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Deferoxamine reduces and nitric oxide synthase inhibition increases neutrophil-mediated myotube injury

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Abstract We tested the contribution of reactive oxygen species (ROS), reactive nitrogen species (RNS) and the $\beta 2$ integrin CD18 to neutrophil-mediated myotube injury. Human myotubes were cultured with human neutrophils in the presence or absence of inhibitors directed against ROS, RNS, and CD18. Muscle injury was assessed by a ^{51}Cr release assay. The inclusion of superoxide dismutase (50–500 U/ml) in the culture medium did not affect myotube injury. A significant protective effect was provided by including catalase (600–2400 U/ml), deferoxamine (1–2 mM), or anti-CD18 antibody (10 $\mu\text{g}/\text{ml}$) in the culture medium. *S*-Ethylisothiourrea (500–1000 μM), an inhibitor of nitric oxide synthase (NOS), significantly increased myotube injury and reduced nitric oxide (NO) in cultures consisting of only myotubes. In conclusion, neutrophil-mediated skeletal muscle injury appears to be largely dependent on CD18-mediated neutrophil adhesion and iron-dependent hydroxyl radical production. In addition, skeletal muscle NOS activity may protect skeletal muscle against the injury caused by neutrophils.

Keywords Skeletal muscle · Injury · Reactive oxygen species · Reactive nitrogen species · Human

Introduction

Reactive oxygen and nitrogen species (ROS and RNS, respectively) are believed to cause both beneficial and deleterious effects in skeletal muscle (Kaminski and Andrade 2001; Rando 2001; Reid 2001). Although skeletal muscle possesses numerous enzymatic and non-enzymatic mechanisms to cope with fluctuations in ROS and RNS concentrations, a high rate of production in skeletal muscle can overwhelm antioxidant defenses and cause muscle dysfunction, membrane damage, protein degradation, and DNA modifications (Rando 2001; Reid 2001; Wink et al. 1996). Indeed, ROS and RNS have been reported to contribute to skeletal muscle injury and necrosis associated with muscular dystrophies (Rando 2001), ischemia-reperfusion (Rubin et al. 1996) and contraction-induced muscle injury (Pizza et al. 1998; Reid 2001; Zerba et al. 1990).

A potential source of injurious ROS and RNS is inflammatory cells (neutrophils and macrophages), which are known to appear in skeletal muscle in the hours to days following injury (Brickson et al. 2001; Frenette et al. 2002; McLoughlin et al. 2003; St. Pierre Schneider et al. 2002). Human neutrophils are capable of producing relatively high concentrations of ROS (Rosen et al. 1995) and have been demonstrated to cause injury to a variety of cell types (Entman et al. 1992; Murphy et al. 1999), including cultured skeletal muscle (Pizza et al. 2001). Although controversial, human neutrophils appear to lack nitric oxide synthase (NOS) activity (Yan et al. 1994) and the ability to produce nitric oxide (NO) (Holm et al. 1999; Miles et al. 1995; Sethi and Dikshit 2000; Yan et al. 1994). On the other hand, human skeletal muscle is known to express high levels of neuronal NOS (nNOS) (Frandsen et al. 1996; Nakane et al. 1993) and to increase production of NO after contraction-induced muscle injury (Radak et al. 1999). Because NO has pro- and antioxidant properties (Wink et al. 1996), an increase in NO in skeletal muscle could augment or attenuate the muscle injury induced by neutrophil-derived ROS.

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The present study was conducted to determine the contribution of ROS, RNS, and the $\beta 2$ integrin CD18, which is required for neutrophil adhesion (Cronstein and Weissmann 1993), to neutrophil-mediated skeletal muscle cell injury. Findings from this study indicate that neutrophil-mediated muscle injury is largely dependent on CD18-mediated neutrophil adhesion and iron-dependent OH production. In addition, skeletal muscle cell NOS activity appears to protect skeletal muscle from injury caused by neutrophils.

Materials and methods

Myoblasts

Human myoblasts (Clonetics Co., San Diego, CA) were seeded at 10,000 cells/cm² in flat-bottom collagen (type I; Sigma, St. Louis, MO) coated 24-well microtiter plates. Myoblasts were grown to ~90% confluence in growth medium (Clonetics) supplemented with 10 ng/ml epidermal growth factor (EGF), 10 μ g/ml insulin, 50 μ g/ml fetuin, 50 μ g/ml bovine serum albumin, 375 ng/ml dexamethasone, 50 μ g/ml gentamicin, and 50 ng/ml amphotericin-B in a humidified, 37°C, 5% CO₂ atmosphere.

