

# Hyperbaric Oxygen Inhibits Reperfusion-Induced Neutrophil Polarization and Adhesion Via Plasmin-Mediated VEGF Release

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**Background:** Ischemia-reperfusion (IR) injury is seen in many settings such as free flap salvage and limb replantation/revascularization. The consequences—partial/total flap loss, functional muscle loss, or amputation—can be devastating. Of the treatment options available for IR injury, hyperbaric oxygen (HBO) is the most beneficial. HBO inhibits neutrophil-endothelial adhesion through interference of CD18 neutrophil polarization in IR, a process mediated by nitric oxide. The purposes of this study were to examine the involvement of vascular endothelial growth factor (VEGF) in the beneficial HBO effect on CD18 polarization and neutrophil adhesion and investigate the effect of plasmin on VEGF expression in skeletal muscle following IR injury.

**Methods:** A rat gracilis muscle model of IR injury was used to evaluate the effect of VEGF in IR, with and without HBO, on neutrophil CD18 polarization and adhesion in vivo and ex vivo. Furthermore, we investigated the effects that plasmin has on VEGF expression in gracilis muscle and pulmonary tissue by blocking its activation with alpha-2-antiplasmin.

**Results:** HBO treatment following IR injury significantly decreased neutrophil polarization and adhesion ex vivo compared with the IR group. Anti-VEGF reversed the beneficial HBO effect after IR with polarization and adhesion. In vivo adhesion was also increased by anti-VEGF. HBO treatment of IR significantly increased the VEGF protein in both gracilis and pulmonary vasculature. Alpha-2-antiplasmin significantly reversed the HBO-induced increase of VEGF in gracilis muscle.

**Conclusions:** These results suggest that HBO inhibits CD18 polarization and neutrophil adhesion in IR injury through a VEGF-mediated pathway involving the extracellular matrix plasminogen system. (*Plast Reconstr Surg Glob Open* 2017;5:e1497; doi: 10.1097/GOX.0000000000001497; Published online 25 September 2017.)

## BACKGROUND

Ischemia-reperfusion (IR) injury refers to a phenomenon commonly seen after the restoration of blood flow after a period of ischemia. In addition to the initial ischemic insult, reestablishment of blood flow often triggers a

deleterious chain of events characterized by vasoconstriction, microthrombi formation, and endothelial damage with subsequent edema and tissue necrosis. Free flap salvage, limb replantation/revascularization, and composite tissue allotransplantation are examples where IR injury can be seen clinically; the consequences might include partial/total flap loss, functional muscle loss, graft rejection, or amputation. These may be associated with additional procedures and health care costs. The mechanisms behind IR injury appear to stem from neutrophil adherence in the microcirculation, with subsequent release of reactive oxygen species.<sup>1-3</sup> This has been demonstrated by

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intravital microscopy of skeletal muscle subjected to IR, where within 5 minutes of reperfusion, neutrophils can be seen to slow in the postcapillary microcirculation, roll along the vessel wall, and eventually adhere to the endothelium.<sup>4-6</sup> The pathophysiology of IR injury in skeletal muscle and therapeutic interventions to mitigate its effects have been appraised by Wang et al.<sup>7,8</sup>

Hyperbaric oxygen (HBO) has been clinically shown to have beneficial effects on the outcome of replanted tissues, and it has been demonstrated experimentally to reduce the deleterious effects of IR, namely with respect to vasoconstriction and neutrophil adherence. HBO can reduce the damage caused from IR injury.<sup>9-11</sup> Part of the beneficial effect of HBO is enhanced wound angiogenesis, predominantly mediated by vascular endothelial growth factor (VEGF). HBO produces a well-documented increase in VEGF mRNA transcription<sup>12</sup> with a subsequent increase in VEGF protein.<sup>13-15</sup> These findings represent a long-term effect of HBO, on the order of days to weeks. Although most studies to date have focused on this long-term effect, HBO also causes early changes that are crucial for tissue survival by preventing neutrophil adherence.

Neutrophils possess a CD18/11b surface adhesion molecule that binds to intercellular adhesion molecule (ICAM) on the endothelial surface. Our group demonstrated with flow cytometry that IR causes a quantitative increase in the number of CD18 molecules<sup>16</sup> and, with confocal microscopy, that there is a qualitative effect on the distribution of the molecules,<sup>17</sup> both occurring within 90 minutes of reperfusion. CD18 becomes polarized, or concentrated on 1 area of the surface, allowing for increased ICAM interaction and therefore increased neutrophil adherence. Further work by our group showed that HBO decreases neutrophil adherence in the microcirculation model<sup>4</sup> and also affects the distribution of CD18 on the neutrophil surface, preventing the polarization necessary for binding to the endothelium.<sup>18</sup> This early benefit of HBO is nitric oxide (NO) and NO synthase (NOS) dependent.<sup>19</sup> An association between NO and HBO has been shown in both the *in vivo* microcirculation model and the *ex vivo* polarization/adherence model. Nitric oxide scavengers and NOS inhibitors reverse the beneficial HBO effect on CD18 polarization and neutrophil adherence *in vivo* and *ex vivo*.<sup>19</sup>

Ischemia causes a decrease of NO and endothelial nitric oxide synthase (eNOS) in the hypoxia-damaged endothelial cells. Hypoxia is a stimulus for the upregulation of (hypoxia inducible factor) HIF-1 $\alpha$  which, when combined with HIF-1 $\beta$ , leads to upregulation of VEGF mRNA. Several investigators have reported increased VEGF mRNA in IR models of liver, lung, spinal cord, skeletal muscle, and heart ischemia.<sup>20-30</sup> Others have explored the effect of VEGF in IR injury either through addition of VEGF to the system or blocking VEGF (myocardial infarction, spinal cord, epigastric, rectus abdominis flaps, and liver ischemia).<sup>23,25,31</sup> Several studies have indicated that the proinflammatory actions of VEGF cause further damage in ischemic tissue. However, some cytoprotective effects were reported but were explained as exogenous VEGF-induced

release of NO from endothelial cells.<sup>32</sup> *In vitro* assays indicate that VEGF significantly increases NO produced by endothelial cells.<sup>33</sup>

The purposes of this study were to examine the involvement of VEGF in the beneficial HBO effect on CD18 polarization and neutrophil endothelial adhesion in the microcirculation of skeletal muscle and to determine if the plasmin fibrinolytic pathway mediates VEGF release.

