloaded cell shortening (as a % of baseline) for all groups. Cell shortening of myocytes alone did not change after 5mins of electrical stimulation (N=6). The addition of WT PMNs caused an approximately 40% reduction in cell shortening within 5mins of PMN exposure (N=7, $P<0.05$). PMNs deficient in NADPH oxidase (the enzyme necessary to generate free radicals) were unable to cause a decrease in the inotropic response of the myocytes (cell shortening levels at 96.8±8.9% of baseline at 5mins, N=6, $P<0.05$ compared to PMN only group). A similar pattern was observed after 10mins of exposure to these PMNs (Figure 5.2).

Myocyte dysfunction is shown in Table 5.1. Control myocytes alone showed no myocyte dysrythmia, but the addition of WT PMNs caused 5 of the 7 myocytes recorded to become dysrythmic. When PMNs from NADPH oxidase deficient mice were added to the myocytes, only 1 of the 6 myocytes recorded showed any dysrythmia.

Rates of contraction and relaxation of myocytes for these groups are shown in Figure 5.3. The maximal rate of contraction and relaxation did not decrease from baseline at 10mins in the control group (N=6, *P*=NS). The addition of WT emigrated PMNs decreased both contraction and relaxation rates by approximately 30% from baseline at 10mins (N=6, $P<0.05$). PMNs from NADPH oxidase deficient mice, however could not affect either contraction or relaxation rates of the myocytes (N=6, *P*=NS).

To ensure that PMNs from NADPH oxidase deficient mice did not produce free radicals, the cytochrome *c* reduction assay was performed on these PMNs and results showed O_2^- levels below the detectable limits of the assay (Figure 5.4). PMNs from WT mice, however showed O_2^- levels at 14.61 ± 1.17nM/10^7 cells/min.

Emigrated PMNs Cause α_4-mediated Free Radical Production in Cardiac Myocytes. Single cell imaging of an adherent WT PMN to a myocyte is shown in Figure 5.5. Fluorescent images at baseline, 5mins and 10mins of PMN exposure are shown. Fluorescence begins at the point of PMN adhesion and spreads with time throughout the myocyte. Figure 5.6 shows quantitative fluorescence intensity changes at 5mins of PMN exposure. Myocyte controls show minimal increase in fluorescence (5.20 ± 1.80 raw intensity units above baseline, N=5). The addition of emigrated WT PMNs caused a 6-fold increase in fluorescence levels over controls (N=6, $P<0.05$). Addition of an anti-CD18 Ab reduced fluorescence levels to 17.20 ± 2.50 units above baseline, although not statistically significantly different from PMN only group (N=6, *P*=NS). Addition of an anti-α_4 Ab, however reduced fluorescence back to control levels (N=4, $P<0.05$ compared to PMN only group). Similar patterns were also seen at 10mins (Figure 5.7).

Extracellular, but not Intracellular SOD Protects Myocytes From PMN-Induced Injury. Unloaded cell shortening using myocytes isolated from SOD over-expressing mice is shown in Figure 5.8. There was no difference in cell shortening between WT and SOD myocytes alone (N=4, *P*=NS). The addition of WT emigrated PMNs caused a reduction in cell shortening in both these myocyte groups. Cell shortening decreased by approximately 30% in both the WT myocyte group (N=4, $P<0.05$ compared to WT control), and in the SOD myocyte group (N=5, $P<0.05$ compared to SOD control), at 5mins of PMN exposure. A similar pattern was observed for both WT and SOD myocytes at 10mins of PMN exposure (Figure 5.9). There was no dysrythmia recorded for WT or SOD myocytes alone (Table 5.2). The addition of WT PMNs caused dysrythmia in 3 of the 4 WT myocytes, but only 1 of the 5 SOD myocytes.

Rates of contraction and relaxation of SOD and WT myocytes are shown in Figure 5.10. Myocytes alone, from either WT or SOD mice, were able to maintain rates of contraction and relaxation at baseline levels throughout the 10min experimental period (N=4, *P*=NS). The addition of WT PMNs caused a 55% reduction in contraction rate in both WT and SOD myocytes, and a 57% and 46% reduction in relaxation rates in WT and SOD myocytes, respectively (N=4 for WT PMN group and N=5 for SOD Myocyte + WT PMN group, $P<0.05$).

Unloaded cell shortening with exogenous SOD treatment is shown in Figure 5.11. 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 (N=4, $P<0.05$). The addition of SOD to the PMNs protected the myocytes from injury (cell shortening at 96.76±2.79% of baseline at 5mins, N=3, $P<0.05$ compared to PMN only group). A

similar pattern was observed at 10mins of PMN exposure (Figure 5.12). There was no dysrythmia recorded for myocytes alone (Table 5.3). The addition of PMNs caused dysrythmia in all of the myocytes recorded. When exogenous SOD was present, none of the myocytes recorded showed any dysrythmia.

Rates of contraction and relaxation of these myocytes are shown in Figure 5.13. Control myocytes maintained both contraction and relaxation rates at baseline levels throughout the 10min experimental period (N=4, *P*=NS). The addition of PMNs alone caused a 40% reduction in contraction rate and a 32% reduction in relaxation rate at 10mins (N=4, $P<0.05$). Exogenous SOD protected against PMN-induced decreases in contraction and relaxation rates (contraction rate at 301.48±83.97μm/sec at baseline and 289.76±99.32μm/sec at 10min, N=3, *P*=NS; and relaxation rate at 201.37±77.82μm/sec at baseline and 198.87±79.59μm/sec at 10mins, N=3, *P*=NS).

5.2 DISCUSSION

A previous chapter in this thesis has shown that emigrated murine PMNs express the α_4-integrin, and use this ligand to mediate PMN-induced myocyte damage. The present results extend this work and, for the first time, show that emigrated PMNs injure cardiac myocytes through an α_4-integrin-coupled free radical pathway. Emigrated PMNs from WT mice in the present study were able to induce myocyte dysfunction. In contrast, emigrated PMNs isolated from NADPH oxidase deficient mice could not affect contractile responses of the myocytes. Cell shortening, rate of contraction, and rate of relaxation were not significantly affected by these PMNs. Furthermore, the number of

myocytes that displayed signs of dysrythmia was dramatically reduced when the PMNs could not generate free radicals.

A previous study has shown the importance of free radical generation in the ability of circulating PMNs to damage cardiac myocytes [25], suggesting that circulating and emigrated PMNs use the same process to cause PMN-induced myocyte injury. Our single cell imaging measurements show that the adhesion of emigrated PMNs to isolated cardiac myocytes caused a dramatic rise in the level of oxidants produced within the myocyte. This observation is in direct agreement with the proposed role of free radicals in circulating PMN-induced myocyte damage. The previous study showed that free radical generation by circulating PMNs was CD18 dependent [25]. Our work demonstrates, for the first time, that the α_4-integrin was coupled to free radical production by emigrated PMNs. The addition of an anti-α_4 Ab inhibited oxidant production. In fact, oxidant levels were at myocyte only control levels even though PMNs continued to adhere to the myocyte via CD18. These findings also suggest that as the PMN emigrates out of the vasculature, the CD18-NADPH oxidase becomes uncoupled or now requires the α_4-integrin. Immunosuppression of the α_4-integrin inhibited all parameters of emigrated PMN-induced myocyte dysfunction, almost certainly by inhibiting oxidative stress. These results provide important new evidence for the functional role of the α_4-integrin in PMN-dependent myocyte injury.

It is well documented that integrins are involved in cell signaling events. Current models postulate that when ligands are engaged, a multitude of different kinases bound to the cytoplasmic tail of integrins, can initiate signaling cascades within the cell [187]. Signaling through the α_4-integrin resulted in protein kinase C (PKC) activation in murine

T cells [188], an event known to phosphorylate a key protein ($p47^{phox}$) in the NADPH oxidase complex. Indeed, $p47^{phox}$ was phosphorylated by purified PKC in a cell-free system [189-191]. $p47^{phox}$ is a cytosolic protein with a Src homology 3 (SH3) domain structural motif involved in specific molecular interactions during signal transduction [192]. $p47^{phox}$ is critical for oxidase activation in intact cells, and the SH3 domain has been implicated in the assembly and maintenance of the NADPH oxidase components in PMNs [193]. We hypothesize that the adhesion of emigrated PMNs to cardiac myocytes causes PKC activation within the PMN and subsequent $p47^{phox}$ phosphorylation. The multi-component NADPH oxidase within the PMN is then assembled for activation, and free radicals can be produced by the PMN. It is possible that this signaling pathway could also be important for the ongoing activation of the NADPH oxidase complex. Although unproven, theoretically this signaling mechanism could be playing a role in the α_4-integrin dependent free radical generation observed in our model.

