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Proclivitiy of Activated Neutrophils to Cause Postischemic Cardiac Dysfunction: Participation in Stunning?

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Summary. **Myocardial stunning is a reversible defect in contractile function provoked by brief episodes of ischemia followed by reperfusion. Many studies have demonstrated the potential involvement of free radicals in the etiology of myocardial stunning. While activated neutrophils have the capacity to release free radicals and evoke contractile dysfunction, it is not clear that this potential is realized in the absence of myocellular damage. Attempts to define the contribution of activated neutrophils to myocardial stunning by removing the cells from the bloodstream are contradictory, and the apparent simplicity of this seemingly logical approach is an illusion. For example, it is not known how many neutrophils are required to induce contractile failure, the site of action within the heart, the mechanisms that may be responsible, or even the time course or process of neutrophil activation. The production of free radicals and endothelial dysfunction may create conditions propitious for neutrophil recruitment. However, because activated neutrophils synthesize and release various mediators that are potentially toxic to myocardium, once the stage is reached for leukocyte accumulation, it may herald the progression from reversible to irreversible cardiac injury.**

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The advent of new techniques to restore coronary blood flow during acute myocardial ischemia has led to the recognition that the recovery of pump function is sluggish, even if the myocardium is still viable, a situation termed the *stunned myocardium* [1]. This review will adhere to the definition of myocardial "stunning," as outlined by Bolli [2,3], as a reversible defect in contractile function observed after a period of ischemia, in the absence of overt myocellular damage or necrosis. The distinction between sublethal or reversible injury and irrevocable cell death is important because, while both situations are associated with mechanical derangements, the mechanisms of contractile dysfunction are probably quite different. The background to the elucidation and recognition of the stunned myocardium has recently been reviewed [3].

Evidence for an important (but not exclusive) role of oxygen-derived free radicals and metabolites in the pathogenesis of myocardial stunning has been elegantly presented by Bolli and colleagues and other investigators [1–3]. It is natural to consider the potential for neutrophil activation as a contributory factor to myocardial stunning, because on a quantitative basis neutrophils are a major potential source of oxygen metabolites. However, free-radical-mediated injury and neutrophil-mediated injury are not necessarily synonymous, and it may be erroneous to equate the two events.

Postischemic contractile abnormalities have been described in isolated buffer-perfused hearts subjected to global ischemia [5–8]. Although it is not clear that this in vitro response mimics myocardial stunning in vivo where the time for contractile function to recover generally takes much longer [9]; nevertheless, it corresponds to a temporary postischemic mechanical abnormality. The in vitro model of stunning is accompanied by a burst of free-radical/oxygen metabolite formation within the first few seconds to minutes of reperfusion [7,10,11], and is ameliorated by various free-radical scavengers [6,7]. Consequently, this response shares many features of myocardial stunning in vivo, and it indicates that neutrophils are not an essential component of some aspects of the contractile defect because it occurs in the absence of any blood elements. Thus a more pertinent issue is whether the activation of neutrophils can exacerbate postischemic cardiac dysfunction in vivo, rather than account for the complete response. Clearly there are other

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sources of free radicals, such as the vascular endothelium [12–14] or myocyte mitochondria [15,16] and additional mechanisms that also contribute to the ultimate response [3].

In reviewing the contribution of activated neutrophils to myocardial stunning, it is apparent that the evidence is fragmentary and incomplete. Many studies have relied upon techniques to deplete neutrophils from the blood and compare the postischemic contractile failure to a control group with neutrophils. Unfortunately, the apparent simplicity of this logic is illusory, and the results are inconsistent, perhaps due, in part, to the inherent difficulty in producing true tissue neutropenia. Therefore it is necessary to examine the issue in a broader context of whether neutrophils have the capacity to influence contractile function, the mechanisms involved, and whether there is a basis to consider these mechanisms as contributing to myocardial stunning. Moreover, myocardial stunning has been described in a variety of settings; after ischemia induced by a single brief occlusion, multiple occlusions or global ischemia during cardioplegic arrest, and during exercise or demand ischemia [3]. It is not inconceivable that differences between the various models may reflect the interplay of different components of injury such that generalized conclusions could be misleading. Recognition of a component of injury attributable to activated neutrophils or other inflammatory cells may be important because this response can become self-perpetuating. Recruitment of inflammatory cells may herald the potential for a reversible injury to be "pushed over the edge" in a transition to lethal damage, due to the variety of injurious mediators produced by activated neutrophils.

