Methylene blue potentiates stimulus-evoked fMRI responses and oxygen consumption during hypoxia

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INTRODUCTION Methylene blue (MB) is a unique auto-oxidizing drug that has a hormetic dose-response with opposite effects at low and high doses (1). At its therapeutic intravenous low doses (0.5-2 mg/kg), MB is an antidote for methemoglobinemia and cyanide poisoning and has potent antioxidant effects (1,2). In animal models, MB has also been shown to improve brain cytochrome c oxidase activity and behavioral memory functions (3), reduces neurobehavioral impairment of Parkinson's Disease (1), cognitive decline in Alzheimer's disease (4), and reperfusion injury in cerebral ischemia (1). We previously reported that MB (0.5 mg/kg) did not have significant effects on arterial oxygen saturation, heart rate and fMRI responses to hypercapnia, but markedly potentiated forepaw-evoked BOLD, CBF and $CMRO_2$ changes in rat brain under normoxia (5). To further evaluate *in vivo* effects of MB under stress conditions, we performed similar measurements under mild hypoxia (15% O_2) herein.

METHODS Male Sprague Dawley rats (200-300 g, n = 6, inhaling 15% O2 throughout the MRI experiments) were initially anesthetized with 2% isoflurane, intubated, mechanically ventilated, and paralyzed with pancuronium bromide (3 mg/kg first dose, 1 mg/kg/hr, ip). MRI measurements were made before and after MB (0.5 mg/kg, iv over 5 mins) injection in the same animals. Bilateral forepaw stimulation used 4 epochs of (96s OFF and 30s ON) of 2 mA, 8 Hz and 1 ms pulse. MRI studies were performed on a 7T/30cm magnet and a 40G/cm BGA12S gradient insert (Bruker, Billerica, MA). Rats were placed in a head holder consisting of ear and tooth bars. Combined CBF and BOLD measurements were made using the continuous arterial spin-labeling technique with TR = 3 s, TE = 20 ms, matrix = 96x96, and FOV = 25.6x25.6 mm (6). $CMRO_2$ was calculated using the biophysical model as descrirbed elsewhere (7). Analysis included whole-brain and forepaw primary somatosensory cortex ROI. Paired t-test was used with $P < 0.05$ indicating statistical significance.

RESULTS Figure 1 shows group-averaged maps of basal CBF, CBF and BOLD fMRI responses to forepaw stimulation before and after MB under hypoxia. CBF images showed heterogeneous blood flow contrast with high CBF in gray matter relative to white matter. fMRI responses to bilateral forepaw stimulation were localized to forepaw primary somatosensory cortex (S1) as expected.

Figure 2 shows the group-averaged results of basal CBF, CBF, BOLD and $CMRO_2$ fMRI responses to forepaw stimulation before and after MB under hypoxia (15% O_2). Data were obtained from the S1 ROIs. Post-MB basal CBF was statistically different (higher) from pre-MB CBF ($P < 0.05$). All CBF, BOLD and $CMRO_2$ evoked responses post-MB were statistically different (higher) from pre-MB ($P < 0.05$).

DISCUSSION The effect of hypoxia on oxygen consumption changes during stimulation is of significant interest from the perspective of energy metabolism *per se* as well as its clinical relevance to hypoxic injury and cerebral ischemia. We found that CBF, BOLD and $CMRO_2$ forepaw-evoked fMRI responses were significantly higher during hypoxia relative to air (5), suggesting that the brain uses more oxygen to perform the same task under hypoxia. Although hypoxia lowered basal oxygen saturation and thus reduced the denominator, it could not account for the substantially larger BOLD fMRI responses to forepaw stimulation. The larger increases in CBF fMRI responses under hypoxia showed that the evoked BOLD responses were driven in large part by stimulus-evoked CBF increases as a result of increased neural activities.

It is conceivable that MB could have a stronger effect under stress conditions where energy substrates are limiting. We thus tested the hypothesis that MB has a stronger effect under mild hypoxic (not ischemic) conditions. Comparison between normoxia and hypoxia data shows that MB under hypoxia induced a larger potentiation of the forepaw-evoked responses and oxygen consumption increases compared to normoxia. Such enhanced potentiation during hypoxia could be one of the mechanisms that accounts for MB's neuroprotective effects in metabolically stressed conditions reported in the literature (1). We predict that more severe (i.e., 9-12% O_2) hypoxia could evoke a larger MB effect.

The novel finding of MB's greater potentiation of evoked fMRI responses under hypoxia is in agreement with previous MB studies. For example, MB can stimulate glucose metabolism in anoxic conditions *in vitro* (8). MB can also prevent brain damage induced by hypoxia-reperfusion injury after cardiac arrest, as shown by a decrease in the plasma level of the astroglial marker of hypoxic brain injury (protein S-100Beta) (9). Brain regions that are more affected by MB would seem to depend on regions that have higher activity-dependent energy demand. For example, memory tasks that evoke greater energy demand in the prefrontal cortex, such as extinction memory, show activation specificity after MB (10). Rats with MB-enhanced extinction memory show relatively greater increases in cytochrome oxidase activity in prefrontal cortical regions thought to contribute to extinction memory than in other regions (10). Similarly, after MB administration, forepaw stimulation results in an even greater fMRI activation of the somatosensory cortex as observed.

In conclusion, we found that MB further potentiated fMRI responses under stress (mild hypoxia) conditions compared to air. This potentiation was activity dependent. These findings have implications in neurological conditions with mitochondrial dysfunction and oxidative stress, such as Alzheimer's and Parkinson's diseases, normal aging, stroke and reperfusion injury.

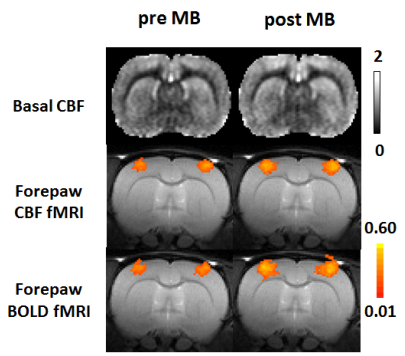

Figure 1. Group-averaged basal CBF, BOLD and CBF fMRI responses to 5% CO_2 and forepaw stimulation for before and after methylene blue (MB).

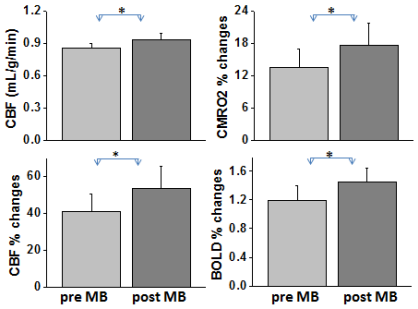

Figure 2. Group-averaged basal CBF, BOLD, CBF and $CMRO_2$ fMRI responses to forepaw stimulation before and after methylene blue (MB) injection in the same animals . Error bars = ± SEM.

References (1) Rojas et al. Prog Neurobiol 96 32-45 (2012). (2) Scheindlin S. Mol Interv 8:268 (2008). (3) Martinez Jr et al.Physiological Psychology 6 387-90 (1978). (4) Medina et al. Brain Pathol 21, 140-9 (2010 (2010). (5) Huang et al, Proc. Soc. Mag. Reson. Med. 20 2166 (2012). (6) Shen et al, JCBFM 23:1479 (2003). (7) Liu et al. MRM 52:277 (2003). (8) Lee RB et al. Arthritis Rheum 46:3190-3200 (2002). (9) Miclescu A et al. Crit Care Med 34: 2806-13 (2006). (10) Gonzalez-Lima F et al. Learn Mem 11: 633-40 (2004).