The addition of an anti-CD18 Ab had only a weak inhibitory effect on oxidant production in myocytes (not significant). These data are consistent with results in a previous chapter where anti-CD18 Ab only partially protected the myocyte from emigrated PMNs by inhibiting the decrease in the rate of contraction and relaxation at 10mins of PMN exposure. The inability of the anti-CD18 Ab to affect the decrease in cell shortening or dysrythmia could be related to the ability of this Ab to only partially inhibit oxidant generation. It is feasible that a minimal level of extracellular free radicals is necessary to affect ion channels (like K^+ or Na^+ channels) on the myocyte surface and thus indirectly affect Ca^{2+}-handling within the myocyte. These alterations may then affect the rate of contraction and relaxation. Damage to mechanisms that control

myocyte contractility, like L-type Ca^{2+} channels in the myocyte plasma membrane, may require a lower level of free radical generation. The ability of extracellular and not intracellular SOD to protect against PMN-induced injury implies that the PMN is causing the injury from the outside of the myocyte and that the production of oxidants within the myocyte is not the critical pathway of myocyte injury.

Although oxidant levels were measured, it is uncertain which molecule had the predominant role in emigrated PMN-induced free radical injury. Over-expression of endogenous Cu/Zn SOD in the myocytes was not able to protect the cell from emigrated PMN-induced decreases in cell shortening, rates of contraction or relaxation, but was able to inhibit the number of myocytes that were dysrythmic. It is possible that scavenging O_2^- was only protective to the resting potential of the myocyte, enabling it to become properly depolarized upon electrical stimulation. The data suggest that the free radical predominantly responsible for the decrease in myocyte contractile properties is not intracellular O_2^-. The addition of exogenous SOD, however resulted in protection against all parameters of myocyte dysfunction induced by the PMNs. This data implies that the PMN is releasing O_2^- upon adhesion to the cardiac myocyte and that this O_2^- then causes detrimental changes to the ionic properties of the myocyte from the outside of the cell.

We can conclude from the present study that emigrated PMN-induced myocyte injury is an O_2^- dependent event. The exact mechanism of free radical injury however, remains unknown. It is conceivable that O_2^- caused direct damage to the myocyte, or alternatively, O_2^- may have reacted with NO to produce $ONOO^-$. Finally, O_2^- may have entered the Fenton reaction to form OH^-. All of these free radical intermediates have the

potential to cause the myocyte dysfunction observed in this thesis. Furthermore, we cannot exclude the involvement of proteases in this PMN-induced myocyte dysfunction. It is well recognized that free radicals can activate certain proteases by inactivating proteinase inhibitors [194; 195], and it has been proposed that proteases play an important role in myocardial injury in myocardial infarction [196-198].

Our results demonstrate that emigrated PMNs, like circulating PMNs, use free radicals to injure cardiac myocytes. However, the generation of free radicals in myocytes induced by emigrated PMNs is coupled in a more dominant manner by the α_4-integrin, not by CD18. This is an important finding as to date most studies I reference have concentrated on the study of circulating PMN-myocyte interactions and have shown that immunosuppression of CD18 could protect the myocyte from free radical-induced injury. Previous studies have not considered the potential role of the α_4-integrin, mainly because the α_4-integrin was not thought to be expressed by PMNs. The interaction of circulating PMNs and cardiac myocytes can never occur in the physiological or pathophysiological state. PMNs must first leave the vasculature and these emigrated PMNs express a different adhesion molecule profile, including the expression of the α_4-integrin [33-35]. Our data show that immunosuppression of the α_4-integrin limits both the generation of free radicals and the subsequent PMN-induced myocyte dysfunction, and provides a rational basis for potential therapies that could benefit patients suffering from pathophysiological conditions like myocardial infarction.

5.3 LIMITATIONS

Since the fluorescent probe DCFH is not specific for O_2^-, we could not confirm

that O_2^- levels in the myocytes were indeed decreased when PMNs were added to the SOD over-expressing myocytes. Although these myocytes have a 10-fold increase in endogenous Cu/Zn SOD [160], ideally we should confirm that O_2^- levels were indeed affected.

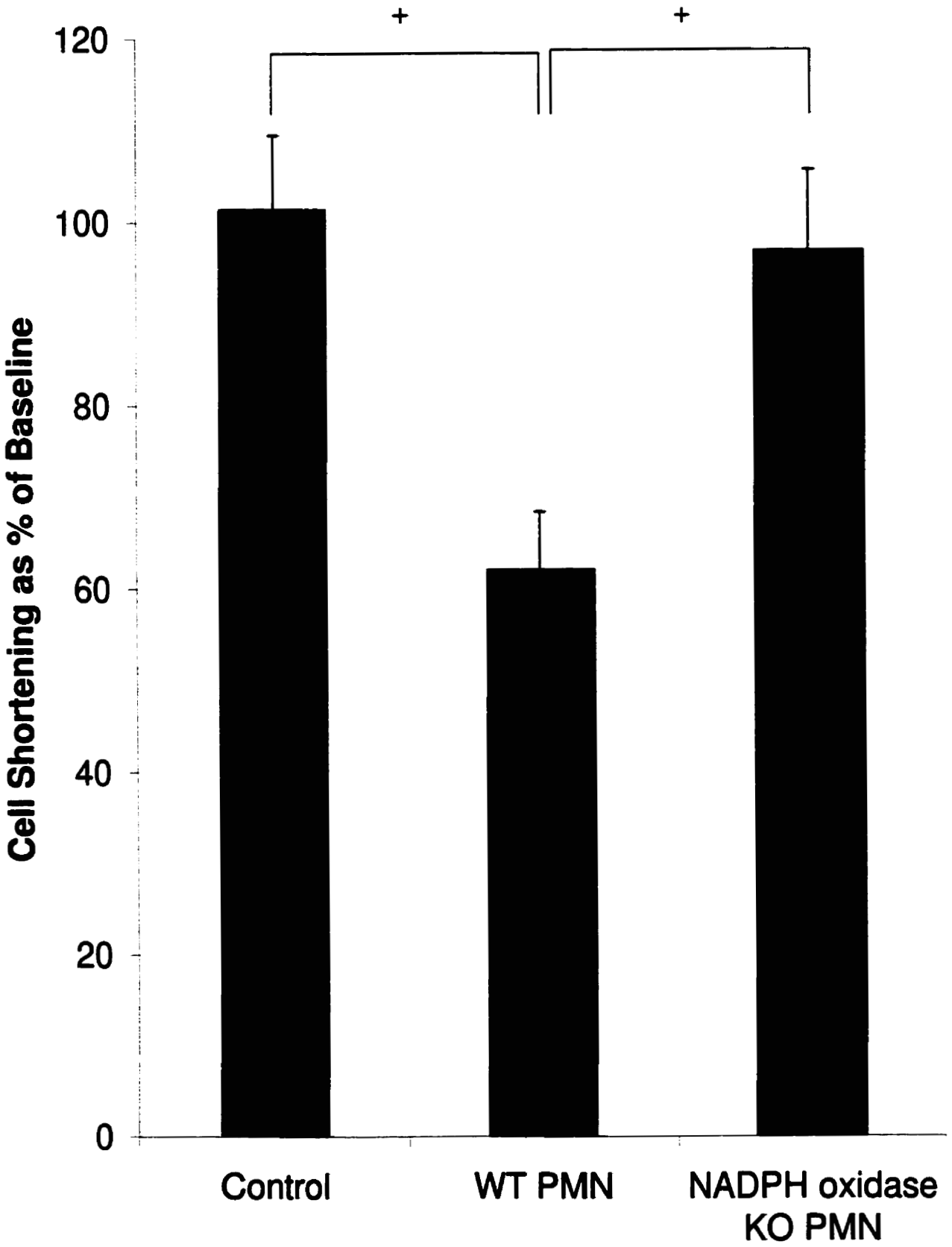

Figure 5.1 Unloaded cell shortening in Control (no PMNs) N=6, WT PMN (N=7), and NADPH oxidase KO PMN (N=6) at 5mins. '+' $P<0.05$