Can Neutrophils Impair Contractile Function?

Quiescent or inactivated neutrophils do not release enzymes and mediators, and apparently do not influence resting cardiac function. Perfusion of isolated hearts with diluted blood or buffer neutrophils does not alter baseline contractile function [8,17,18], despite the long transit times of leukocytes through the microcirculation [19] and the potential for diminished perfusion. This is probably because of substantial capillary reserve in the heart, where it is suggested that only 50–60% capillaries are perfused during normal cardiac function [20,21]. Moreover, removal of leukocytes from blood perfusing hearts in situ is also without effect on baseline cardiac function [22–25]. Changes in contractile function elicited by neutrophils could be secondary to intravascular sequestration or entrapment, thereby causing perfusion defects and local ischemia, or by neutrophil-derived products directly depressing contractile function. Consequently, Kraemer and coworkers [26,27] studied the interaction of neutrophils with papillary muscles mounted in organ baths and devoid of a vascular component. In this setting, where the direct influences of neutrophils on contractility can be assessed, nonstimulated cells do not impair cardiac function. Thus, resting neutrophils do not alter myocardial contractility in vitro or in vivo.

Activation of neutrophils by chemotactic factors leads to the production and/or release of a host of different mediators, many of which could influence myocardial contractility. These mediators include lipid peroxidation products of arachidonic acid [28]; 1-0-alkyl-2-aryl-sn-glyceryl-3-phosphorylcholine, or platelet activating factor (PAF), which promotes contractile dysfunction either directly [29] or through a mechanism dependent upon the presence of blood elements [30]; metabolites of oxygen including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hypochlorite anions ($HOCl^-$), each of which has been identified as a negative inotropic species [7,26,31,32]; and proteases such as collagenase and elastase, which may compromise structural and cellular integrity [33,34] to impair cardiac function.

Although activated neutrophils elaborate multiple mediators capable of compromising contractility, the question remains as to whether this potential is actually realized in a dynamic setting of cell-tissue interactions. This was addressed directly by the addition of neutrophils to isolated papillary muscles and monitoring changes in contractile function [26,27]. Activated neutrophils provoked a concentration-dependent decline in papillary muscle function, which developed over 5–10 minutes (Figure 1). PAF, leukotrienes, superoxide anions, or hypochlorite anions were excluded from mediating this response. The decrease in function was attributed to the release of hydrogen peroxide because catalase attenuated the neutrophil-induced contractile derangement while exogenous hydrogen peroxide mimicked the effect [26]. Monoclonal antibodies to the CD18 adhesion complex of the neutrophil abolished contractile failure provoked by activated neutrophils [27]. Adherent neutrophils also produce substantially more H_2O_2, and for longer periods of time, than cells in suspension [35]. Prevention of adhesion with monoclonal antibodies to the CD11/CD18 adhesion complex suppressed this augmented H_2O_2 release [36] and could account for the beneficial effects of the antiadhesion antibodies.

Activated neutrophils also adhere to isolated myocytes that have been primed with a cytokine such as