## METHODS

### Animal Model

This animal model and protocol were approved by the University Institutional Animal Care and Use Committee. The animals were allowed to recover for at least 4 days upon arrival in the Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility. The animals were dual housed with 12/12 light/dark cycle and received food and water *ad libitum*.

The Wistar rat gracilis skeletal muscle model of IR injury was used for this study.<sup>4</sup> Male Wistar rats were chosen to parallel previous models of IR injury at our facility. Briefly, the animals were anesthetized with sodium pentobarbital (50 mg/kg), administered intraperitoneally. The gracilis muscle flap was isolated and raised on its vascular pedicle in all groups. Ischemia was induced by placing a vascular clamp across the pedicle for 4 hours. Reperfusion began with the removal of the vascular clamp and progressed for up to 2 hours.

### HBO Treatment

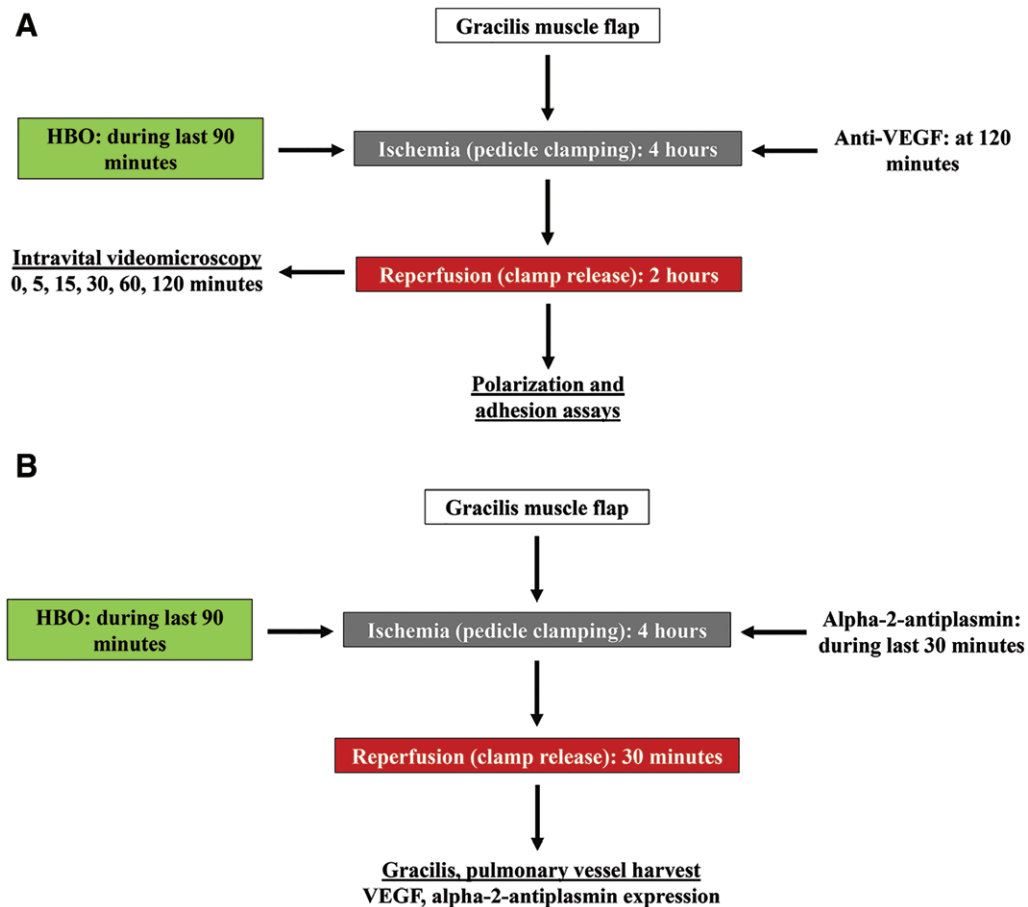
HBO treatment consisted of 100% oxygen at 2.5 atmospheres absolute during the last 90 minutes of ischemia (Hyperbaric Research Chamber Model 1300, Sechrist Industries, Inc., Anaheim, Calif.).

### In Vivo Neutrophil Adhesion

Twenty-four male Wistar rats (130 $\pm$ 40 g) were randomly assigned to one of 5 groups: (1) nonischemic control (NIC); (2) NIC + anti-VEGF; (3) IR; (4) IR-HBO; and (5) IR-HBO + anti-VEGF. The monoclonal antibody anti-VEGF (167  $\mu$ g/kg) (Calbiochem/EMD Biosciences, La Jolla, Calif.) was infused into the contralateral femoral vein 30 minutes before HBO treatment (*i.e.*, at 120 minutes ischemia). Intravital videomicroscopy was used to view and quantify adherent leukocytes at the following time points postreperfusion in gracilis muscle venules (reported as change from baseline): baseline, 5, 15, 30, 60, and 120 minutes. A schematic diagram of the procedure is shown in Figure 1A.