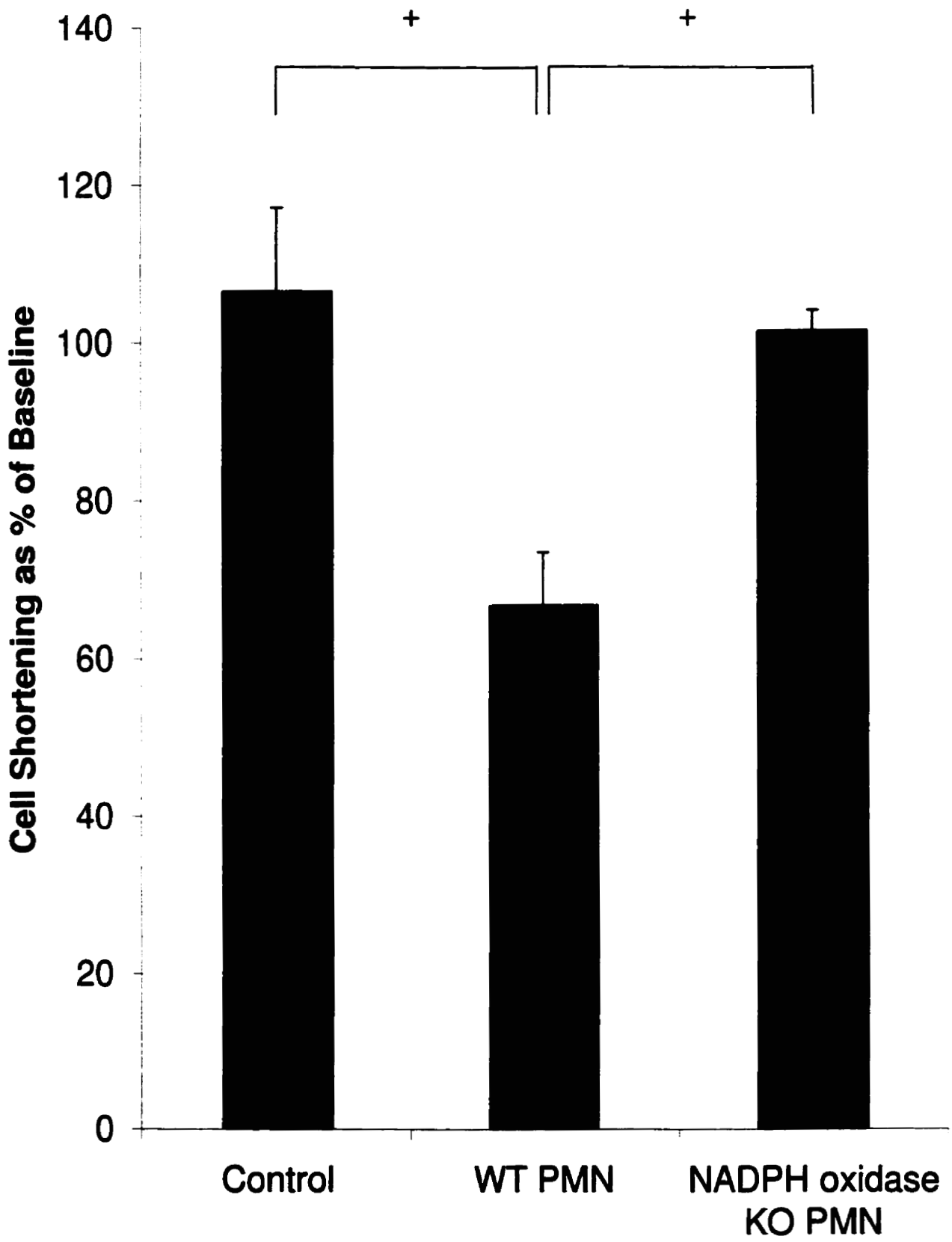

Figure 5.2 Unloaded cell shortening in Control (no PMNs) N=6, WT PMN (N=7), NADPH oxidase KO PMN (N=6) at 10mins. '+' P<0.05

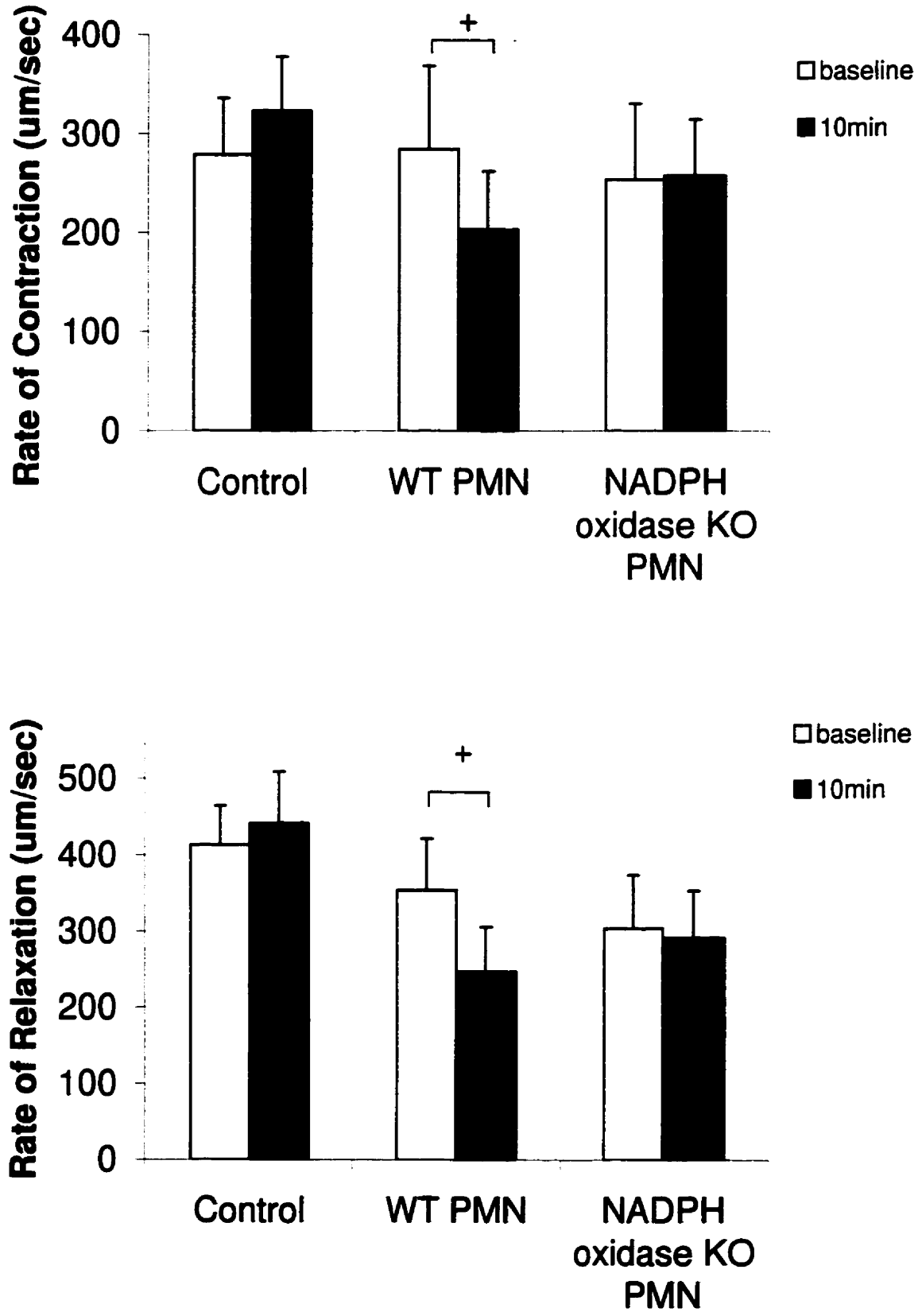

Figure 5.3 Change in rate of contraction and relaxation at 10mins in Control (no PMNs) N=6, WT PMN (N=7), and NADPH oxidase KO PMN (N=6). '+' $P<0.05$