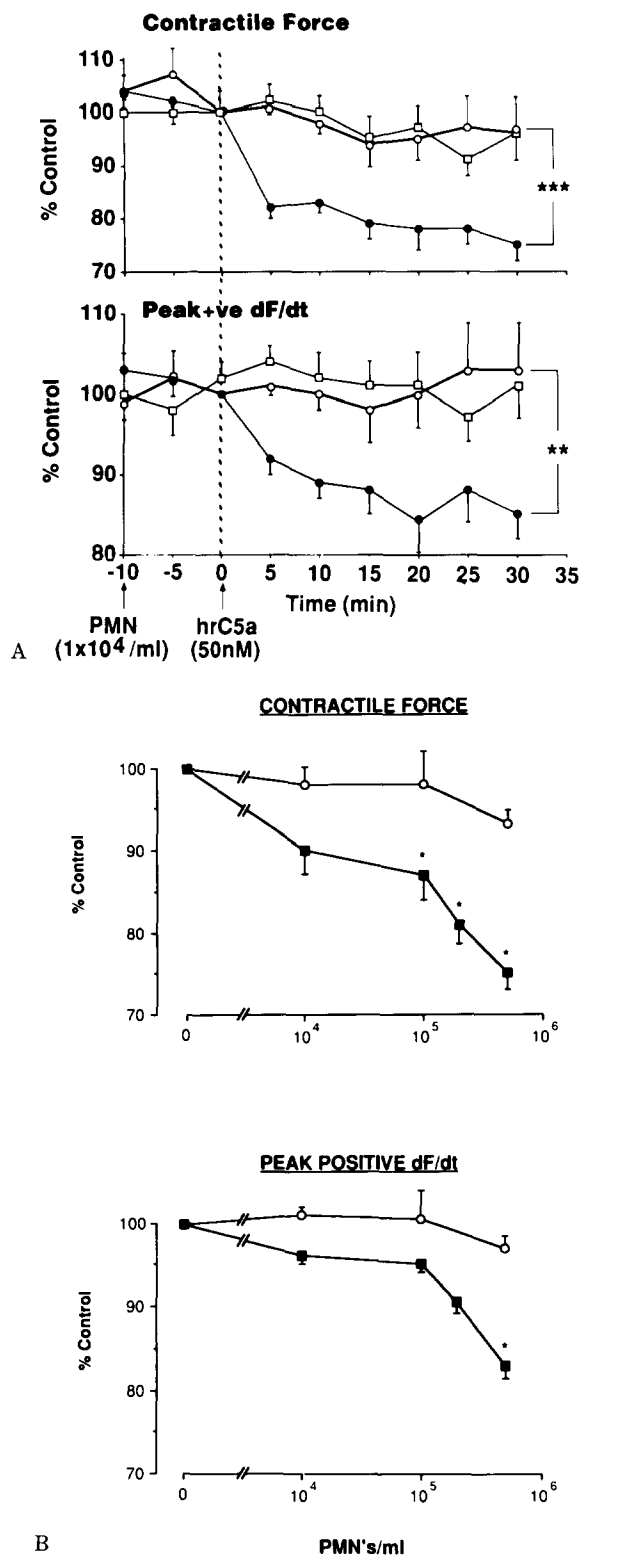

Fig. 1. *A: Time-dependent reduction in rabbit papillary muscle function by human neutrophils (PMNs) activated with human recombinant (hr) C5a (●---●, n = 18), while either PMNs (□---□) or hrC5a (○---○) alone were without effect. Data from Kraemer et al., [27]. * * $p < 0.01$, * * * $p < 0.001$, repeated measures ANOVA. B: Concentration-dependent reduction in rabbit papillary muscle function by the cumulative addition of rabbit neutrophils (□---□, n = 18) versus time-related control papillary muscles in the absence of PMNs (○---○, n = 10). The response was allowed to develop over 10 minutes before the next concentration of cells was added. Data from Kraemer et al. [26]. * $p < 0.01$*

interleukin-1 or tumor necrosis factor, and express intercellular adhesion molecule-1 (ICAM-1) [36]. Adhesion is mediated by an interaction between CD18 and ICAM-1, and results in the generation and release of H_2O_2 by the neutrophils, which is associated with contracture of the myocytes [36]. Thus, neutrophils have the capacity to interact directly with myocytes.

These studies support the suggestion that activated neutrophils can release mediators in amounts sufficient to provoke cardiac dysfunction. The nature of this functional defect—whether it reflects overt damage of the endocardial covering of the muscle, as has been described with activated eosinophils [37]; oxidant-induced injury to the myocytes, perhaps interfering with calcium transport [38], or by compromising either cellular integrity or the contractile apparatus by interactions between oxidants and neutrophil-derived proteases [33]—remains to be elucidated. Importantly, it is not clear if the functional derangement reflects a reversible phenomenon, which is mandatory for the definition of stunning, or it represents irreversible overt damage to the muscle, and thus, a different phenomenon. In this context it should be noted that isolated myocytes with attached neutrophils acquired the rounded configuration of damaged cells [36].