To initiate differentiation into myotubes, growth medium was exchanged for Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% heat-inactivated fetal bovine serum and 2 ng/ml EGF (differentiation medium). Differentiation medium was changed every other day for 3 days. Hematoxylin-eosin staining and light microscopy observations of myotubes revealed numerous multinucleated cells (>4 nuclei). On the 4th day, myotubes were washed with Earle's balanced salt solution (EBSS) and radioactively labeled for 24 h in a humidified, 37°C, 5% CO₂ atmosphere. On the 5th day myotubes were cultured with human neutrophils.

Neutrophils

Human neutrophils were obtained from heparinized venous blood of healthy volunteers ($n=6$) after obtaining verbal and written consent in accordance with institutional guidelines. Neutrophils were isolated by density gradient centrifugation as previously described (Pizza et al. 2001). Briefly, whole blood was layered over neutrophil isolation medium (Accurate, Westbury, NY) and centrifuged to isolate neutrophils from mononuclear and red blood cells. The neutrophil layer was aspirated and washed in calcium and magnesium free Hanks' balanced salt solution (HBSS) and centrifuged. Residual red blood cells were lysed using an ammonium chloride solution. Lastly, neutrophils (5×10^6) were suspended in EBSS (600 μ l) supplemented with 0.25% FBS and 400 μ M L-arginine (co-culture medium).

For the muscle injury assay, neutrophils were stimulated in vitro by adding *N*-formyl-methionyl-leucyl-phenylalanine (fMLP; 2×10^{-6} M final concentration 0.02% v/v DMSO) to the neutrophil suspensions just before culturing them with myotubes. fMLP, upon binding to cell surface receptors, promotes ROS production from neutrophils via activation of NADPH oxidase (Dalpiaz et al. 2003). Cultures consisting of only neutrophils were stimulated with fMLP just prior to adding them to flat-bottom collagen-coated tissue culture plates. We have previously reported that stimulating human neutrophils with fMLP (2×10^{-6} M) results in a fourfold increase in neutrophil-derived superoxide anion (O₂⁻) production as measured by the cytochrome-*c* assay (Pizza et al. 2001).

Experimental design

Each subject's neutrophils were used across all experimental conditions. To assess the contribution of O₂⁻ to myotube injury,

superoxide dismutase (SOD; CuZn, Sigma Co., St. Louis, MO) at 50, 250, or 500 U/ml (30, 150, and 300 total units/well, respectively) was included in cultures consisting of both neutrophils and myotubes. The contribution of hydrogen peroxide (H₂O₂) was determined by including 600, 1200, or 2400 U/ml (360, 720 or 1440 total units/well, respectively) of catalase (bovine liver; Sigma) in co-cultures. To test the contribution of iron-mediated hydroxyl radical ([•]OH) generation, we preincubated myotubes with 0.5, 1.0 or 2.0 mM of the iron-chelator deferoxamine mesylate (Sigma) for 2 h. Pretreatment of myotubes and not neutrophils with deferoxamine mesylate was performed because previous investigators have demonstrated that the catalytic iron for [•]OH production in co-cultures is derived from target cells and not neutrophils (Gannon et al. 1987). These concentration ranges were selected based upon previous studies demonstrating the efficacy of SOD (Hansen and Stawski 1994; Lieser et al. 1995; Murphy et al. 1999), catalase (Lieser et al. 1995; Murphy et al. 1999; Weiss et al. 1981) and deferoxamine mesylate (Gannon et al. 1987; Hansen and Stawski 1994; Murphy et al. 1999; Varani et al. 1996) in reducing neutrophil-mediated cellular injury in vitro. We also tested whether the myotube injury caused by neutrophils was dependent on CD18-mediated neutrophil adhesion by including 10 μ g/ml of an anti-CD18 antibody (MHM23; DAKO Co., Carpinteria, CA) in co-cultures. The influence of RNS was determined by removing L-arginine from the co-culture medium and by culturing neutrophils with myotubes in the presence of an NOS inhibitor. *S*-Ethylisothiourea (S-EITU) (1, 500, or 1000 μ M) was used because of its high inhibitory potency for human inducible NOS (iNOS) and nNOS (Garvey et al. 1994), the two isoforms likely present in our neutrophil and myotube cultures. For all experimental conditions, neutrophils were preincubated for 10 min with the selected inhibitors prior to culturing them with myotubes. Furthermore, all of the inhibitors were present in the co-culture medium throughout the 24-h coincubation.