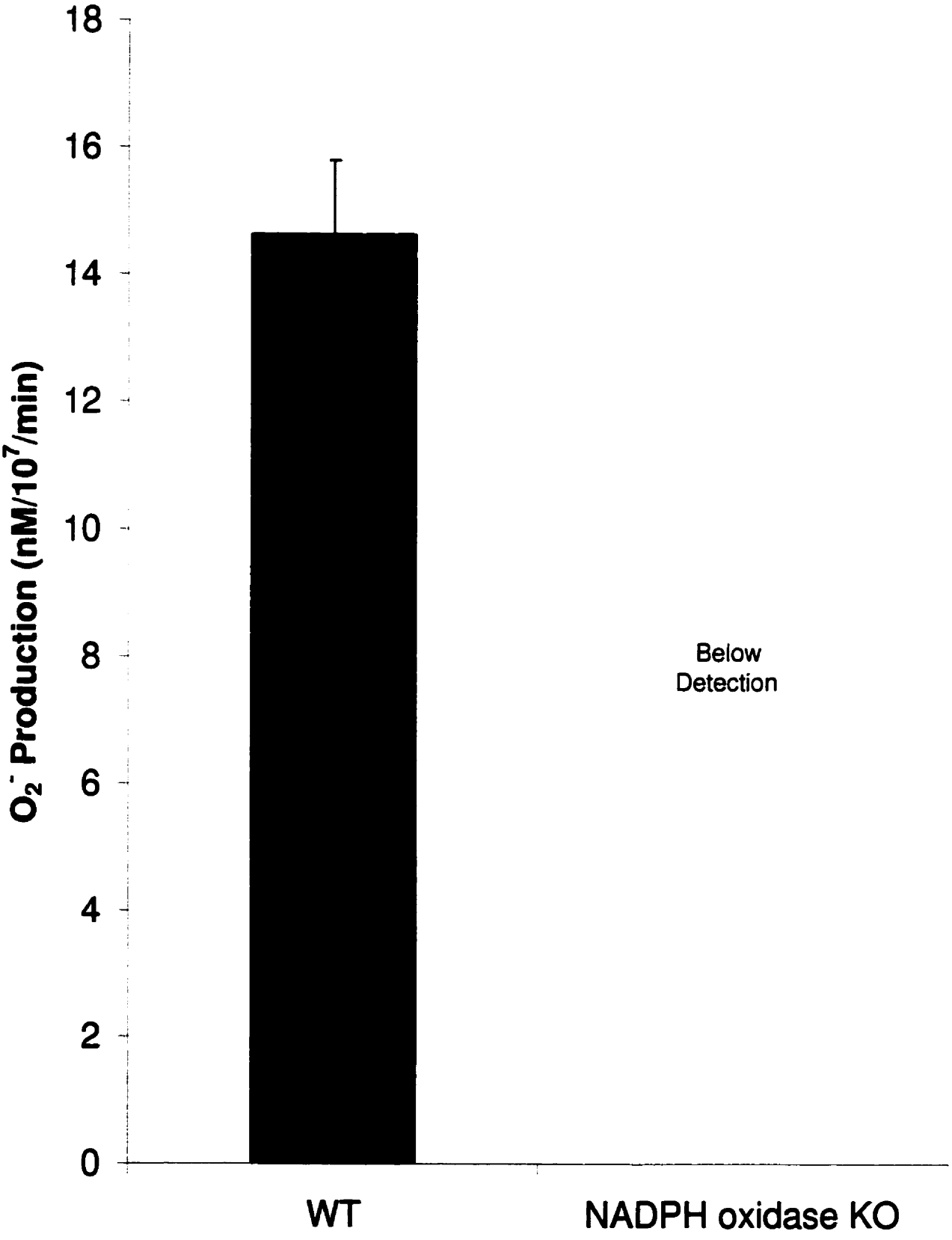

Figure 5.4 O_2^- production in emigrated PMNs from WT (N=4) and NADPH oxidase KO (N=2) mice

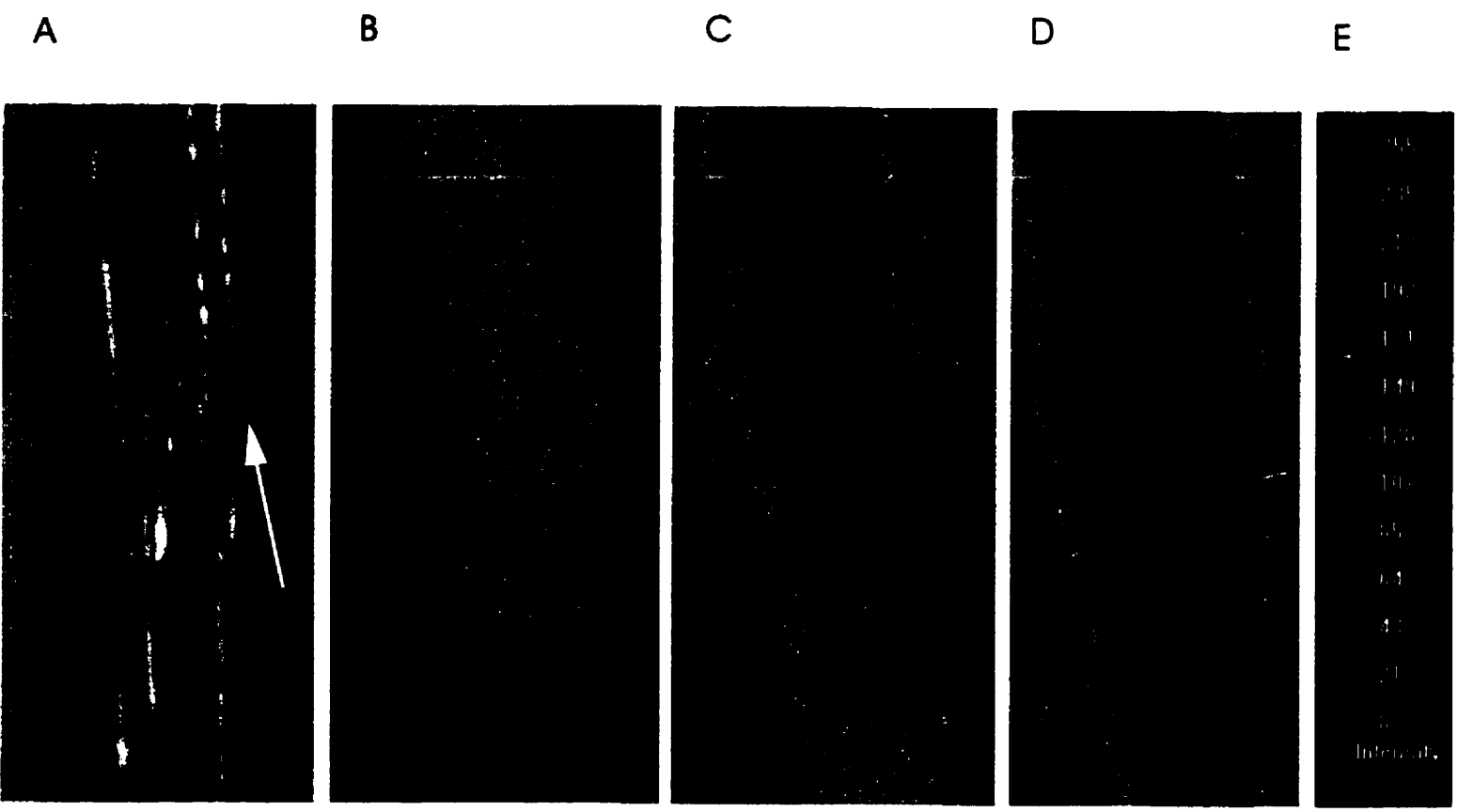

Figure 5.5 Representative fluorescence images of an adherent WT PMN to a WT myocyte. A) phase contrast photo (PMN marked with arrow), B) fluorescence image immediately following adherence of PMN to the myocyte (baseline), C) at 5mins, D) at 10mins, and E) color bar.

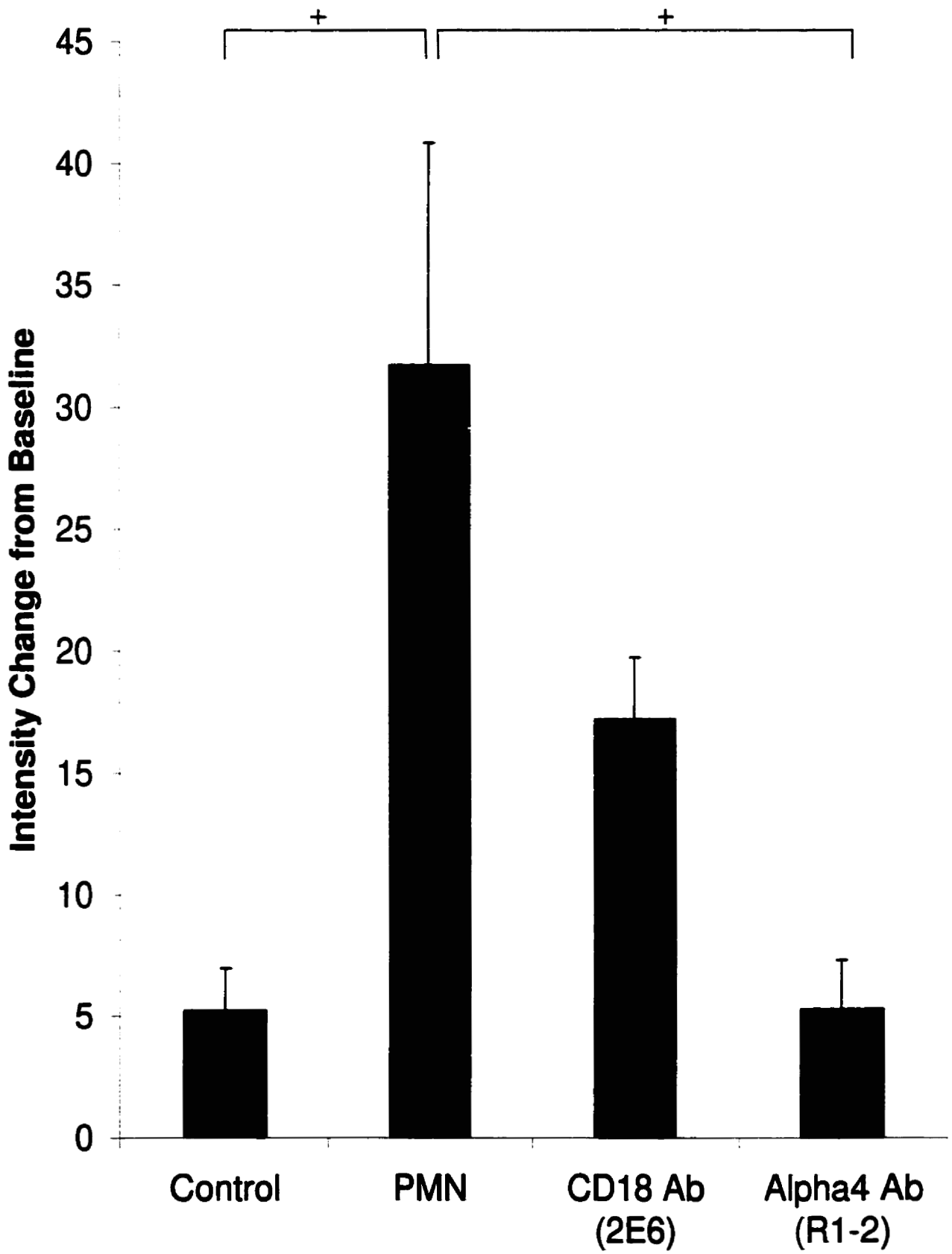

Figure 5.6 Change in fluorescence intensity for Control (no PMNs) N=5, PMN (PMN only) N=6, CD18 Ab (PMNs + anti-CD18 Ab 2E6, 8ug/ml) N=6, and Alpha4 Ab (PMNs + anti-alpha4 Ab R1-2, 10ug/ml) N=4 at 5mins. '+' $P<0.05$