### Ex Vivo CD18 Polarization and Adhesion Assays

Twenty-two male Wistar rats (396 $\pm$ 22 g) were randomly assigned to one of the same 5 groups outlined above. The same systemic anti-VEGF administration procedure was used as for the videomicroscopy procedure. Following reperfusion, 1 mL of heparinized whole blood was obtained from the gracilis flap through the epigastric vein with the tip of the catheter at the opening of the gracilis pedicle. The femoral vein was clamped during withdrawal to ensure that the sample was from the gracilis microvasculature and not from the systemic circulation. The plasma was separated by centrifugation and used as the activat-



**Fig. 1.** Schematic diagrams of gracilis muscle flap assays. A, In vivo neutrophil adhesion and ex vivo polarization/adhesion assays. B, VEGF and alpha-2-antiplasmin protein expression.

ing agent in the adhesion and polarization assays. A schematic diagram of the procedure is shown in Figure 1A.

Polarization and adhesion methods were published previously.<sup>17,18</sup> Briefly, normal neutrophils were combined with activated plasma and sICAM (in tubes or on coverslips) for 30 minutes at 37°C. The neutrophils were labeled with fluorescein isothiocyanate anti-rat CD11b (0.10 µg; BD Pharmingen, Torreyana, Calif.) for 30 minutes at 37°C and fixed with 1% paraformaldehyde in phosphate-buffered saline for confocal microscopic analysis. Twenty consecutive, isolated neutrophils from each animal were photographed on the confocal microscope (Carl Zeiss, Inc., N.Y.) and a Z-stack analysis (LSM 510 software) was performed. Based on the mean intensity, percentage of polarized cells was determined for each experimental animal (polarized cells/20 cells analyzed × 100 = percentage polarized cells). The adherent neutrophils on the coverslips were counted, and percentage adherent neutrophils was calculated in a similar fashion.

#### VEGF and Alpha-2-Antiplasmin Protein Expression

Fifty-six male Wistar rats (318±7g) were randomly assigned to 1 of the 7 groups: (1) NIC; (2) IR; (3) IR-HBO; (4) NIC-HBO; (5) IR-HBO + Alpha-2-antiplasmin; (6) IR-HBO + saline (vehicle control); and (7) NIC + Alpha-2-antiplasmin.

A local, gracilis flap was raised and allowed to stabilize. An injection of alpha-2-antiplasmin (0.8 mg/kg × 0.1 mL; Sigma Chemical Co., St. Louis, Mo.) or saline (0.1 mL) was completed during the last 30 minutes of ischemia with flow stopped. This local injection was accomplished through the epigastric artery with a 30-gauge blunted needle with the femoral artery clamped. The injection was visible as it washed blood out of the gracilis microvasculature into the femoral vein. Thirty minutes of no flow were allowed for tissue uptake of the alpha-2-antiplasmin. Following tissue uptake, the clamp was released, and after 30 minutes of reperfusion, the anterior gracilis muscle and the pulmonary vasculature were harvested, weighed, and frozen with liquid nitrogen. A schematic diagram of the procedure is shown in Figure 1B. The tissues were stored at -70°C for western blot analysis of VEGF protein.

For alpha-2-antiplasmin western blot analysis, a similar procedure was performed with 12 Wistar rats randomly assigned to 1 of the 2 groups: NIC-saline and NIC-Alpha-2-antiplasmin. Endogenous alpha-2-antiplasmin, estimated from the NIC-saline group, was set at 100%. This method and measurement was performed to ensure that the alpha-2-antiplasmin was delivered through the gracilis pedicle artery and crossed the vascular barrier.

Tissue samples were prepared for western blot analysis (n = 8/group). The frozen tissue samples were homog-

enized and protein was isolated and quantified using the Bradford Assay (BioRad, Hercules, Calif.). Total protein volume was normalized to 20  $\mu\text{g}$  per sample. The detection of VEGF and alpha-2-antiplasmin protein was performed using standard western blot techniques. Briefly, the normalized protein samples were run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel by electrophoresis, transferred to the nitrocellulose membrane, and then probed with monoclonal anti-VEGF antibody (R&D Systems, Minneapolis, Minn.) and anti-alpha-2-antiplasmin (Fitzgerald Industries International, Concord, Mass.). Chemiluminescent detection was employed using the ECL Plus kit (Amersham). Glyceraldehyde-3-phosphate dehydrogenase was utilized as a housekeeping protein for standardization. Semiquantitative analysis of chemiluminescent intensity between the treatment groups was conducted using the Typhoon Variable Wavelength Imager (Amersham). Intensity was expressed as a percentage of intensity of the nonischemic control.

### Statistics

The results are reported as mean  $\pm$  standard error of the mean of the raw data. Analysis of variance for repeated measures was used to evaluate the statistical difference between the groups/time levels/measures, and a Duncan's Post Hoc analysis was performed to determine between-group differences. Arc sine transformations of the raw percentages were performed to meet the assumptions of analysis of variance. A  $P \leq 0.05$  was considered significant.