Neutrophils and Coronary Vascular Resistance

Another means by which neutrophil activation could impair myocardial contractility is indirectly via an effect on coronary perfusion. Isolated hearts perfused with a buffer containing neutrophils exhibit vasoconstriction accompanied by a decrease in left ventricular developed pressure when the neutrophils are stimulated with phorbol myristate acetate [39]. The vasoconstriction was attributed to the production of oxygen metabolites by the neutrophils [39]. Subjecting the neutrophil-perfused heart to a period of hypoxia followed by reoxygenation leads to neutrophil activation and tissue sequestration [8,17], accompanied by vasoconstriction during hypoxia and compromised recovery of contractile function upon reoxygenation [8]. Isolated rat hearts perfused with blood diluted 1:1 with Kreb's buffer and subjected to 30 minute isch-

emia showed a 62% decrease in capillary perfusion and a 76% increase in coronary vascular resistance at reperfusion, whereas perfusion with blood depleted of leukocytes gave a 33% reduction in perfused capillaries and only a 5% increase in vascular resistance [18]. The increased vascular resistance associated with whole blood perfusion was attributed to microvascular plugging and/or vasoconstrictor production by the neutrophils [18]. Thus, various studies using isolated perfused hearts concluded that activated neutrophils could impair coronary perfusion, and the diminished perfusion was associated with a decline in contractile function. However, these studies do not permit differentiation between contractile failure secondary to inadequate perfusion and a direct negative inotropic effect of neutrophils or neutrophil-derived mediator(s).

Capillary plugging

Neutrophil entrapment within the microcirculation has been observed in the reperfused myocardium in vivo and is suggested to account for the capillary no-reflow phenomenon, where flow is not restored uniformly to the postischemic region [40], while leukocyte depletion reduces the extent of no reflow [41,42]. However, no reflow only occurs within regions of the myocardium that are irreversibly injured [43], so by definition is unlikely to be a significant factor in the stunned myocardium. Furthermore, 12 minutes of coronary occlusion followed by 1 hour of reperfusion was associated with a paradoxical 40% decrease in myocardial neutrophil content [44], indicating capillary entrapment does not occur in this setting.

Neutrophil-mediated vasoconstriction

The ability of neutrophils to influence vascular tone directly has been addressed by adding neutrophils to organ baths containing rings of vascular tissue [45–50]. The neutrophil-induced vasoconstriction that is observed upon activation of the leukocytes has been variously attributed to oxygen metabolites, in particular, superoxide anions, which degrade endothelial-derived relaxing factor [EDRF: 47], the transcellular metabolism of neutrophil-derived leukotriene A_4 to leukotriene C_4 by the endothelium [49]; or the release of an unidentified peptide [50]. Stimulated neutrophils release a vasoconstrictor principle that is heat stable and produces contraction of vascular smooth muscle in the absence of neutrophils [50]. This material also augments platelet-mediated contractions [50], attributed primarily to serotonin and thromboxane A_2 [51,52]. Isolated, coronary arteries perfused ex vivo after being subjected to 15 minutes hypertension in vivo to induce neutrophil and platelet adhesion to the endothelium, exhibited a fourfold increase in sensitivity to the vasoconstrictor effects of serotonin that was reversed by a combination of thromboxane and leukotriene receptor blockade [56]. Thus, activated neutrophils can promote vasoconstriction directly by the release of vasoactive mediators, and indirectly by potentiating the activities of other vasoconstrictor agents. The significance of these neutrophil-mediated changes in vascular tone are unknown at this time, but if occurring locally in the reperfused myocardium might create areas of hypoperfusion, which, in turn, would promote regional wall-motion abnormalities.

Vascular stunning

While dramatic vasoconstriction is not observed in stunned myocardium, resting subendocardial blood flow is diminished after a single 15-minute ischemic episode followed by reperfusion [9,53,54]. In this setting, the reative hyperemic response following a 40-second occlusion and the maximal vasodilator responses to adenosine or papaverine are also impaired, giving rise to the term *microvascular stunning* [54]. These vascular derangements do not correlate with the contractile dysfunction [54], and it is unlikely that changes in coronary tone contribute to the functional derangement. Moreover, changes in vascular function are not apparent after 10 minutes of ischemia and reperfusion [55], suggesting that different models of stunning, while sharing a common defect, may reflect different events that culminate in injury. Vascular stunning and a diminished coronary vascular reserve is apparently associated with more "severe" forms of myocardial stunning and may represent the beginning of a transition into lethal injury. The possibility that defects in subendocardial perfusion and vascular reserve are linked to neutrophil recruitment has not been addressed.