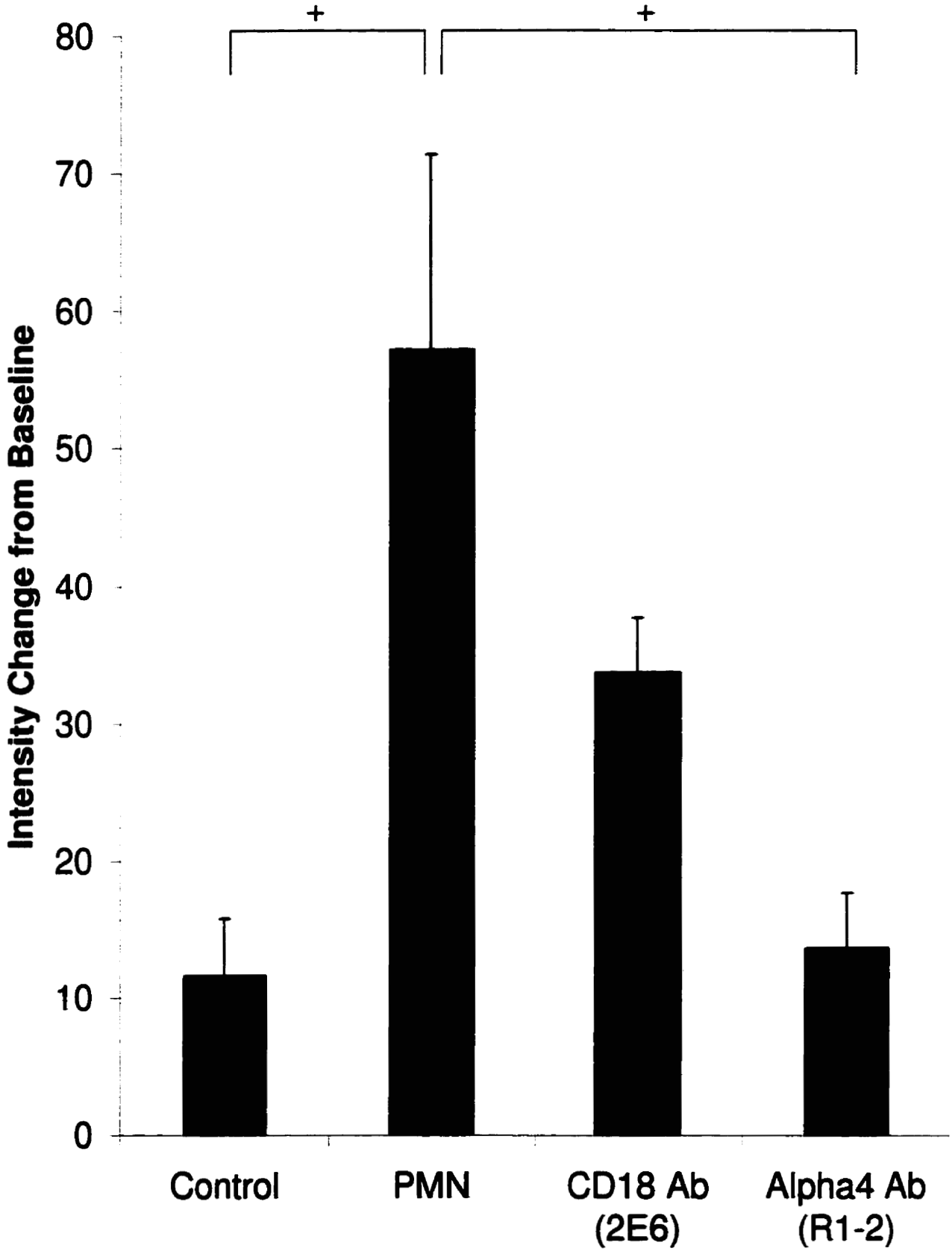

Figure 5.7 Change in fluorescence intensity for Control (no PMNs) N=5, PMN (PMN only) N=6, CD18 Ab (PMNs + anti-CD18 Ab 2E6, 8ug/ml) N=6, and Alpha4 Ab (PMNs + anti-alpha4 Ab R1-2, 10ug/ml) N=4 at 10mins. '+' $P<0.05$

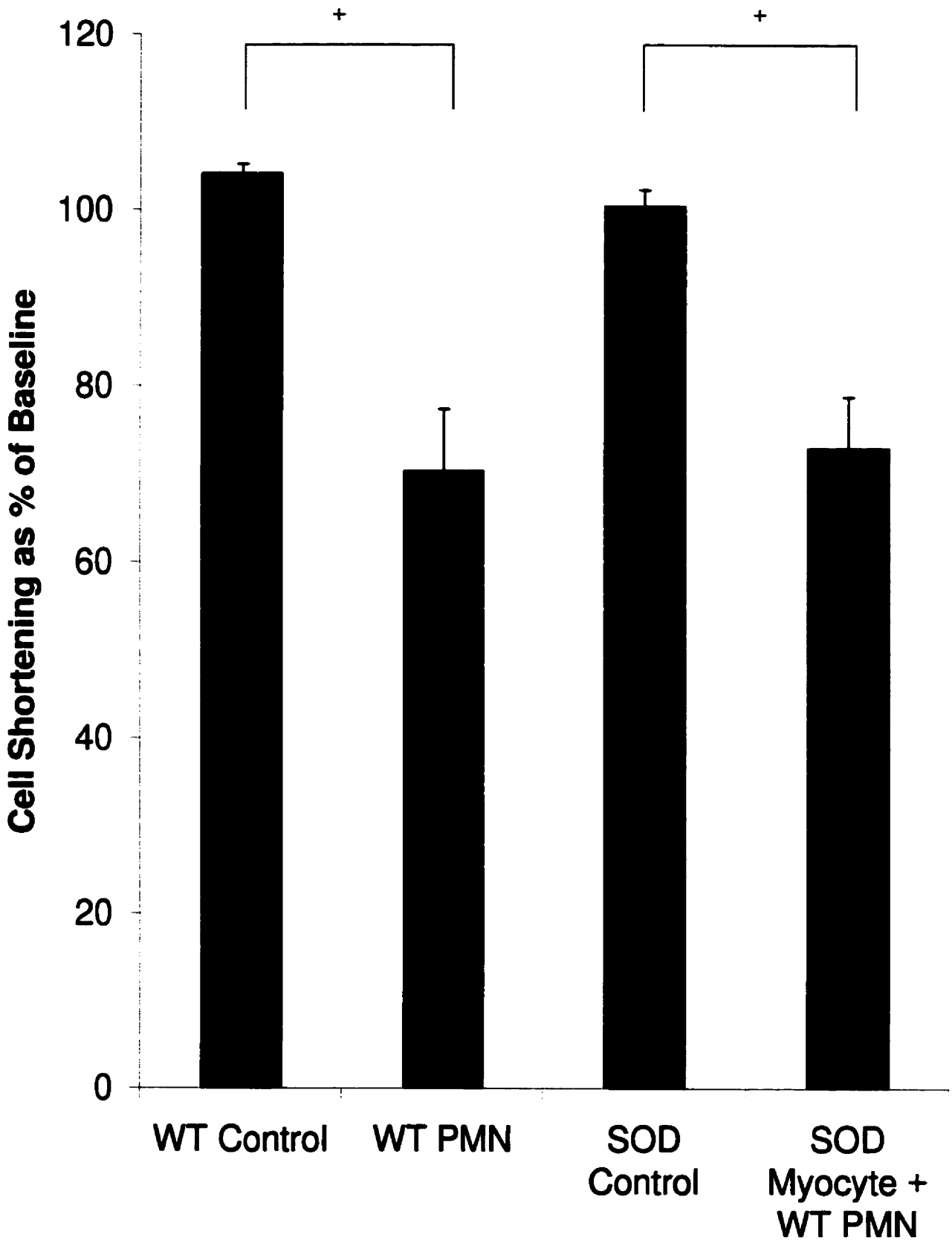

Figure 5.8 Unloaded cell shortening for WT Control (no PMNs) N=4, WT PMN (WT myocyte + WT PMN) N=4, SOD Control (no PMNs) N=4, and SOD Myocyte + WT PMN (N=5) at 5mins. '+' P<0.05

