Response to the Letter by Edwards et al

We first acknowledge the profound contribution that Edwards, Felétoú, and Weston have made over many years to our understanding of the role of endothelial-derived hyperpolarizing factors in the regulation of vascular function in health and disease. It is clear from the expansive literature as well as from the recent extremely scholarly review by these investigators that the subject of endothelial-derived hyperpolarization and the factors that mediate this response are complex. The responses are broadly divided into the “classical” endothelial-derived hyperpolarizing pathways, which, for the most part, involve Ca^{2+}-activated K^+ channels of intermediate conductance-dependent and Ca^{2+}-activated K^+ channels of small conductance-dependent hyperpolarization of endothelial cells, efflux of K^+, or coupling of vascular smooth muscle through myoendothelial junctions. Another broad class of what we refer to as “alternate” pathways involves the release of endothelial-derived mediators resulting from Ca^{2+} activation that mediate hyperpolarization through K_{ATP} or Ca^{2+}-activated K^+ channels of large conductance channels.

As is highlighted in our article and by the work of others, it appears that hydrogen sulfide mediates its vasorelaxant effects by mechanisms that involve both classic and alternate pathways. The predominant mechanism appears to involve the alternate pathway. The evidence supporting the latter include that: although nitric oxide synthase and cyclooxygenase inhibition have little effect on membrane potential in mesenteric vessels, both glibenclamide and the cyclooxygenase inhibitor propargylglycine markedly attenuate relaxation and change in membrane potential; and mesenteric vessels from CSE knockout mice have markedly blunted vasorelaxant responses to acetylcholine without significant changes in Em. Together, these results suggest that endogenous CSE-derived hydrogen sulfide is mediating its hyperpolarizing effects through the K_{ATP} channel. Furthermore, exogenous hydrogen sulfide causes significant vasorelaxation and change in Em in endothelial-denuded mesenteric vessels, a process that is blocked by glibenclamide. Moreover, hydrogen sulfide enhances K_{ATP} currents in vascular smooth muscle cells from rat mesenteric arteries that are sensitive to glibenclamide. With regard to the classic pathway, we also present evidence that both endogenous and exogenous hydrogen sulfide can activate and sulphydrate IK channels. For example, endothelial cell hyperpolarization is absent in response to acetylcholine in endothelial cells from CSE^{-/-} mice, and hyperpolarization is significantly attenuated in human aortic endothelial cells preincubated with TRAM-34 but not iberiotoxin.

We are in complete agreement with Edwards, Felétoú, and Weston that acetylcholine-dependent relaxation represents overlapping combined effects of separate endothelial-dependent vasorelaxant pathways. However, precise inhibition of specific pathways in knockout mice, as well as more sensitive and specific inhibitors (not to mention more sophisticated myography), have allowed us to more accurately estimate the contribution of each of the pathways to cholinergic relaxation. Careful comparison of Figure 1A and Figure 2D clearly demonstrates an attenuation of endothelial-dependent relaxation in homozygous CSE^{-/-} mouse mesenteric arteries in both publications. As the investigators point out, there are quantitative differences in the responses. Methodological differences, including the groups performing the experiments, equipment, and mesenteric artery size (Yang et al have used the third-order mesenteric arteries), make these experimental results difficult to compare. Furthermore, Yang et al used methacholine to stimulate the endothelium as compared to the present article in which we used acetylcholine. Although methacholine has better potency in vivo because of relative resistance to acetylcholine-esterase, ex vivo responses may account for the differences. Despite the difference, the similarities are informative of the contribution of CSE to endothelial relaxation. In addition to the original Figure 1A (CSE^{-/-} without endothelial nitric oxide synthase/cyclooxygenase inhibitors (NCI), CSE^{-/-} with NCI, CSE^{-/-} with NCI) in our present article, we share results in a further group, CSE^{-/-} without NCI, to demonstrate the presence and contribution of nitric oxide synthase and cyclooxygenase in CSE^{-/-} arteries. Maximum percent-relaxations achieved in mesenteric arteries of CSE^{-/-} without NCI, CSE^{-/-} with NCI, CSE^{-/-} without NCI, and CSE^{-/-} with NCI were 68.87%±4.861%, 51.11%±2.373%, 48.51%±8.086%, and 25.09%±1.291%, respectively. Thus, nitric oxide synthase and cyclooxygenase together contribute similarly to the vasorelaxation response in CSE^{-/-} and CSE^{-/-} mice.

Our data strongly support an endothelial-dependent relaxation and hyperpolarization mechanism involving the K_{ATP} channel in the mesenteric artery. Evidence in the literature also supports K_{ATP} channel activation independent of cGMP/PKG or cyclooxygenase pathways (ie, a pathway that is sensitive to glibenclamide). Furthermore, review of the literature in mesenteric arteries (including the publications listed by the author) show significant methodological differences, which might well explain the discrepant results. These include differences in vessel preparation and tone for measurement of relaxation and membrane potential, pharmacological agents used for preconstriction, concentration of glibenclamide used, and repetitive use of vessel preparations in the presence of multiple inhibitors. We recognize and agree with the investigators that, as expected from pathway analysis, blockade or knockout of CSE will lead to an increase in homocysteine and diminished levels of L-cysteine. Some studies have shown oxidative stress and decreased endothelial-derived hyperpolarizing factor-mediated effects through Ca^{2+}-activated K^+ channels of intermediate conductance and Ca^{2+}-activated K^+ channels of small conductance, as well as decreased nitric oxide availability. Sex differences in homocysteine levels in CSE^{-/-} mice make it more difficult to determine this potential contribution; similar changes in blood pressure in both sexes indicate that this effect may be less important. Moreover, administration of L-methionine to mice for 6 weeks causes homocysteinemia but does not increase blood pressure in wild-type mice. Although hyperhomocysteinemia may potentially contribute to oxidative stress in vivo, ex vivo experiments without hyperhomocysteinemic plasma, the contribution of homocysteine to the endothelial dysfunction (in
presence of active or inhibited nitric oxide synthase/cyclooxygenase pathways) remains unclear. In our hands, reactive oxygen species levels are not different in CSE/H11002/H11002 as compared to wild-type arteries (online Figure VI in the original publication).

In conclusion, we again recognize the outstanding contribution of the investigators over many years in defining the important endothelial-derived hyperpolarizing factor vasoregulatory mechanism. We also acknowledge that the field of hydrogen sulfide-mediated vascular effects is yet young, and we expect lively debate regarding its mechanisms of action to continue.

Asif K. Mustafa
Gautam Sikka
Solomon H. Snyder
Dan E. Berkowitz
Johns Hopkins Medicine
Baltimore, MD
E-mail dberkow1@jhmi.edu

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