## RESULTS

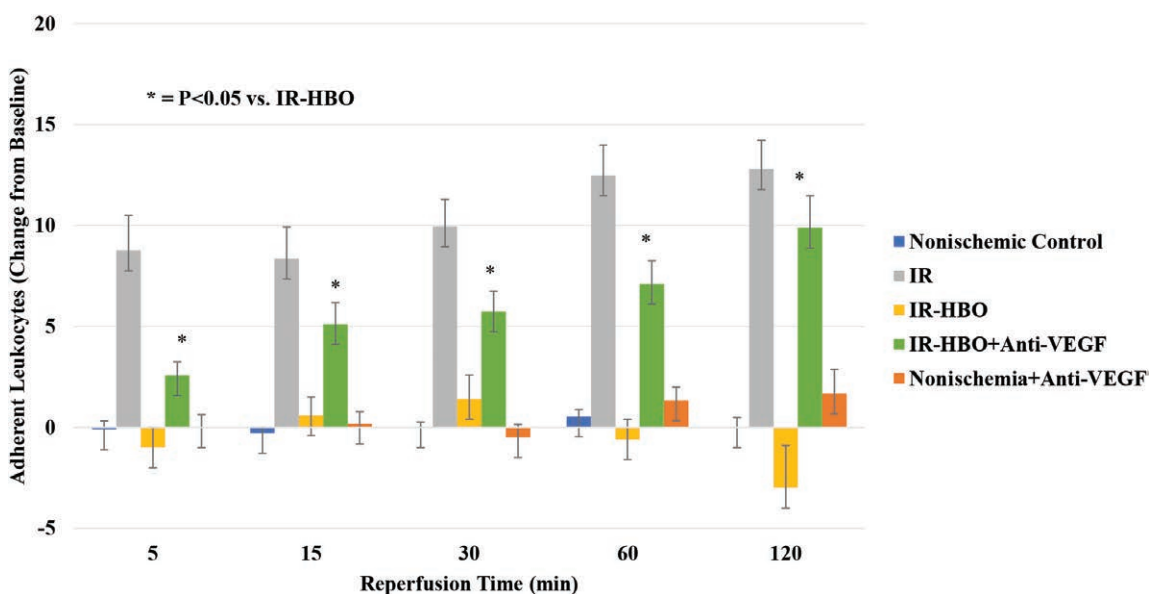
### In Vivo Neutrophil Adhesion

The in vivo model of IR showed a significant increase in the neutrophil-endothelial adherence, which was significantly

reduced from baseline by HBO treatment. Figure 2 demonstrates that when anti-VEGF infusion was added to the IR-HBO group, it significantly increased adherent neutrophils at 5, 15, 30, 60, and 120 minutes when compared with IR-HBO ( $9.89 \pm 1.58$  versus  $-3.0 \pm 2.1$ ;  $P < 0.05$  at 120 minutes post-perfusion). These data show that the HBO mechanism in IR involves VEGF in the reduction of neutrophil-endothelial adhesion in the microcirculation. The raw data for each group are represented in Table 1.

### Ex Vivo CD18 Polarization and Adhesion

The ex vivo polarization and adhesion assays were developed to isolate the cellular interactions with activated plasma from experimental animals. Figure 3 demonstrates that polarization of the heterodimer CD18 was significantly increased in the IR group compared with NIC ( $43.89 \pm 12.07\%$  versus  $6.23 \pm 1.71\%$ ;  $P < 0.05$ ). This IR-induced polarization was significantly reduced by HBO treatment to  $5.48 \pm 2.93\%$  versus IR  $43.89 \pm 12.07\%$ ,  $P < 0.01$  (Fig. 3). When the IR-HBO group was infused with anti-VEGF, the percentage of CD18 polarization significantly increased to  $36.00 \pm 7.31\%$  ( $P < 0.01$ ). These data show that VEGF could be a signaling molecule in the HBO mechanism in IR injury by inducing CD18 polarization. The resultant neutrophil adhesion was similarly changed. Figure 4 shows that neutrophil adhesion was significantly increased in the IR cohort compared with NIC ( $16.69 \pm 2.18\%$  versus  $3.33 \pm 1.71\%$ ;  $P < 0.05$ ). HBO treatment reduced neutrophil adhesion induced by IR injury from  $16.69 \pm 2.18\%$  to  $1.64 \pm 0.35\%$  ( $P < 0.05$ ). Anti-VEGF infusion also significantly reversed the HBO effect on adhesion (IR-HBO + anti-VEGF versus IR-HBO,  $24.94 \pm 6.32\%$  versus  $1.64 \pm 0.35\%$ ;  $P < 0.05$ ). Blocking the endogenous VEGF involvement in polarization and adhesion with a monoclonal antibody against VEGF effectively reversed the beneficial HBO effect.



**Fig. 2.** The number of adherent leukocytes (expressed as change from baseline) from intravital videomicroscopy of gracilis muscle venules. Nonischemic control (blue), nonischemia + anti-VEGF (orange), IR (grey), IR-HBO (yellow), IR-HBO + anti-VEGF (green). \* $P < 0.05$  vs. IR-HBO.

**Table 1. The Number of Adherent Leukocytes (Expressed as Change from Baseline) from Intravital Videomicroscopy**

Group	No. Vessels	Reperfusion Time (min)	Change in Adherent Leukocytes (SEM)
1	18	5	-0.11 (0.43)
	17	15	-0.29 (0.29)
	17	30	0 (0.27)
	17	60	0.53 (0.35)
	16	120	0 (0.49)
2	6	5	0 (0.63)
	6	15	0.17 (0.60)
	4	30	-0.5 (0.65)
	3	60	1.33 (0.67)
	3	120	1.67 (1.20)
3	17	5	8.76 (1.74)
	17	15	8.35 (1.57)
	19	30	9.95 (1.35)
	17	60	12.47 (1.51)
	18	120	12.78 (1.43)
4	9	5	-1.0 (1.00)
	9	15	0.60 (0.90)
	9	30	1.40 (1.20)
	9	60	-0.60 (1.00)
	9	120	-3.00 (2.10)
5	18	5	2.58 (0.66)*
	19	15	5.11 (1.07)*
	19	30	5.74 (1.01)*
	19	60	7.11 (1.14)*
	19	120	9.89 (1.58)*

Group 1, nonischemic control; group 2, nonischemic control with anti-VEGF; group 3, 4 hours of ischemia with reperfusion; group 4, IR plus HBO during the last 90 minutes of ischemia; group 5, IR plus HBO with anti-VEGF.

