definitively proven, and no RCT of that size could definitively prove, that this therapy should not be considered in further RCTs.

The growing array of RCTs of exercise interventions that demonstrate no difference contrasts sharply with the equally growing sense among clinicians that this is something that should be done. To advance, we need trials big enough to give either a compelling “yes” or a compelling “no” about specific timing and dose of rehabilitation in the ICU. As Popper argued, compelling refutation of hypotheses is essential for the advancement of science (15). ■

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Compartmentalization Is Key in Limiting Nitric Oxide Scavenging by Cell-Free Hemoglobin

In 1994, Lancaster proposed that nitric oxide (NO) could not possibly function as the endothelium-derived relaxing factor because of the proximity of so much NO-scavenging Hb located adjacent to the endothelium (1). The primary reaction whereby Hb destroys NO

activity is known as NO dioxygenation, where NO reacts with oxygenated Hb to form ferric Hb (methemoglobin) and nitrate. This reaction is rate-limited by the time it takes for NO to diffuse to the heme pocket and is thus extremely rapid, governed by a rate constant of $6-8 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ (2, 3). Through several mechanisms (Figure 1), it turns out that compartmentalization of Hb in the

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Figure 1. Under physiological conditions, Hb compartmentalization into red cells creates a number of biophysical and biochemical barriers (cell-free zone, intrinsic membrane barrier, and unstirred layer) that slow down dioxygenation reactions between Hb and endothelial-derived nitric oxide (NO). These barriers are disrupted by hemolysis, with the released Hb (tetramers and dimers) in the intra- and extravascular space rapidly oxidizing NO to nitrate, leading to inhibition of NO signaling. This pathway has been demonstrated in various pathologic situations, including hemolytic and critically ill diseases and toxicity associated with cell-free Hb therapeutics and transfusion with stored red cells. Schaer and colleagues show that haptoglobin prevents free Hb-dependent increases in blood pressure via binding to Hb dimers and sequestering these complexes in the vascular lumen (i.e., preventing diffusion of Hb into the vessel wall) and suggest the potential for therapeutics that prevent Hb extravasation (11). EC = endothelial cell; NOS = nitric oxide synthase; OxyHb = oxygenated hemoglobin. Adapted by permission from Reference 19.

red blood cell greatly reduces the rate of NO scavenging, so that NO can, indeed, function as the endothelium-derived relaxing factor (4–7).

The importance of compartmentalization in limiting NO scavenging by Hb has been appreciated for some time by those developing hemoglobin-based oxygen carriers (HBOCs) or blood substitutes that are composed mainly of cell-free Hbs. Early studies testing these products demonstrated increased vasoconstriction and hypertension, underscoring key differences between erythrocytic and cell-free Hb (8). In 2002, Gladwin and colleagues showed that cell-free Hb released upon chronic hemolysis in sickle cell disease lowers NO bioavailability and suggested that loss of NO homeostasis contributes to several pathologies including pulmonary and systemic hypertension, thrombosis, and renal failure. This concept has been extended to other hemolytic anemias and disease settings, including those in which red cells are exposed to mechanical stress (e.g., during cardiopulmonary bypass, extracorporeal membrane oxygenation circuits, and hemodialysis), malaria, and transfusion of older stored blood (9). In addition to loss of red cell intrinsic barriers of limiting NO–Hb reactions, extravasation of cell-free Hb into the interstitial space between the lumen and smooth muscle cells, positioning Hb in closer proximity to the source of NO (endothelium), has also been proposed as a likely factor in increased NO scavenging (Figure 1) (8, 9). In the case of HBOCs, it was noted that increases in blood pressure varied inversely with the size of the HBOC; this was attributed to larger HBOCs being less likely to extravasate (8).

In humans and many other species, haptoglobin (Hp) is the main plasma protein for binding and removing cell-free Hb (10). Hp binds

to Hb dimers with very high affinity and is removed from plasma by monocytes and Kupffer cells in the liver (10). Haptoglobin prevents free Hb-dependent hypertension. In this issue of the *Journal*, Schaer and colleagues (pp. 1111–1122) present data suggesting the primary and, in fact, archetypical function of Hp and similar proteins is to prevent Hb extravasation, rather than simple removal from the plasma (11). This notion is based on the fact that Hb bound to Hp reacts with NO just as fast as cell-free Hb (12) and that, because of blocked renal clearance, Hb bound to Hp is cleared slower than cell-free Hb (13).

Using a rat model, Schaer and colleagues show that Hp attenuates increases in mean arterial pressure when coin infused with cell-free Hb compared with infusion of cell-free Hb alone (11). They also show that Hp abrogates vasoconstriction by cell-free Hb in isolated porcine aortic rings and reverses cell-free Hb-mediated inhibition of NO-induced vasorelaxation in porcine and canine rings. Importantly, this study shows that the Hb–Hp complex still consumes NO, but compartmentalization in the lumen and prevention of extravasation prevented hypertensive effects. Prevention of Hb extravasation by Hp was verified using fluorescence microscopy of functionalized Hbs in rats and through microscopy and electron paramagnetic resonance detection of methemoglobin in the vasculature (11).

The maintenance of normal NO-associated reactivity resulting from addition of Hp in the presence of cell-free Hb was recapitulated when large, synthesized Hb–albumin conjugates were employed (11), supporting the hypothesis that an increase in size of Hb-bound species prevents extravasation and preserves NO-dependent vasoactivity. Further support for this idea was obtained by examining the vasoactivity of glutaraldehyde-polymerized

Hbs, where size was found to be inversely proportional to vasoactivity (11). Importantly, the avian plasma Hb binding protein PIT54 was shown to function similarly to Hp, abrogating loss of NO-dependent vasodilation resulting from the presence of chicken Hb in porcine aortic rings (11). That PIT54 and Hp are structurally and evolutionarily distinct suggests separate evolution of an archetypal function to mitigate deleterious effects of hemolysis.

The striking importance of preventing Hb extravasation by Hp and other complexes in maintaining NO vasoactivity as demonstrated by Schaer and colleagues is a major, novel contribution to the field (11). However, others have demonstrated that exclusion of red cells from a region near the endothelium called the cell-free zone (Figure 1) also plays an important role in limiting NO scavenging by Hb, and it seems unlikely that Hp–Hb complexes will be excluded from the cell-free zone because of their size (14). Further work should examine the interplay between the cell-free zone and Hp–Hb compartmentalization and how this modulates NO-dependent vasoactivity in a variety of vascular beds. In addition, although striking effects are seen in terms of vasoactivity (11), one wonders how other positive effects of NO are affected by the addition of Hp to cell-free Hb in the lumen, including effects on inflammation, inhibition of platelet activation and aggregation (15, 16), and blood cell adhesion to the endothelium (17). Further work is needed to explore these questions.

A major strength of the work by Schaer and colleagues is their use of several different animal models (11), improving the likelihood of successful translation to humans. Given the number of conditions in which red cell hemolysis occurs, combined with the positive effects of Hp observed by Schaer and colleagues (11), perhaps it is time for a clinical trial examining whether Hp coinfusion can improve outcomes in conditions associated with hemolysis. As mentioned by Schaer and colleagues, the antioxidant effects of Hp when bound to Hb may also be beneficial (11). Particular conditions that may be best suited for a trial might be those in which there is acute hemolysis, such as in transfusion of older stored blood or in use of extracorporeal circulation devices. Positive results in these acute settings could lead to studies in hematological diseases characterized by pulmonary hypertension associated with increased hemolysis (18). ■

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