Neutrophils and Contractile Failure in Vivo

Few studies have addressed directly whether the local activation of neutrophils in vivo is accompanied by contractile dysfunction. Based on the apparent importance of components of complement as neutrophil chemoattractants in the setting of myocardial ischemia and reperfusion [57,58], the chemotactic complement fragment C5a was injected into the coronary artery of anesthetized open-chest pigs [55–58]. C5a transiently evoked coronary vasoconstriction, compromised contractile function, and promoted myocardial leukocyte sequestration [59,60]. The derangements in cardiac function were attributed to the peptido leukotrienes, such as leukotriene D_4, and to thromboxane A_2, mediators released by C5a, because

the response was ameliorated by a combination of antagonists to these eicosanoids [61]. These antagonists did not attenuate the leukocyte accumulation in the heart [61], and the leukocytes and/or other blood elements have been proposed as the source of the vasoconstrictor material [61,62]. However, myocardial leukocyte uptake could be an epiphenomenon of unknown significance. Leukotriene B_4 is another chemotactic agent that induced equivalent leukocyte sequestration to C5a when injected into a coronary artery, but did not cause vasoconstriction or contractile dysfunction [63]. Moreover, while neutrophil depletion with filters prevented vasoconstriction in response to C5a, hearts previously perfused with filtered blood remained refractory to the C5a-induced thromboxane A_2 production and vasoconstriction, even when perfused with whole blood never exposed to filters [64]. Pigs rendered severely neutropenic with cyclophosphamide were equisensitive to C5a-induced ischemia as normal pigs [65]. Therefore cells within the heart, rather than circulating blood elements, appear to be responsible for the response to C5a, and neutrophil trapping is an epiphenomenon for the acute response. The source(s) of the vasoconstrictor material elicited by C5a remain to be defined and could be tissue leukocytes (i.e., macrophages) that synthesize both leukotriene D_4 and thromboxane A_2, in contrast to the circulating neutrophils [66]. Because the predominant effect of C5a is on tissue cells, rather than the neutrophils, it remains to be determined if activation of leukocytes in blood perfusing the heart can promote contractile failure.

Does transient ischemia and reperfusion create conditions propitious for neutrophil activation?

The adhesion of neutrophils to the vascular endothelium is a prerequisite for their recruitment and accumulation in tissues. The site specificity of neutrophil adhesion implies that it results from a change in the status or function of the endothelium. Normally, endothelial cells elaborate and release various mediators that oppose neutrophil adhesion, including EDRF or nitric oxide [66], prostacyclin, and adenosine [67–69]. Although endothelial cells are relatively resistant to ischemic damage [70], endothelial dysfunction expressed as an increased permeability to macromolecules and impaired relaxations to agonists that operate through the release of EDRF, has been described after 15 minutes of ischemia followed by reperfusion [71], a classical setting of myocardial stunning. The formation of oxygen metabolites at reperfusion may account, at least in part, for the impaired endothelial-dependent relaxations, because they can be reproduced with exogenous free-radical generating systems such as xanthine-xanthine oxidase [50]. In addition, oxygen metabolites inhibit prostacyclin production [72] and deplete endothelial adenosine triphosphate [73], a major source of adenosine, while the administration of superoxide dismutase prior to reperfusion preserves endothelial function after longer periods of ischemia [74–76]. Thus, brief periods of ischemia followed by reperfusion promote endothelial dysfunction, with impaired production of various autacoids that normally attenuate neutrophil attachment.

Oxygen metabolites, in particular H_2O_2, not only provoke a loss of EDRF, PGI_2, and adenosine, but also induce endothelial cells to rapidly express GMP-140 [77] and PAF [78], two molecules that are intimately involved in neutrophil adhesion. Cooperative interactions between these adhesion molecules could lead to the early recruitment of neutrophils. In addition, H_2O_2 activates CD11b/CD18-dependent cell adhesion [79].

The significance of these functional changes of the endothelium to neutrophil activation and accumulation, or indeed to myocardial stunning, is not clear, but serves to highlight changes that could favor neutrophil recruitment. However, these changes alone may not be sufficient to promote neutrophil accumulation, and the production of an agent to activate the leukocytes may also be required.

To date, there is a paucity of direct evidence demonstrating the formation of a chemotactic agent after brief ischemic episodes. Preliminary studies in patients undergoing coronary-artery balloon-angioplasty induced ischemia for a duration of only 1.5 minutes was associated with neutrophil activation attributed to the local release of an undefined chemoattractive substance(s) [80]. Using accumulation of Clq, a subunit of the first component of complement, in ischemic canine myocardium as a measure of complement activation and equating Clq localization with severity of ischemia, Rossen and colleagues [57] showed some local complement activation after 15 minutes of ischemia that did not achieve statistical significance ($p = 0.09$).