\* $P < 0.05$  versus group 4.

SEM, standard error of the mean.

### VEGF Protein Expression

In the gracilis muscle, the percentage of VEGF protein in the IR-HBO group was significantly increased compared with IR and NIC-HBO ( $134.69 \pm 7\%$  versus  $90.59 \pm 2.87\%$  and  $101.38 \pm 7.41\%$  of NIC,  $P < 0.05$ ; Fig. 5). The percentage of VEGF was, again, significantly increased in the pulmonary tissue (Fig. 6) from IR-HBO compared with IR and NIC-HBO ( $112.4 \pm 7.8\%$  versus  $88.8 \pm 4.1\%$  and

$89.9 \pm 7.2\%$  of NIC;  $P < 0.05$ ). Additionally, to examine the contribution of the extracellular matrix and the plasminogen system to this increased VEGF in the gracilis muscle we injected alpha-2-antiplasmin into the pedicle artery of the gracilis muscle 30 minutes before HBO treatment. The plasmin blocker, alpha-2-antiplasmin, significantly reversed the HBO induced increase of VEGF in gracilis muscle ( $98.95 \pm 10.65\%$  versus  $134.69 \pm 7\%$  of NIC;  $P < 0.05$ ; Fig. 5). A similar reversal was seen in pulmonary vasculature ( $95.11 \pm 7.32\%$  versus  $112.4 \pm 7.8\%$  of NIC) but did not reach significance (Fig. 6).

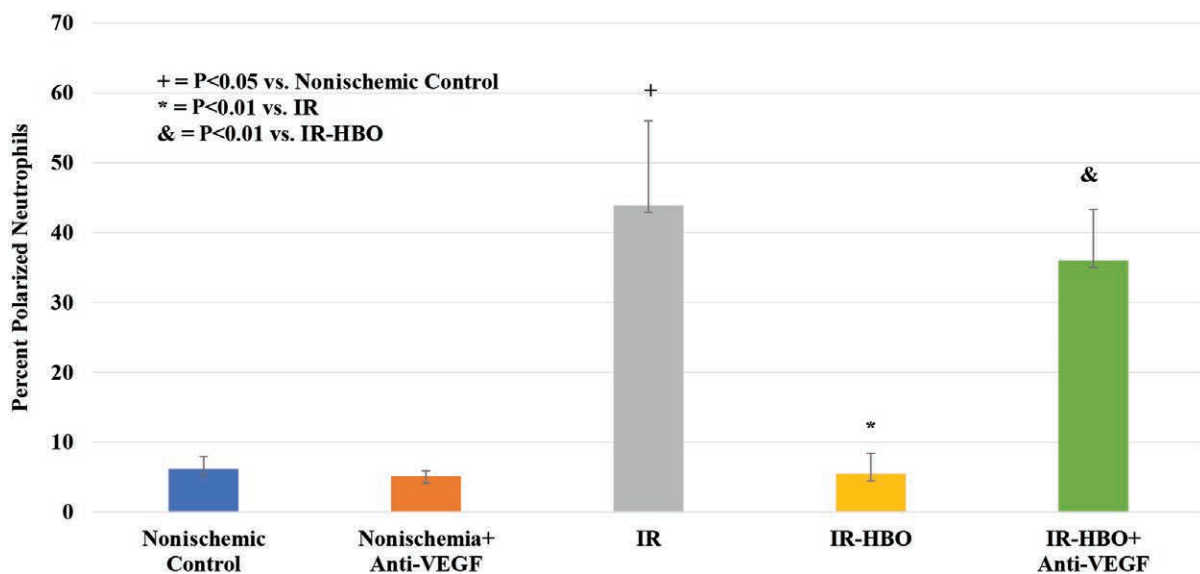
### Alpha-2-Antiplasmin Protein Expression

The injection of alpha-2-antiplasmin was successfully delivered to the gracilis muscle through the epigastric artery to the gracilis pedicle artery. Based on the amount of protein demonstrated by western blot, the exogenous alpha-2-antiplasmin crossed the vascular barrier and was taken up by the extracellular matrix (ECM). Figure 7 shows a 50% increase in alpha-2-antiplasmin protein ( $P < 0.05$ ) available to block plasmin following the injection and 30-minute uptake by the gracilis muscle.

