LETTER

Reply to Bell et al.: Nrf2-dependent and -independent mechanisms of astrocytic neuroprotection

We appreciate the comments of Bell et al. (1), as they highlight advances from our recent publication that broaden astrocytic neuroprotective mechanisms (2). Although we agree that nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent gene transcription in astrocytes is a highly important neuroprotective mechanism, as confirmed by us and others (2, 3), we disagree that physiological levels of hydrogen peroxide (H_2O_2) act to activate Nrf2. In our manuscript, neurons were protected from oxidative stress when H_2O_2 was produced enzymatically in astrocytes. This effect was Nrf2-independent at low H_2O_2 concentrations sufficient for neuroprotection. Although the comments by Bell et al. (1) suggest that one Nrf2 activity assay that we used (a luciferase reporter utilizing the antioxidant response element from the *NQO1* gene) may be insensitive to H_2O_2-mediated Nrf2 activation, we also show that enzymatic H_2O_2 failed to evoke expression of the Nrf2-regulated gene heme oxygenase-1 (*Hmox1*), siRNA targeted against Nrf2 failed to abrogate the protective effect of H_2O_2, and two separate microarray strategies failed to show up-regulation of Nrf2-regulated genes at protective levels of H_2O_2 production. Of note, we reconfirm our astrocyte toxicity results in response to exogenous H_2O_2 using an alternative cell death measure (Fig. 1).

Bell et al. (1) show that exogenous H_2O_2 (25–100 μM) increases mRNA levels of Nrf2-regulated genes, *Srxn1* and *Hmox1* (1), and suggest that these H_2O_2 levels are physiological based on a rat brain microdialysis study measuring H_2O_2 during cerebral ischemia that reported concentrations of 25–60 μM basally, 110 μM during ischemia, and 160 μM during reperfusion (4). These are likely overestimations of physiological H_2O_2 levels. First, basal brain oxygen levels are estimated to be ∼30–50 μM and decrease to near zero during oxygen glucose deprivation (OGD) (5). Thus, the maximum concentration generated if all oxygen were converted to H_2O_2 (before enzymatic H_2O_2 decomposition) would be 30–50 μM basally and 0 μM in the ischemic core. Second, brain dialysate H_2O_2 measurements are likely elevated by damage from probe insertion and metal contaminants in the artificial cerebral spinal fluid that cause autoxidation. Third, using catalase inactivation as a measure, intracellular H_2O_2 has been estimated at picomolar concentrations (6). Thus, the levels of H_2O_2 used by Bell et al. (1) are unproven to be physiological.

Discrepancies between the two studies are likely caused by differences in H_2O_2 generation/delivery (Fig. 2). We expressed an intracellular H_2O_2-generating enzyme (D-amino acid oxidase) that metabolizes an exogenous substrate (D-alanine), allowing for control of H_2O_2 amplitude and duration (1). This is in contrast to the bolus addition of H_2O_2, which also oxidizes extracellular and plasma membrane molecules that may not be relevant to intracellular H_2O_2 signaling. Thus, the ability to finely control H_2O_2 production in astrocytes is an important tool for understanding the dual role of H_2O_2 as a signaling molecule and toxin (2).

Bell et al. (1) show that sublethal OGD conditions neurons against a subsequent lethal ischemic insult and that this effect is dependent on astrocytic Nrf2 (1). However, no evidence is provided that H_2O_2 is responsible for the Nrf2-dependent preconditioning effect or that H_2O_2 can oxidize Nrf2's cytoplasmic inhibitor Keap1 or activate Nrf2 through another mechanism.

Our results reinforce the role of Nrf2 in the brain but highlight Nrf2-independent mechanisms of astrocyte-dependent neuroprotection. We broaden the pool of astrocyte-dependent therapeutic targets that may yield clinical benefit beyond Nrf2 and suggest that an intense search for Nrf2-independent targets is appropriate.

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The authors declare no conflict of interest.

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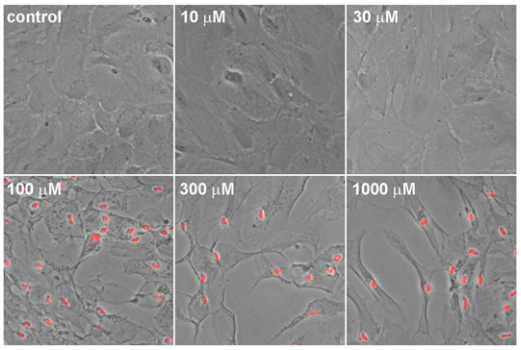

Fig. 1. Exogenous H_2O_2 > 30 μM is toxic to cultured astrocytes. Primary rat cultured astrocytes were treated with exogenous H_2O_2 in minimal essential medium + 10% horse serum for 6 h at the indicated concentrations. The astrocytes were incubated with 4 μM ethidium homodimer-1 (EH-1; shown in red) for 15 min, which only stains the nuclei of dead cells. Images are shown as the overlay of EH-1 fluorescence over the corresponding phase contrast field.

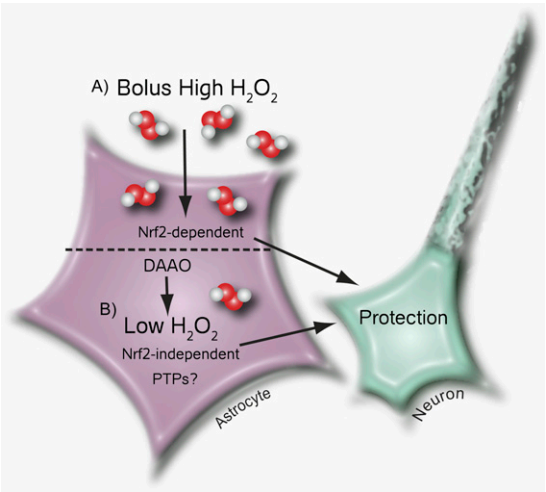

Fig. 2. Schematic representation showing that astrocyte-specific neuroprotective cascades are differentially regulated by high-bolus H_2O_2 vs. enzymatic low-level H_2O_2 generation. (*A*) Bell et al. (1) show that exogenous ≥25 μM H_2O_2, which is likely outside of a physiological range, stimulates Nrf2-dependent gene transcription in astrocytes. Thus, they assume that H_2O_2 is responsible for the Nrf2-dependent neuroprotective preconditioning effect in their OGD model (1). (*B*) In contrast, Haskew-Layton et al. show that enzymatic low-level H_2O_2 production in astrocytes (produced through the flavoenzyme D-amino acid oxidase; DAAO) initiates a neuroprotective cascade that is independent of Nrf2 but may involve protein tyrosine phosphatase inhibition (2). The discrepant findings between the two groups highlight the differential effects of bolus addition vs. enzymatic H_2O_2 production on cellular functioning.