Muscle injury assay

Muscle injury was assessed by a standard chromium-51 (⁵¹Cr) release assay. Previous investigators have reported that ⁵¹Cr is rapidly taken up by cells and eventually becomes bound to cytoplasmic adenine nucleotides, cytoplasmic proteins, and membrane constituents (Chopra et al. 1987). Thus, the release of ⁵¹Cr from labeled cells is considered to be the gold standard for assessing cell injury in vitro. Specifically, the ⁵¹Cr assay has been reported to be a more sensitive indicator of cell injury relative to other commonly used techniques such as quantifying the release of lactate dehydrogenase (Chopra et al. 1987).

In the present study, myotubes were radioactively labeled for 24 h by incubation with differentiation medium supplemented with 24 μ Ci/well of ⁵¹Cr (Na₂⁵¹CrO₄; ICN, Costa Mesa, CA). After the 24-h labeling period myotubes were washed with EBSS. Neutrophils (5×10^6 cells/well) in the presence or absence of SOD, catalase, deferoxamine mesylate, anti-CD18 antibody or S-EITU were then introduced to the plate. The plate was centrifuged at 50 \times g for 1 min to promote adhesion of neutrophils to myotubes. Neutrophils were cultured with skeletal myotubes for 24 h in a humidified, 37°C, 5% CO₂ atmosphere.

Each plate contained wells for experimental conditions, maximal ⁵¹Cr release, and background release. Maximal ⁵¹Cr release was induced by incubating myotubes with a 0.5% Triton X-100 (Sigma) solution for 10 min. Background release was determined by incubating myotubes with co-culture medium in the presence and absence of SOD, catalase, deferoxamine mesylate, S-EITU or anti-CD18 antibody. An aliquot from each well was aspirated and centrifuged to precipitate detached myoblasts and non-adherent neutrophils. After centrifugation, radioactivity was counted by gamma-counter. An injury index was calculated using the mean of triplicates in the following equation: myotube injury index = $(e-b)/(m-b)$, where e = mean experimental release, b = mean background release, and m = mean maximal release. Myotube injury indices for control experiments were calculated using background release

wells containing unsupplemented co-culture medium. Although none of the experimental conditions significantly affected background release (data not reported), myotube injury indices for experimental conditions were calculated using mean background release wells containing the appropriate concentration of SOD, catalase, deferoxamine mesylate, S-EITU, or anti-CD18 antibody. The intraassay percent coefficient of variation across all experiments was 3.8%, indicating an acceptable degree of assay repeatability.

Nitric oxide chemiluminescence

Conditioned media from cultures consisting of either neutrophils ($5 \times 10^6/600 \mu\text{l}$) or confluent myotubes (24-well plates; $6 \text{ cm}^2/\text{well}$) were collected after a 24-h incubation (humidified, 37°C , 5% CO_2 atmosphere) period. Nitrite, nitrate and nitrosothiols (NOx) in the medium were first reduced to NO using vanadium (III) chloride at $>90^\circ\text{C}$. NO concentrations were then measured using a chemiluminescent analyzer (NOA 280i; Sievers Instruments, Boulder, CO). The analyzer was calibrated using $100 \mu\text{l}$ of sodium nitrate (50 nM–10 mM) and the samples ($100 \mu\text{l}$) were analyzed in triplicate.

Statistics

The injury indices for each experimental condition were statistically analyzed by a repeated measures analysis of variance (ANOVA). NOx concentrations in cultures consisting of either neutrophils or myotubes were analyzed by an ANOVA. The Newman-Keuls post hoc test was used to determine differences between means when the observed *F*-ratio was significant ($P \leq 0.05$). All data are represented as the mean \pm SE.