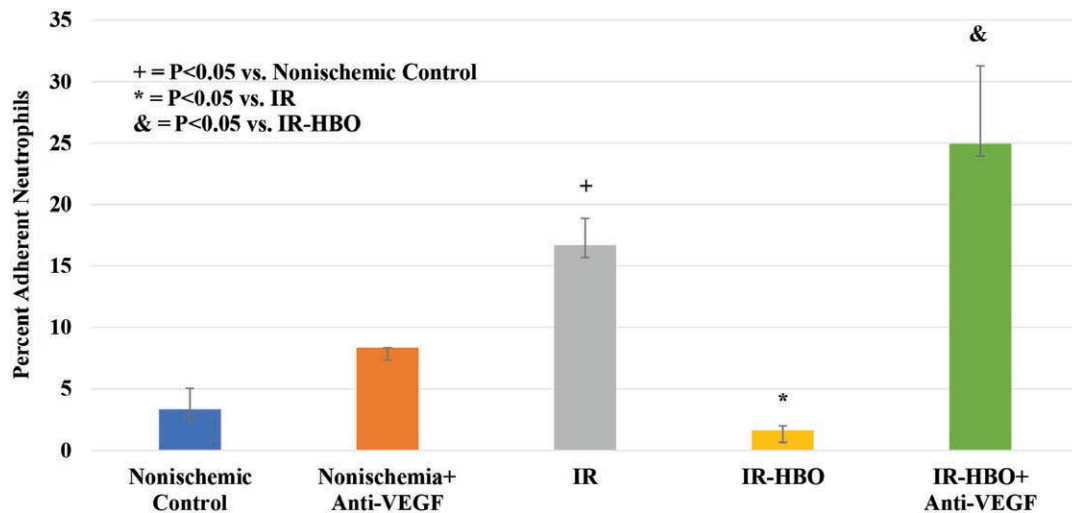
## DISCUSSION

VEGF<sub>165</sub> is the most functional form of VEGF with effects including angiogenesis, lymphangiogenesis, vasodilation, increased vascular permeability, and prevention of apoptosis.<sup>34,35</sup> VEGF can be secreted by the cell or bound to the ECM. The bound forms can be cleaved by various proteases to release them from the ECM.<sup>36</sup> Both of the large forms, VEGF<sub>189</sub> and VEGF<sub>206</sub>, are biologically active and may release VEGF<sub>165</sub> upon breakdown of the ECM.

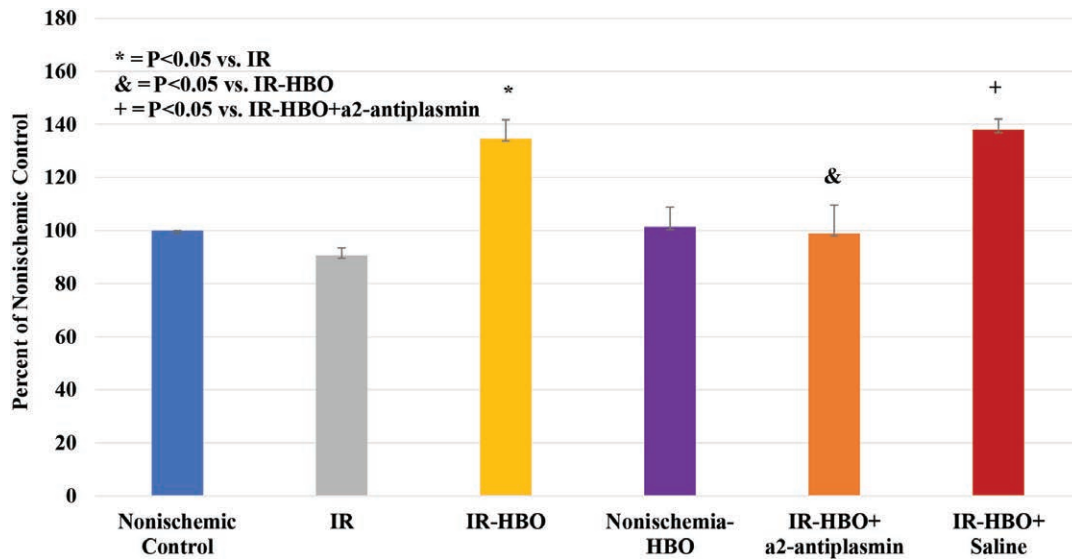
Plasmin is a protein found in the fibrinolytic pathway. The inactive precursor, plasminogen, is activated by specific tissue-type or urokinase-like (tPA or uPA) plasminogen activators. The activated plasmin degrades fibrin and participates in the degradation of the extracellular matrix



**Fig. 3.** Percentage of CD18 polarized neutrophils ex vivo. Nonischemic control (blue), nonischemia + anti-VEGF (orange), IR (grey), IR-HBO (yellow), and IR-HBO + anti-VEGF (green). + $P < 0.05$  vs. nonischemic control, \* $P < 0.01$  vs. IR, & $P < 0.01$  vs. IR-HBO.



**Fig. 4.** Percentage of adherent leukocytes ex vivo. Nonischemic control (blue), nonischemia + anti-VEGF (orange), IR (grey), IR-HBO (yellow), and IR-HBO + anti-VEGF (green). +*P* < 0.05 vs. nonischemic control, \**P* < 0.05 vs. IR, &*P* < 0.05 vs. IR-HBO.



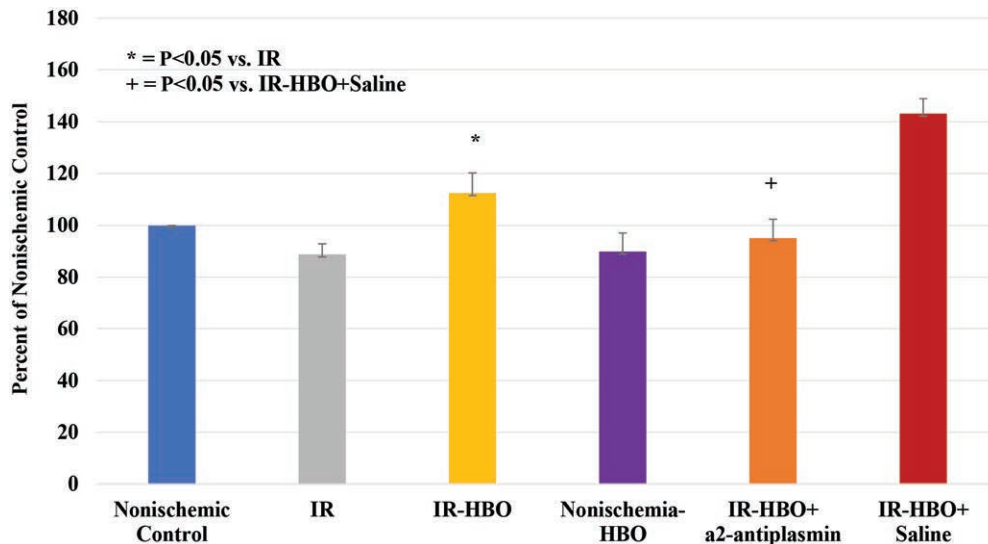
**Fig. 5.** VEGF protein expression from gracilis muscle measured by western blot (expressed as percentage of nonischemic control). Nonischemic control (blue), IR (grey), IR-HBO (yellow), nonischemia-HBO (purple), IR-HBO + alpha-2-antiplasmin (orange), and IR-HBO + saline (red). *N* = 8/group, \**P* < 0.05 vs. IR, &*P* < 0.05 vs. IR-HBO, and +*P* < 0.05 vs. IR-HBO + alpha-2-antiplasmin.