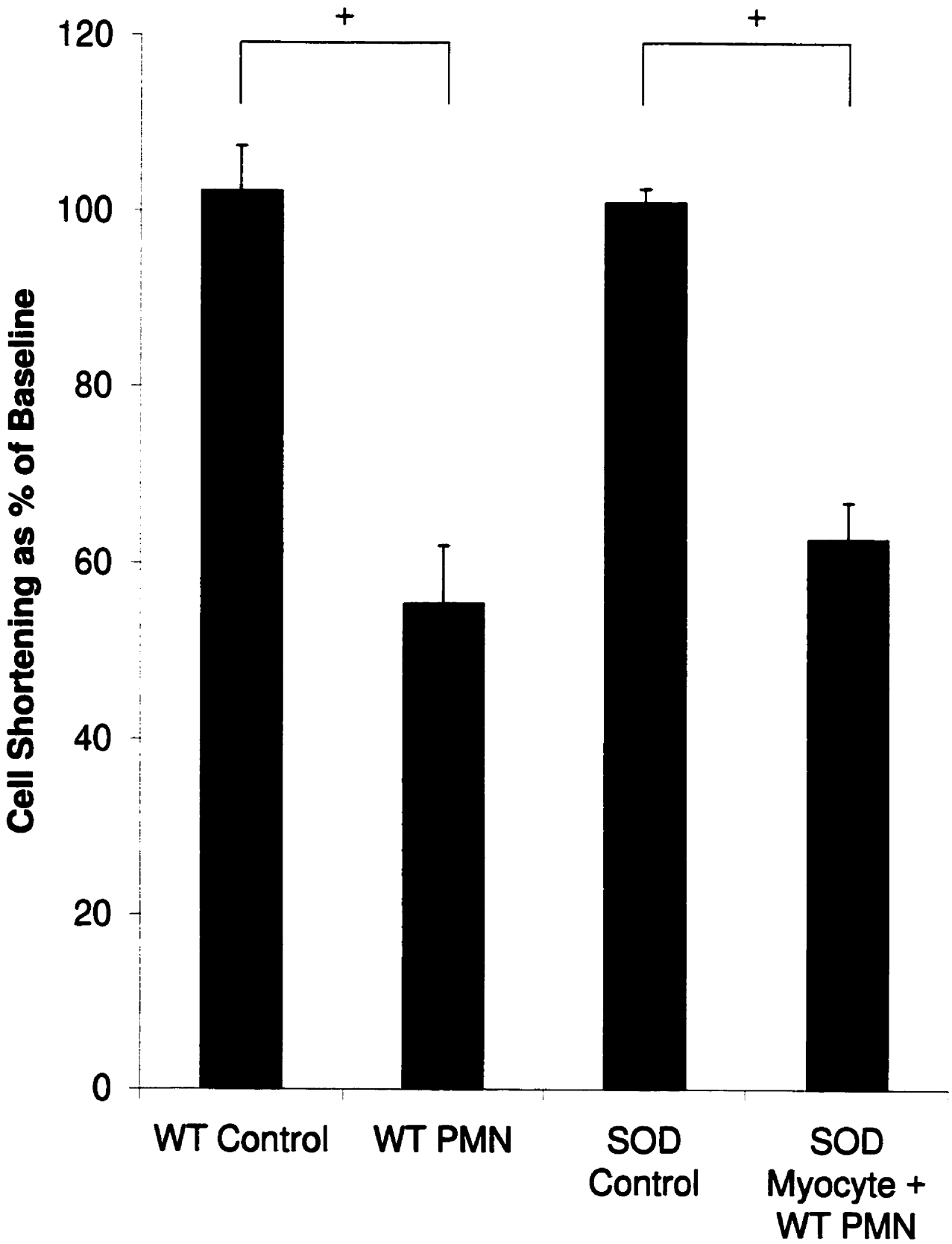

Figure 5.9 Unloaded cell shortening for WT Control (no PMNs) N=4, WT PMN (WT myocyte + WT PMN) N=4, SOD Control (no PMNs) N=4, and SOD Myocyte + WT PMN (N=5) at 10mins. '+' $P<0.05$

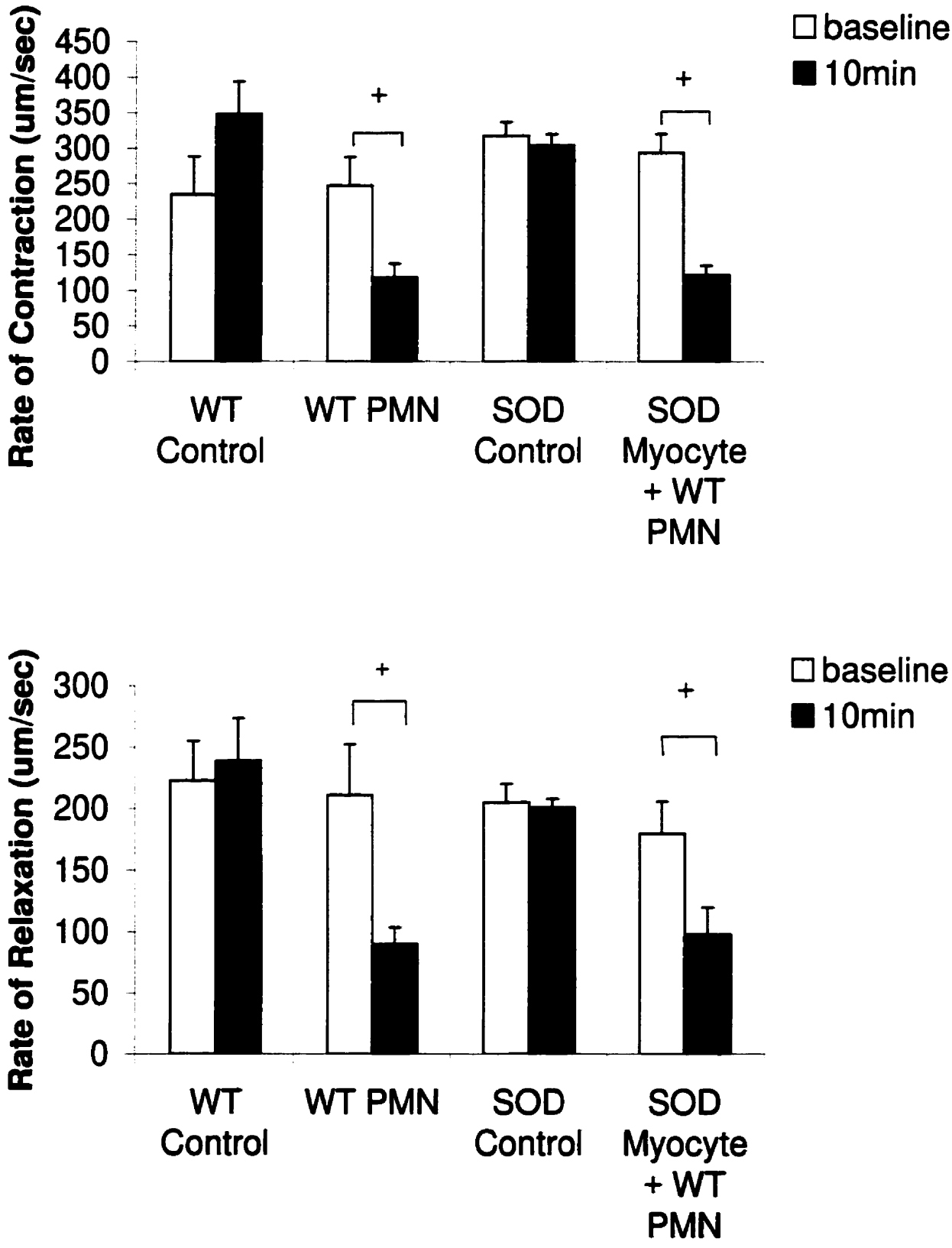

Figure 5.10 Change in rate of contraction and relaxation in WT Control (no PMNs) N=4, WT PMN (WT myocyte + WT PMN) N=4, SOD Control (no PMNs) N=4, and SOD Myocyte + WT PMN (N=5) at 10mins. '+' $P<0.05$