Results

The inclusion of SOD (50–500 U/ml) in the co-culture medium did not significantly affect the injury index (Fig. 1a). The failure of SOD to influence the injury index was not attributed to a suboptimal concentration of SOD because SOD ($<300 \text{ U/ml}$) abolished the detection of O_2^- in cultures consisting of neutrophils stimulated with fMLP (data not reported). A significant protection against myotube injury, however, was observed when catalase was added to the co-culture medium (Fig. 1b). Specifically, 600, 1200, and 2400 U/ml of catalase resulted in a 13%, 32%, and 47% reduction in the injury index relative to the control condition. The reduction in the injury index with catalase treatment may indicate that H_2O_2 and/or downstream ROS contributed to the myotube injury caused by neutrophils. Interestingly, a comparable degree of protection was afforded by treatment with deferoxamine. The 40% and 55% reduction in the injury index associated with 1.0 and 2.0 mM of deferoxamine, respectively (Fig. 1c), may indicate that the hydroxyl radical was the dominant ROS that caused myotube injury. The neutrophil-mediated myotube injury was also largely dependent on CD18 as indicated by the 63% reduction in the injury index with anti-CD18 antibody treatment (Fig. 2). As expected, qualitative observations revealed that the anti-CD18 antibody also abolished neutrophil adhesion to myotubes.

Although the removal of L-arginine from the co-culture medium had no significant effect on the injury

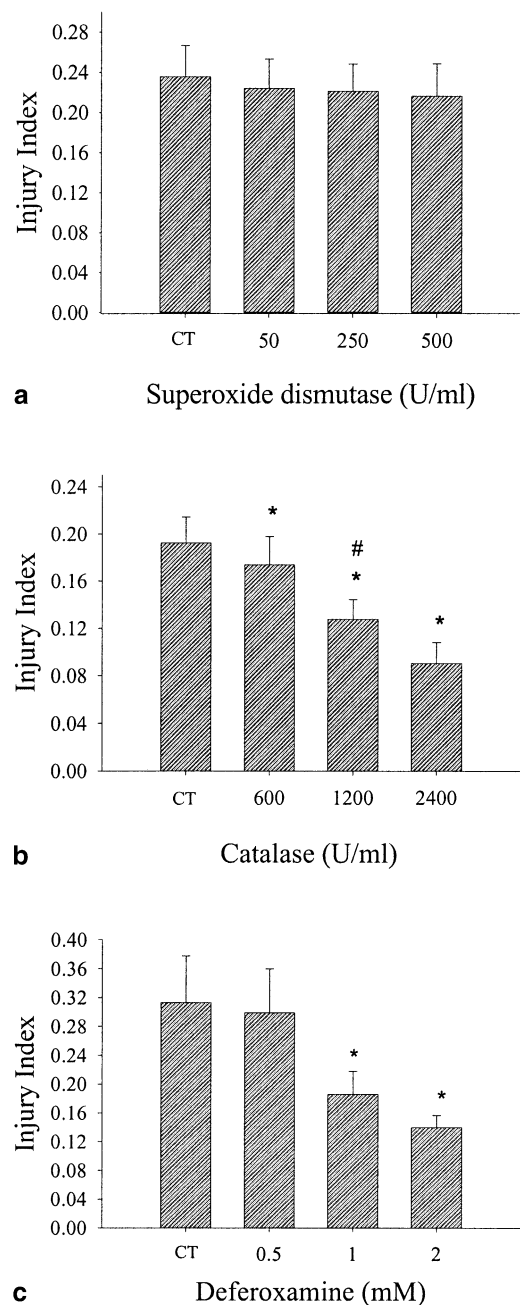


Fig. 1a–c Injury indices for control (CT) and experimental conditions examining the contribution of ROS to neutrophil-mediated skeletal myotube injury. **a** Neutrophils were preincubated with superoxide dismutase (50, 250 or 500 U/ml) for 10 min prior to culturing with skeletal myotubes. **b** Neutrophils were preincubated with catalase (600, 1200 or 2400 U/ml) for 10 min prior to culturing with myotubes. * $P \leq 0.05$ compared to CT. # $P \leq 0.05$ compared to 600 and 2400 U/ml. **c** Myotubes were preincubated for 2 h with deferoxamine (0.5, 1, or 2 mM) prior to culturing them with neutrophils. * $P \leq 0.05$ compared to CT and 0.5 mM. All conditions are represented as means \pm SE ($n=6$)

index (Fig. 3a), NOS inhibition via S-EITU treatment increased the injury index by 21% (500 μM) and 23% (1000 μM) (Fig. 3b). The increased injury index with S-EITU treatment may indicate that NO and/or downstream

