Bradley I. Goetz *et al.* reply:

We recently proposed that nitrite-mediated vasodilation involves a reaction between NO and nitrite-bound methemoglobin, Hb(Fe(III))-NO_2^-, to form N_2O_3, thus facilitating export of NO activity from the red blood cell[1].

Schwab *et al.* challenge our recent work by suggesting that the nitrite binding affinity to methemoglobin is very low and that nitrite is not a physiological vasodilator. We maintain that (i) numerous studies now confirm that dietary, physiological and pharmacological levels of nitrite vasodilate and mediate hypoxic NO signaling[2–11] and (ii) the binding of nitrite to methemoglobin is complex and includes an O-bound nitrito species that has NO_2 radical character, and the affinity is high under certain conditions. This hypothesis was proposed based on a number of experimental observations, including rapid reductive nitrosylation, gas-phase N_2O_3 formation, density functional theory (DFT)calculations and evidence of an unusual electronic configuration of nitrite bound to methemoglobin measured by EPR spectroscopy. The formation of an O-bound nitrito species has now been independently confirmed by other groups using DFT calculations and X-ray crystallography[12].

Schwab *et al.* challenge our proposed mechanism based on their calculation of a lower affinity of nitrite for methemoglobin. We propose that the difference between our results and theirs is due to experimental conditions used. Support for a low dissociation constant and the effects of experimental conditions is given in Figure 1, which provides evidence for complex behavior of the nitrite-methemoglobin interaction where both pH and other buffer conditions modulate affinity. Schwab *et al.* argue that our error in determining the dissociation constant was due to a failure to observe a low-spin nitrite-bound methemoglobin EPR signal. However, this does not affect our calculation of the dissociation constant, as we measured the disappearance of the high-spin methemoglobin signal as an indicator of the formation of nitrite–methemoglobin. We have found that at least part of the reason that we did not observe the low-spin nitrite–methemoglobin signal is due to unexpected saturation of the low-spin signal when using phosphate buffer and scanning at 5 K (rather than 77 K using HEPES buffer, as used by Schwab *et al.*).

The nitrite reaction with methemoglobin is clearly more complex than suggested by Schwab *et al.*, and we hypothesize that this complexity is related to the predominance and properties of the *O*-nitrito species.

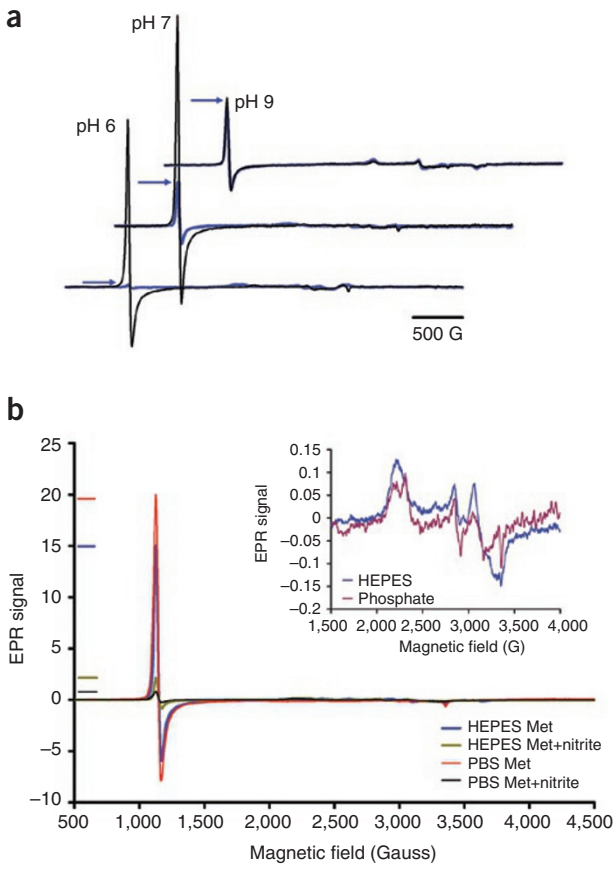

Figure 1 EPR of methemoglobin–nitrite under different conditions. (**a**) pH dependence. Methemoglobin (MetHb; 50 μM) was prepared in 0.05 M HEPES buffer at various pH, and 2.5 mM nitrite was added. Spectra before (black) and after (blue) nitrite addition are shown. Blue arrows indicate the height of the $g = 6$ peak for the MetHb samples after nitrite addition. Parameters used for the spectroscopy were as described previously[1]. The degree of disappearance of the low-field EPR signal corresponding to high-spin MetHb after nitrite addition is greatest at pH 6 and not detectable at pH 9. There is very little change in the spectrum at pH 9, which confirms the pH dependence of nitrite binding. Notably, the EPR signal nearly completely disappears at pH 6 after nitrite addition. If the dissociation constant were 1.8 mM, as suggested by Schwab *et al.*, then one would expect there to still be 21 μM (slightly less than half the original concentration) after nitrite addition, which clearly is not the case here. (**b**) Buffer dependence. Nitrite (10 mM) was added to MetHb prepared in either 0.05 M HEPES (initial MetHb = 39 μM) buffer or phosphate-buffered saline (initial MetHb = 39 μM) at pH 7.4. The high-spin MetHb signal appears to be substantially more sensitive to nitrite addition in PBS than in HEPES buffer (peak heights for the $g = 6$ signals are indicated next to the ordinate axis). The inset shows the high-field region where 5 mM nitrite was added to 50 μM MetHb in the two buffers (HEPES and phosphate). The shape of the low-spin nitrite-bound hemoglobin signal is different in HEPES than in 0.05 M phosphate buffer, particularly around 2,200 G.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturechemicalbiology/.

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