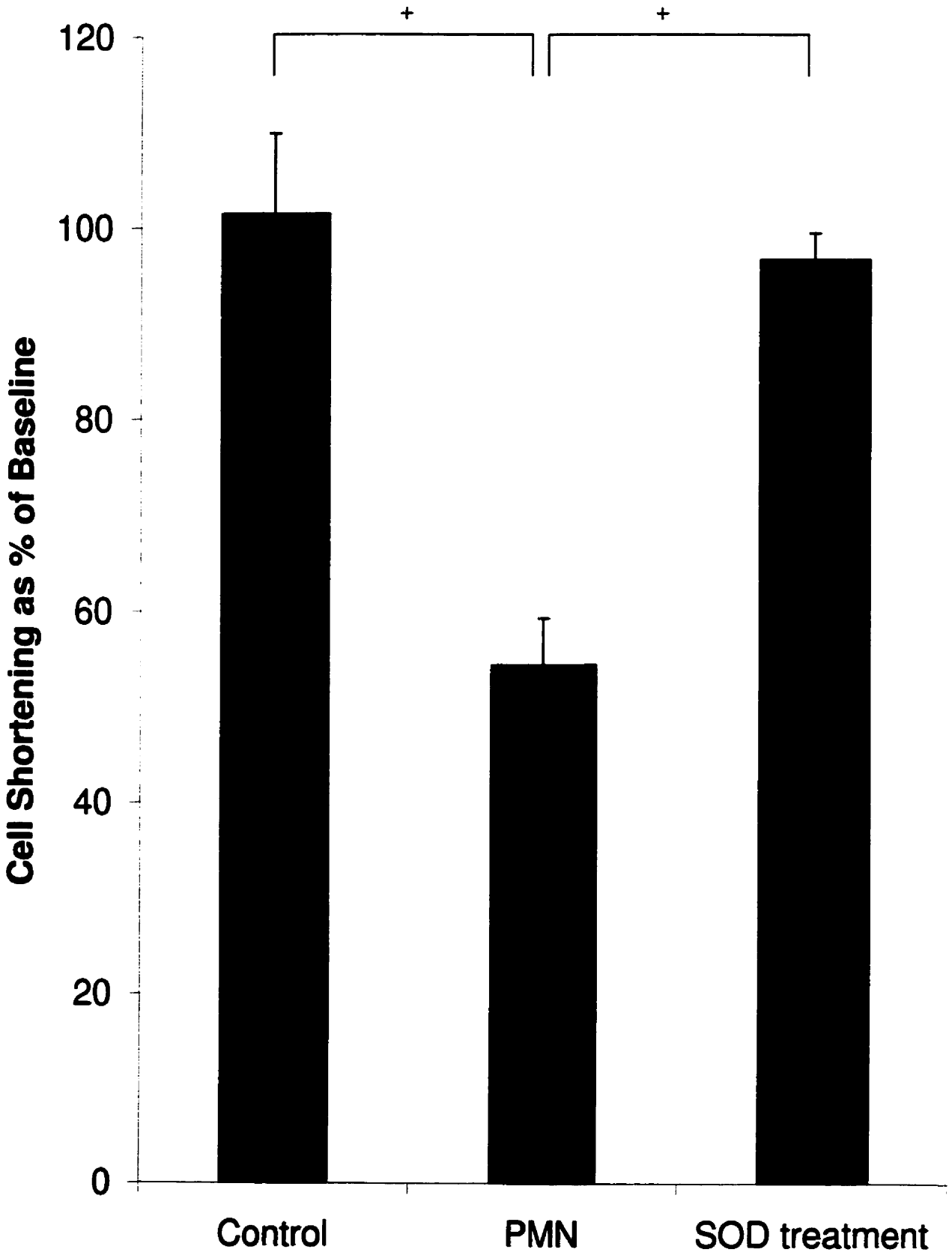

Figure 5.11 Unloaded cell shortening in Control (no PMNs) N=4, PMN (PMN only) N=4, and SOD treatment (PMNs + SOD, 300U/ml) N=3 at 5mins. '+' $P<0.05$

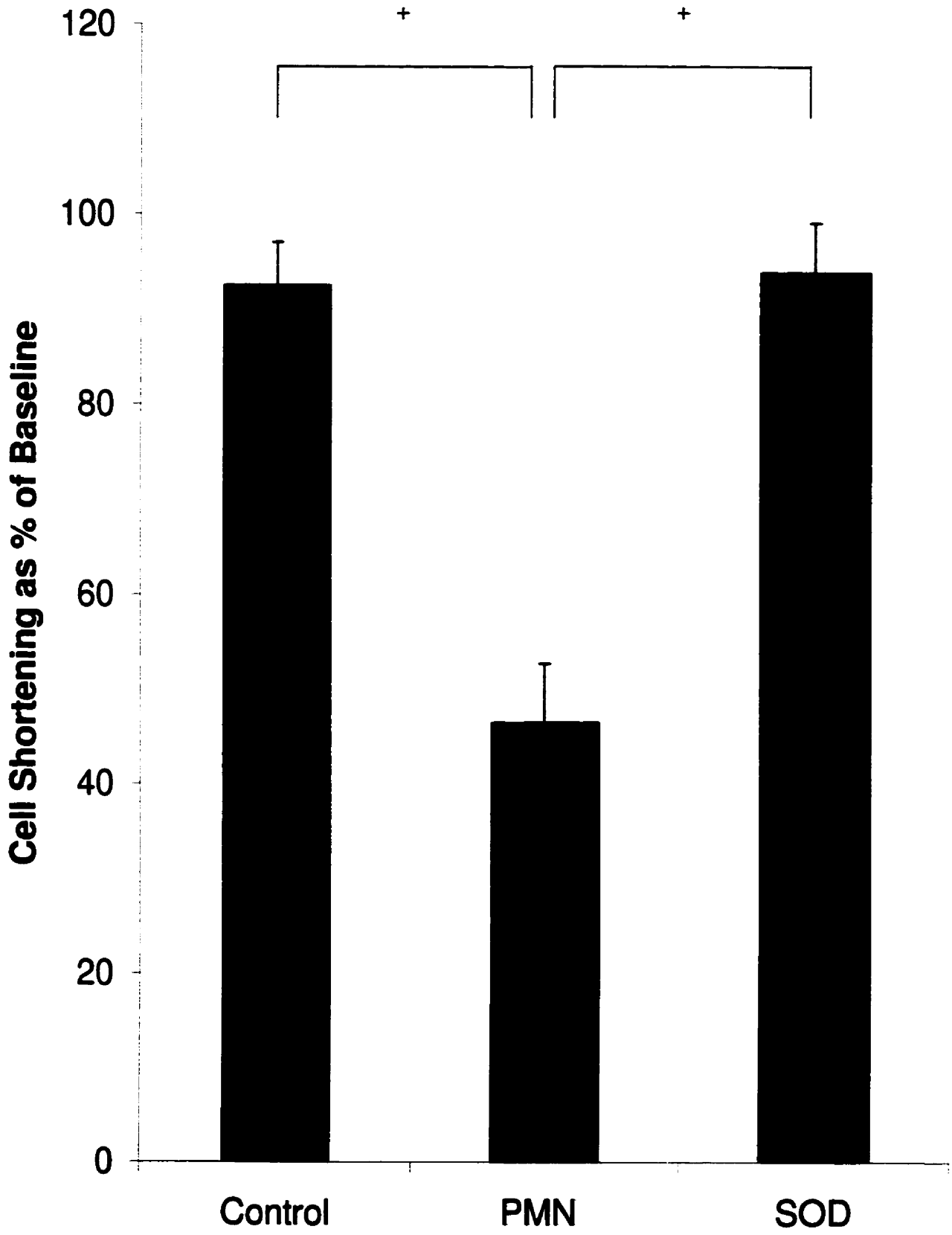

Figure 5.12 Unloaded cell shortening in Control (no PMNs) N=4, PMN (PMN only) N=4, and SOD treatment (PMNs + SOD, 300U/ml) N=3 at 10mins. '+' P<0.05