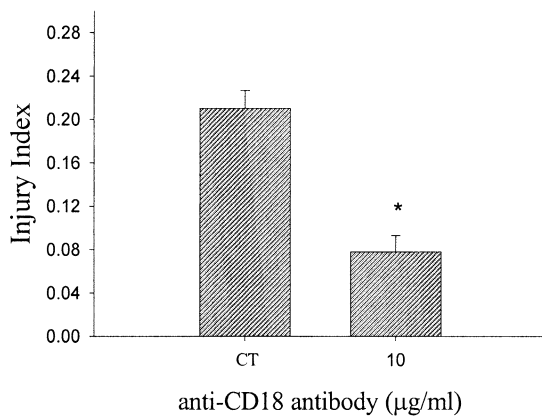


Fig. 2 Contribution of the β_2 integrin (CD18) to neutrophil-mediated myotube injury. Injury index for control (CT) and experimental conditions in which neutrophils were preincubated with anti-CD18 antibody (10 $\mu\text{g/ml}$) for 10 min prior to culturing them with myotubes. *Significantly different compared to CT. All conditions are represented as means \pm SE ($n=6$)

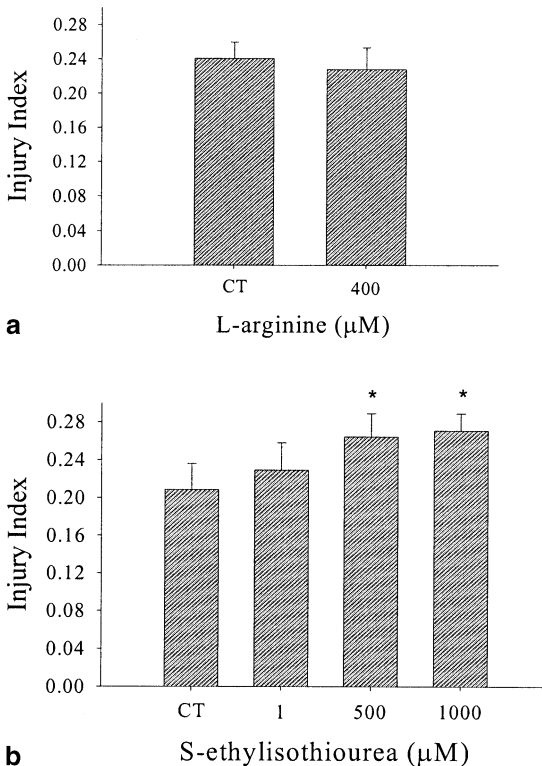


Fig. 3a, b Injury indices for control (CT) and experimental conditions examining the contribution of RNS to neutrophil-mediated skeletal myotube injury. **a** CT conditions lacking L-arginine supplementation compared to conditions supplemented with 400 μM L-arginine. **b** Neutrophils were preincubated with S-ethylisothiourea (1, 500 or 1000 μM) for 10 min prior to culturing with skeletal myotubes. *Significantly different from CT. All conditions are represented as means \pm SE ($n=6$)

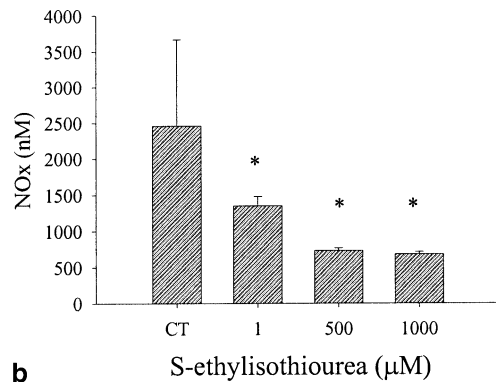
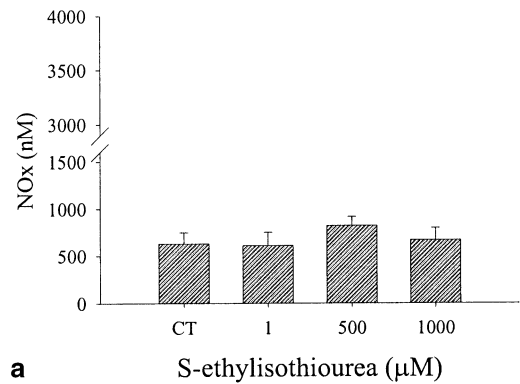