by activating matrix metalloproteinases (MMPs) thereby releasing the bound stores VEGF.<sup>36-38</sup> The release of bioactive VEGF from the extracellular matrix may then stimulate increased eNOS expression and activity to produce NO.<sup>26</sup>

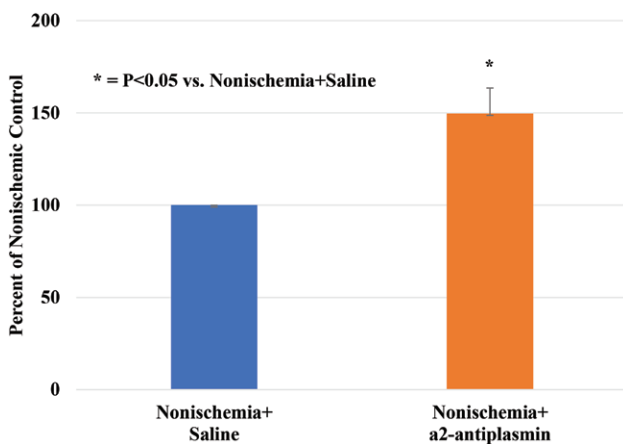
Furthermore, HBO has been reported to increase the protein concentration of tPA, uPA,<sup>39</sup> which would enhance activation of plasmin leading to ECM release of bioactive VEGF.<sup>36</sup> If the HBO-induced increase of tPA and uPA activates more plasmin than the alpha-2-antiplasmin available to inactivate it, then more stored VEGF would be released. Other investigators reported increased levels of plasmin-alpha-2-antiplasmin, a stable inactive complex, in myocardial infarction, acute stroke, and unstable angina.<sup>40,41</sup> Also,

Pepper et al.<sup>42</sup> reported that VEGF induces expression and synthesis of tPA and uPA from endothelial cells increasing the pool of activated plasmin. Bioactive VEGF targeting endothelial cells to increase eNOS activity and increased NO could account for the HBO-induced increases reported by Baynosa et al.<sup>12</sup>

These findings taken together suggest a relationship between HBO administration, the fibrinolytic pathway, VEGF release from the ECM, and NO production. At the molecular level, the beneficial effects of HBO are mediated by neutrophil CD18 surface adhesion molecules. Using an IR model in rat skeletal muscle, we propose that this HBO-induced release of VEGF from ECM, targets endothelial cells to increase eNOS activity, and release NO



**Fig. 6.** VEGF protein expression from pulmonary vasculature measured by western blot (expressed as percentage of nonischemic control). Nonischemic control (blue), IR (grey), IR-HBO (yellow), nonischemia-HBO (purple), IR-HBO + alpha-2-antiplasmin (orange), and IR-HBO + saline (red). N = 8/group, \* $P < 0.05$  vs. IR and + $P < 0.05$  vs. IR-HBO + saline.



**Fig. 7.** Alpha-2-antiplasmin protein expression in gracilis muscle (expressed as percentage of nonischemic control). Nonischemia + saline (blue) and nonischemia + alpha-2-antiplasmin (orange). \* $P < 0.05$  vs. nonischemia + saline.

