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Protocol Optimisation for a Fluorescent Nitric Oxide Indicator in Rat Mesenteric Arteries *Ex Vivo*

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Endothelial cell (EC) dysfunction is an early hallmark of cardiovascular disease, associated with reduced bioavailability of nitric oxide (NO) and raised vasoreactivity. Despite this, the current techniques do not appear to reliably measure NO synthesis in intact arteries. Here, we optimise a novel protocol to extend the use of DAR-4M AM, a sensitive cell-trappable NO dye, into living cells of rat small mesenteric arteries. By using confocal fluorescence microscopy, we found that in cell-free chambers, Krebs buffer containing Cu_2FL2E (1 μM FL2E and 2 μM Cu^{2+}), or DAR-4M AM (5 μM, Cu^{2+}-free), the addition of the NO-donor molecule, S-nitroso-N-acetylpenicillamine (SNAP, 10 μM) caused a time-dependent and significant increase in fluorescence compared to baseline. This was further enhanced by the addition of 1 μM and 10 μM Cu^{2+}, for each dye respectively (by increasing NO-availability from SNAP). To assess biological relevance, wire myography showed that 10 μM SNAP fully reversed contraction to phenylephrine in rat mesenteric arteries. However, in the same system, >2 μM Cu^{2+} caused vascular smooth muscle (VSM) and EC dysfunction in mesenteric arteries. Next, using confocal microscopy and pressure myography, we showed that both Cu_2FL2E and DAR-4M AM could be loaded into arterial cells, and each also labelled the elastin. The role of this elastin label was assessed by co-loading Alexa Fluor 633 hydrazide (AF-633, 1 μM). However, despite many types of approaches, we were unable to measure increases in fluorescence to either ACh (1 μM) or SNAP (10 μM) when cells were loaded with Cu_2FL2E. Instead, we turned our attention to DAR-4M. Increases in the ratio of DAR-4M and AF-633 were visualised in pressurised, isolated rat mesenteric arteries stimulated with the EC-dependent agonist ACh (1 μM) or SNAP (10 μM). The addition of either drug evoked an accumulating, time-dependent (and by 20 mins) significant increase in fluorescence in both EC and VSM above baseline. This response did not require the addition of Cu^{2+}, thus simplifying the protocol and more reducing the possibility of cellular damage. These experiments will be repeated in the presence of L-NAME, an NO synthase inhibitor, to further consolidate the link between DAR-4M T fluorescence and NO production. Moving forward, these preliminary data will facilitate the advance of our understanding of vascular function, and potentially elucidate the basal vs. stimulated NO release conundrum.