Fig. 4a, b Nitric oxide chemiluminescence for control (CT) and experimental conditions containing neutrophils or skeletal myotubes. Neutrophils (**a**) ($n=5$) or skeletal myotubes (**b**) ($n=6$) were treated with fMLP and S-ethylisothiourea (1, 500 and 1000 μM) for 24 h and analyzed for nitrite, nitrate, and nitrosothiol concentrations (NOx). *Significantly different from CT. All conditions are represented as means \pm SE

RNS protect myotubes from injury caused by neutrophils. In an effort to delineate the source of the protective NOx, we quantified NOx concentrations in cultures consisting of either neutrophils or myotubes in the presence of fMLP and S-EITU. Neutrophil only cultures produced low levels of NOx that were not influenced by fMLP (data not reported) or S-EITU (Fig. 4a). Treatment of myotube cultures with fMLP and S-EITU resulted in significant reductions in NOx concentrations. Specifically, 1, 500, and 1000 μM of S-EITU caused a 45%, 70%, and 72% reduction in NOx concentrations, respectively (Fig. 4b). Taken together, these data indicate that a substantial reduction in myotube NOS activity exacerbates neutrophil-mediated myotube injury.

Discussion

The observations in the current study confirm our previous report (Pizza et al. 2001) by demonstrating that neutrophils injure cultured myotubes. The present study extends our prior observations by demonstrating that neutrophil-mediated myotube injury was largely depen-

dent on CD18-mediated neutrophil adhesion and on an apparent increase in iron-dependent OH radical production. Considering that skeletal myotubes in culture appear to possess the requisite antioxidant defenses, including: catalase, glutathione peroxidase and SOD (Franco et al. 1999), these findings may indicate that neutrophil-derived ROS injure skeletal muscle by overwhelming antioxidant defenses. In addition, myotube NOS activity appears to provide modest protection for myotubes against injury caused by neutrophils.

Contribution of ROS

Neutrophils, a potent source of ROS, have been demonstrated to cause injury to cultured endothelial cells and cardiac myocytes (Entman et al. 1992; Hansen and Stawski 1994; Murphy et al. 1999). The present study extends these observations by demonstrating that ROS also contribute to neutrophil-mediated myotube injury. Specifically, the significant reduction in the injury index with catalase treatment (Fig. 2b) implicates H₂O₂ as an injurious oxidant in our neutrophil and myotube cultures. In addition to its role as a potent oxidizing agent, its ability to participate in iron-dependent chemistry (e.g., Haber-Weiss chemistry), resulting in the production of the OH radical, potentiates the ability of H₂O₂ to mediate cellular injury. This latter role of H₂O₂ in the cascade of ROS production appears to be a vital mechanism for neutrophil-mediated myotube injury.

Considering that a comparable degree of protection was observed between the catalase and deferoxamine treatments (Fig. 1b, c, respectively), it appears that the predominant means for H₂O₂-mediated injury was via iron-dependent OH generation. This interpretation is consistent with previous studies that have implicated iron-dependent OH production in neutrophil-mediated injury to cultured endothelial cells and cardiac myocytes (Gannon et al. 1987; Hansen and Stawski 1994; Varani et al. 1996) and with the observations of Fantani and Yoshioka (1993), who reported that deferoxamine administration prevented reoxygenation injury in postischemic skeletal muscle in vivo. Because neutrophil-derived ROS can permeate plasma membranes (Gabbita et al. 2000) and release ferric iron from ferritin (Biemond et al. 1984) and myoglobin (Prasad et al. 1989), neutrophil-initiated iron-dependent OH generation may serve as a potential mechanism whereby neutrophils could injure skeletal muscle cells.

Contribution of neutrophil adhesion

The CD11b/CD18 (Mac-1) is a leukocyte adhesion molecule expressed on the neutrophil surface, consisting of a 160-kDa α -chain (CD11b) noncovalently linked to a 95-kDa β -chain (CD18) (Cronstein and Weissmann 1993). Expression of the CD18 subunit, in particular, appears to be largely responsible for regulating the

adhesion properties of neutrophils (Entman et al. 1990). Data from the present study (Fig. 2) are in accordance with several studies reporting a protective effect of CD18 inhibition against neutrophil-mediated cellular injury to cultured cardiac myocytes and endothelial cells (Entman et al. 1992; Hansen and Stawski 1994; Lieser et al. 1995). Furthermore, considering that CD18-dependent neutrophil adhesion appears to function as a trigger for the release of ROS (Shappell et al. 1990), myotube protection through CD18 inhibition may be provided through a reduction in neutrophil adhesion-dependent ROS generation.