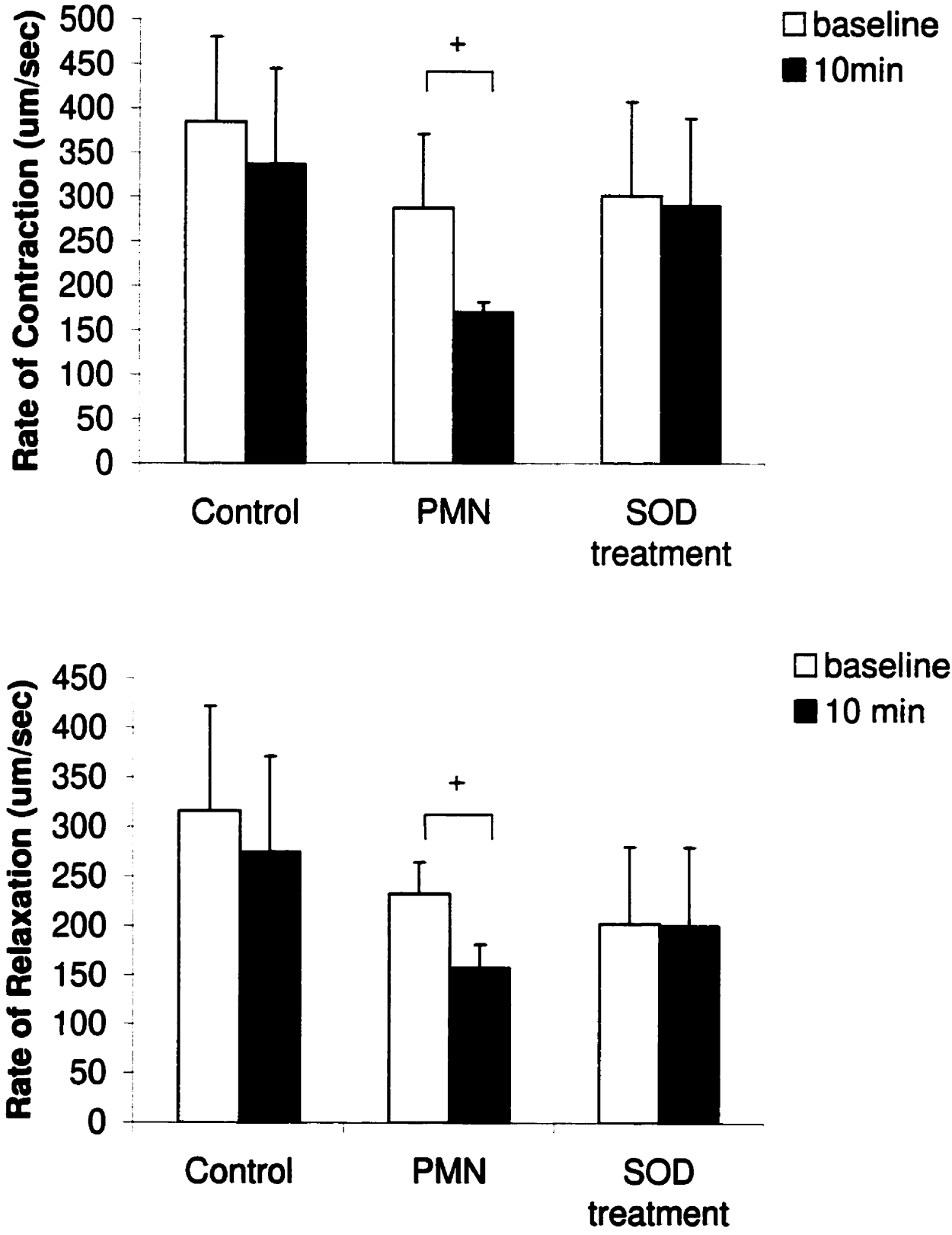

Figure 5.13 Change in rate of contraction and relaxation in Control (no PMNs) N=4, PMN (PMN only) N=4, and SOD treatment (PMNs + SOD, 300U/ml) N=3 at 10mins. '+' $P<0.05$

Group	Baseline (N)	Dysrythmia (N)	% Dysfunction
Control	6	0	0%
WT PMN	7	5	71%
NADPH oxidase KO PMN	6	1	17%

*Functional observations of myocytes in Control (no PMNs), WT PMN, and NADPH oxidase KO PMN

Group	Baseline (N)	Dysrythmia (N)	% Dysfunction
WT Control	4	0	0%
WT PMN	4	3	75%
SOD Control	4	0	0%
SOD Myocyte + WT PMN	5	1	20%

*Functional observations of myocytes in WT Control, WT PMN, SOD Control, and SOD Myocyte + WT PMN.

Group	Baseline (N)	Dysrythmia (N)	% Dysfunction
Control	4	0	0%
PMN	4	4	100%
SOD treatment	3	0	0%

*Functional observations of myocytes in Control (no PMNs), PMN (PMN only), and SOD treatment (PMNs + SOD, 300U/ml).

CHAPTER 6

SUMMARY AND CONCLUSIONS

Only recently has the study of the α_4-integrin in PMN adhesion been investigated. Since the first discovery of increased levels of this ligand on transmigrated human PMNs in 1995 [34], interest in α_4–dependent PMN adhesion has slowly increased. Although information remains limited, Reinhart *et al* have previously shown that rat PMNs also express the α_4-integrin after emigration [33]. PMNs isolated from the circulation adhered to cardiac myocytes via the β_2-integrin CD18, but once they emigrated, the PMNs utilized the α_4-integrin, in conjunction with CD18, to firmly adhere. Prior to the series of experiments in this thesis, it was unclear if α_4-integrin expression on PMNs occurred in mice, and what biological significance could be attributed to this process in any species.

The work presented in the first part of this thesis was conducted to determine if the α_4-integrin also played a role in PMN adhesion in the murine system. We found that murine PMNs isolated from the circulation had very low surface expression of α_4-integrin, and this level increased after emigration. Moreover, as was the case in the rat system, murine circulating PMNs adhered avidly to cardiac myocytes via CD18. Following emigration however, the PMNs adhered via both CD18 and α_4-integrin.

This thesis was the first to show that upon adhesion, emigrated murine PMNs can impair contractile responses of electrically stimulated cardiac myocytes through a predominantly α_4-integrin-dependent pathway. We found that emigrated PMNs require a respiratory burst to induce contractile impairment in cardiac myocytes and that the free radical involved is most likely PMN-derived $O_2^{\cdot-}$. Most importantly, we are the first to show that emigrated PMN-induced oxidative injury to cardiac myocytes is coupled to the engagement of the α_4-integrin.

Adhesion of PMNs to cardiac myocytes is necessary for PMN-induced injury since myocytes in the presence of emigrated PMNs did not show impairment unless at least one PMN was adherent to the myocyte. It is also interesting that only one PMN was necessary to induce injury, highlighting the need for absolute inhibition of PMN adhesion to limit myocyte injury. Previous studies have focussed on CD18 as the adhesion molecule of interest in hopes of inhibiting circulating PMN-myocyte interactions, however a circulating PMN will never interact with a cardiac myocyte. Only recently has the importance of emigration in PMN-myocyte interactions been addressed [33]. The discovery of a new adhesion molecule on emigrated PMNs will prove vital to the design of effective therapies for PMN-induced pathologies of the heart.

Although emigrated PMNs use both CD18 and the α_4-integrin to adhere to cardiac myocytes, immunosuppression of the α_4-integrin protected myocytes from emigrated PMN-induced injury. Our data suggest that therapies involving CD18 inhibition would only be effective at limiting PMN recruitment in the vasculature. Indeed, if one could anticipate the onset of an inflammatory state, anti-CD18 therapies may prove effective. However, after PMNs have already infiltrated the myocardium anti-CD18 therapies will no longer protect against additional myocardial injury.

It appears that a PMN, either from the circulation or after emigration, will use the same mechanism to injure cardiac myocytes. Although a PMN can produce a multitude of toxins, it induces myocyte injury through a free radical-dependent pathway. Emigrated PMNs that are deficient in NADPH oxidase, and thus unable to mount a respiratory burst, cannot injure myocytes. Furthermore, through fluorescence microscopy, we observed an elevated level of oxidants in the myocyte within 5mins of

PMN adhesion. Immunosuppression of the α_4-integrin alone inhibited the oxidative stress within the myocyte following adhesion of the emigrated PMN, while an anti-CD18 Ab did not statistically reduce the free radical levels observed in the myocyte. These are the first data to imply a role for the α_4-integrin in controlling the production or release of free radicals by the emigrated PMN, further emphasizing the vital role of the α_4-integrin in PMN-myocyte interactions.

There is extensive information on the destructive effects of free radicals on many cell types, including cardiac myocytes. Exogenous free radicals affect Ca^{2+} handling, ion channel function, and cell viability. It remains unclear, based upon the data from this thesis, which exact mechanism of injury was responsible for the impairment to myocyte contractility observed. Additional studies involving simultaneous fluorescence measurements of intracellular Ca^{2+} and electrophysiological response of single cardiac myocytes should be conducted in the presence of adherent emigrated PMNs to address this question.

Our data suggest that the free radical predominantly responsible for the decrease in myocyte contractile properties caused by emigrated PMNs is the O_2^-. Over-expression of endogenous O_2^- scavenger SOD was unable to protect the myocyte from the emigrated PMN, but exogenous SOD protected the myocytes from all of the PMN-induced injury observed with PMNs alone. The data show that the emigrated PMNs injure the myocytes from the outside of the cell, possibly altering ion channel activity or membrane integrity which in turn results in myocyte dysfunction.

The importance of PMNs in myocardial pathologies like IR has been well documented. Since adhesion is critical to the ensuing injury, the development of anti-

adhesive therapies has been proposed as a strategy to limit myocardial injury. Before an effective intervention can be developed however, we must have a complete understanding of the adhesive properties of the PMN. Although CD18 is important in PMN-endothelium interactions, the study herein shows that we must acknowledge that the α_4-integrin plays a vital role in emigrated PMN-myocyte interactions, and thus effective therapies for myocardial I/R must consider both of these ligands.

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APPENDICES

Appendix A: This thesis is partially based upon the following manuscript:

Betty Y. Poon, Chris A. Ward, Wayne R. Giles, and Paul Kubes. Emigrated neutrophils regulate ventricular contractility via α_4-integrin. *Circ. Res.* 1999; 84: 1245-1251.