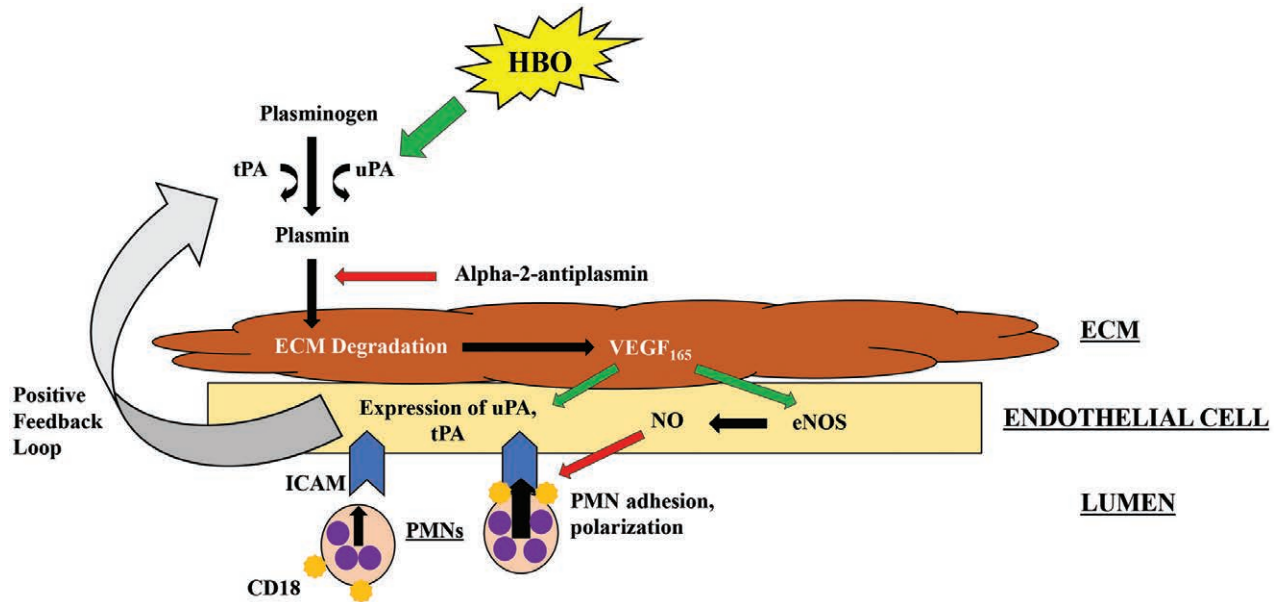
that disrupts the actin cytoskeleton of neutrophils inhibiting CD18 polarization and neutrophil-endothelial adhesion. In this study, the monoclonal antibody, anti-VEGF, significantly blocked the HBO-induced beneficial effect of reversing CD18 polarization and resultant neutrophil-endothelial adhesion in both the in vivo and ex vivo models of IR.

Our data also demonstrated that HBO treatment of skeletal muscle IR caused a significant increase in VEGF protein in the gracilis muscle expressed as percentage of control. When alpha-2-antiplasmin was infused locally to the gracilis muscle, the HBO-induced increase in VEGF protein was significantly reduced. The alpha-2-antiplasmin vehicle, saline, did not alter the HBO-induced increase of VEGF protein. Also, alpha-2-antiplasmin injection did not significantly alter the VEGF protein measured from the

nonischemic control. The results in the pulmonary vasculature were similar, suggesting that the beneficial effects of HBO are systemic; however, the reduction of HBO-induced VEGF with alpha-2-antiplasmin did not reach significance. Although our assays for measuring VEGF was not specific for the 165 species, we believe our data reflect physiologic conditions more appropriately since other biologically active isoforms are present and may also mediate the beneficial effects of HBO following IR injury. In addition, injection of alpha-2-antiplasmin into the pedicle artery of the gracilis muscle increased the amount of gracilis muscle alpha-2-antiplasmin by 50%, a significant increase over saline. This was to ensure that our injection of alpha-2-antiplasmin crossed the vascular barrier and was taken up by the surrounding tissue.

As plasminogen is converted to plasmin in the fibrinolytic pathway, alpha-2-antiplasmin inactivates it to prevent breakdown of the ECM until the level of alpha-2-antiplasmin is exhausted. Then, plasmin begins the breakdown of ECM to release VEGF<sub>165</sub> from the bound VEGF isoforms.<sup>37</sup> In cases of myocardial infarction, acute stroke and unstable angina increased levels of the stable and active complex, plasmin-alpha-2-antiplasmin were shown.<sup>40,41</sup> HBO may be acting by increasing the tPA and uPA<sup>39</sup> to increase plasmin concentration beyond the ability of alpha-2-antiplasmin to inactivate it. This leads to increased VEGF in the muscle that increases eNOS activity and NO production that protect the microvasculature as well as stimulate endothelial cells to produce more tPA and uPA,<sup>42</sup> which can act as a positive feedback loop leading to more plasmin production. The proposed model for the changes induced by HBO therapy following IR injury in skeletal muscle is seen in Figure 8.

Skeletal muscle IR injury represents a challenge in free flap salvage, limb replantation/revascularization, and composite tissue transplantation, given the potential for significant morbidity and the paucity of treatment op-



**Fig. 8.** Proposed model for HBO-induced changes following IR injury in skeletal muscle. HBO stimulation of plasmin production via tPA and uPA permits VEGF release from the ECM, which produces multiple downstream effects including augmenting NO production to inhibit neutrophil adhesion/polarization and stimulating further expression of tPA/uPA. A positive feedback loop is established to produce more plasmin beyond the ability of alpha-2-antiplasmin inactivation. Red arrows represent inhibitory pathways; green arrows represent stimulatory pathways.

tions. However, access to hyperbaric treatment is limited, as many of the facilities performing the procedures mentioned above do not have associated hyperbaric chambers. Still, HBO therapy represents a promising intervention for skeletal muscle IR injury. In skeletal muscle, it appears that the NO-dependent beneficial HBO effect in IR injury on neutrophil CD18 polarization and adhesion involves VEGF and the plasminogen system. Specifically, the HBO-induced VEGF increase occurs through plasmin degradation of the ECM. This pathway represents 1 potential mechanism in the HBO pathway following IR injury. However, other mechanisms may be involved in upregulating NO and mediating neutrophil polarization/adhesion. Many aspects of the pathway have yet to be elucidated but are promising areas of inquiry. The relative activity of plasmin versus MMPs in extracellular matrix degradation, specifically with respect to VEGF release, remains unknown. MMPs and their inhibitors, tissue inhibitors of metalloproteinases, may represent an entirely unique pathway or influence the plasmin-mediated pathway for VEGF release. Alternatively, plasmin could degrade VEGF inhibitors. The present study also does not examine downstream aspects of the pathway such as eNOS activity following plasmin or VEGF blockade. Further research is needed to confirm these findings in other models, identify other contributing mechanisms, and elucidate clinical applications.

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