Electron paramagnetic spin resonance studies of either myocardial tissue [9] or spin-trap agents perfused through the heart [11,81,82] demonstrate free-radical production at reperfusion that is suppressed by the administration of superoxide dismutase and catalase [7,83]. Because some of the spin-trap agents used remain confined to the vascular comparment [82,83], it appears that oxygen metabolites such as superoxide anions may be released into the bloodstream. Petrone et al. [84] described the production of a neutrophil

chemotactic factor in plasma exposed to superoxide anions. This factor did not cause neutrophil degranulation or free-radical generation, but was suggested to account for the ability of superoxide dismutase to attenuate neutrophil accumulation at sites of inflammation [84]. The identity of this factor, or whether it is actually formed in situ, remains unknown. While these observations suggest that elements necessary for neutrophil activation and sequestration may be present in some situations that provoke myocardial stunning, assessments of III-indium-labeled neutrophils, as mentioned above, failed to show neutrophil accumulation after 12 minutes of ischemia and reperfusion in the anesthetized dog [44]. Indeed, a paradoxical decrease in myocardial neutrophil content was observed for reasons that are not clear.

Neutrophil Depletion and Myocardial Stunning

Four studies have addressed the influence of neutrophil depletion on myocardial stunning in the anesthetized dog, using either filters to remove cells from blood entering the coronary circulation [22,24,25] or antiserum to canine neutrophils to effect a systemic neutropenia [23]. A further study used $f(ab')_2$ fragments of a monoclonal antibody to the CD11b/CD18 adhesion complex on the leukocyte to prevent neutrophil accumulation [85]. In some cases a reduction in the degree of stuning was noted [22,25], while no benefit was observed in other studies [23,24,85]. While it can be argued that differences in dogs, diet, severity of ischemia, anesthesia, specificity of cell depletion etc. could account for some of these differences, two other points may be particularly noteworthy. The first pertains to the number of activated neutrophils that are required to provoke cardiac dysfunction. In vitro experiments [8,26] and the in vivo study of Engler and Covell [22] suggest that activation of only a few neutrophils may be necessary to evoke contractile dysfunction. Since the techniques to remove neutrophils are incomplete and probably activate the cells, the remaining cells may exert a greater influence than would be anticipated on a quantiative basis, and obscure interpretation of the studies. It should be noted that the absolute number of neutrophils in the tissue may not relate to leukocyte-mediated injury. Rather, the microvascular location and the state of activation may be more important determinants [86,87].

The second issue is that both studies that reported a beneficial effect of neutrophil depletion used Leukopak® filters to remove the cells [22,25], while Jeremy and Becker [24] used Imugard IG500 filters. Therefore it is appropriate to ask if the Leukopak® filters somehow confer a protective effect on the postischemic myocardium independent of their ability to remove neutrophils. This has not and cannot now be addressed since Leukopak® filters are no longer available. However, it has been noted that leukocyte depletion with Pall filters desensitizes the heart to the coronary vasoconstrictor and negative inotropic actions of C5a, which persist during subsequent perfusion with normal blood, and are thus independent of leukocyte depletion [64]. These findings indicate that filtered blood confers some ill-defined protective effect on the heart. This is potentially important because it suggests that there is an endogenous factor(s) that can be "activated" or "formed" within the tissue to provide persistent protection against the ischemia provoked by C5a. Moreover, because filtering blood solely at reperfusion attenuates stunning [25], it indicates that any endogenous protectant can prevent injury from occurring after an ischemic event. This is an area that warrants further investigation.

Summary

The participation of neutrophils in myocardial stunning remains uncertain. While it appears that neutrophils have the potential to elicit contractile dysfunction, it is not evident that this potential is realized after transient ischemic periods not associated with myocellular necrosis. Other mechanisms may account for the beneficial effects of filtering the blood of neutrophils on the stunned myocardium. However, this review is filled with disclaimers and qualifiers indicating large gaps in our knowledge concerning the time course of neutrophil activation, number of cells necessary to elicit functional derangements, the site of action of neutrophils within the heart, the mechanisms responsible for neutrophil-induced dysfunction, etc. Merely depleting circulating blood of neutrophils is not an adequate means to define their contribution to postischemic contractile dysfunction, and different approaches to this issue are mandated.

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