Contribution of RNS

The increase in the injury index with NOS inhibition (Fig. 3) suggests that NO and/or downstream RNS protects skeletal muscle from injury caused by neutrophils. This interpretation is in agreement with several in vitro studies which reported a significant protective role of NO against neutrophil-mediated cellular injury (Casini et al. 1997; Hansen and Stawski 1994; Murphy et al. 1999; Wink and Mitchell 1998). The mechanism for the protection in the present study may be related to NO's ability to inhibit neutrophil adhesion, reduce neutrophil-derived ROS production, interrupt lipid peroxidation chain reactions, and/or scavenge other cytolytic molecules (Clancy et al. 1992; Kubes et al. 1991; Sethi and Dikshit 2000).

Despite the evidence that NO can regulate several events associated with inflammation, the production of NO by human blood neutrophils is controversial. The low, but detectable, levels of NO_x in our neutrophil cultures were not increased with fMLP treatment or reduced by S-EITU (Fig. 4a). These observations are consistent with previous investigators who have reported that NO production by human blood neutrophils is not influenced by fMLP or by inhibitors of NOS activity (Holm et al. 1999; Padgett and Pruett 1995). In contrast, Schmidt et al. (1989) reported increased neutrophil-derived NO after fMLP stimulation. Human blood and extravasated neutrophils have also been reported to have low or undetectable NOS (constitutive and inducible) mRNA, protein, and/or activity (Miles et al. 1995; Tse et al. 2001; Yan et al. 1994). Thus, the NO_x concentrations observed in our neutrophil cultures may have been produced via a NOS-independent mechanism. This interpretation is consistent with previous investigators who have suggested that human blood neutrophils can synthesize nitrite and nitrate via NOS-independent mechanisms such as from exogenous N^G-hydroxy-L-arginine (an intermediate in the biosynthesis of NO from L-arginine) and via a reaction between D- or L-arginine and H₂O₂ (Holm et al. 1999; Nagase et al. 1997; Tse et al. 2001).

Because S-EITU reduced NO_x concentrations in myotube cultures (Fig. 4b) and increased neutrophil-mediated myotube injury (Fig. 3b), the protective NO_x in cultures consisting of both neutrophils and myotubes

appears to have been derived from myotube NOS activity. This interpretation is consistent with Wehling et al. (2001), who reported that the normalization of NOS expression in dystrophin-deficient muscle (mdx mice) reduced muscle injury and inflammation associated with muscular dystrophy. The protection afforded by increased NOS expression in mdx muscle was attributed to NO inhibition of macrophage-mediated muscle injury (Wehling et al. 2001). In contrast, Zhuang et al. (2001) reported that the susceptibility of cultured skeletal myotubes to oxidative injury was independent of NOS expression. However, as suggested by Zhuang et al. (2001), the nature of the oxidative stress implemented in their culture system (i.e., paraquat administration) may not reflect physiological relevant oxidative stress. Based on our observations and those of Wehling et al. (2001), NOS activity in skeletal muscle may play an important role in coping with oxidative stress imposed by myeloid cells.

Conclusion

In the context of skeletal muscle injury, neutrophils are thought to contribute to the phagocytosis of injured tissue (Papadimitriou et al. 1990). Because neutrophils can release ROS during phagocytosis (Kobayashi et al. 1998), it is possible that neutrophil-derived ROS exacerbate preexisting muscle injury in vivo by damaging previously uninjured muscle. The data provided in the present study lend credence to our hypothesis by demonstrating that ROS contribute to neutrophil-mediated injury to healthy skeletal muscle cells. Based on the observed increase in neutrophil-mediated myotube injury with NOS inhibition, our results may also indicate that a decrease in skeletal muscle NOS activity may increase the susceptibility of skeletal muscle to oxidative stress imposed by neutrophils. However, because cell culture studies cannot replicate the milieu of skeletal muscle in vivo, further work is needed to determine if similar mechanisms are operating in vivo.

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