CYANIDE-INSENSITIVE MITOCHONDRIAL RESPIRATION IN PLACOPECTEN MAGELLANICUS AND MYTILUS EDULIS: IMPLICATIONS FOR REOXYGENATION INJURY

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Reoxygenation following transient ischemia is injurious to aerobically-poised tissues [Cadenas, Ann. Rev. Biochem. 58:79-110, 1989]. According to the current paradigm for the metabolic pathology responsible for this reoxygenation tissue damage (RTD) [reviewed by McCord, N. Engl. J. Med. 312: 159-163, 1985], xanthine dehydrogenase is irreversibly converted during hypoxia into xanthine oxidase (XOD). Upon reperfusion, XOD uses oxygen as a cofactor to generate superoxide radicals (O_2^-), reactive univalent reductants of oxygen that overwhelm cellular antioxidant defenses and injure the tissue. Although O_2^- can damage cells directly, they also undergo secondary reactions in the presence of transition metals to yield hydroxyl radicals ($\cdot OH$), one of the most reactive compounds found in biological systems [Halliwell and Gutteridge, Free Radicals in Biology and Medicine, Clarendon Press, 1990].

The above model has been derived from mammalian tissues where fluctuating O_2 availability is pathological. This is not the case; however, for intertidal marine invertebrates which thrive in a habitat characterized by oxygen denial and resupply as the tides ebb and flow. Conversely, numerous subtidal species succumb to these conditions. As such, these animals are ideal comparative cases in which to examine resistance and susceptibility to RTD. Among molluscs examined, XOD is found in several aerobically-poised subtidal species susceptible to RTD, but not in euryoxic intertidal species [Dykens and Shick, Comp. Biochem. Physiol., 91C: 35-41, 1988].

Although the evidence is compelling that RTD is mediated by oxy-radicals, reported XOD activities can generate less than 0.2% of the radical flux observed by electron spin resonance (ESR) in reoxygenated mammalian myocardium [Zweier, J. Biol. Chem. 263: 1353-1357, 1988]. In both mammalian and molluscan tissues, observed XOD activities probably account for O_2^- production on the order of nmols$\cdot$min^{-1}; at pH 7.8 and in air-saturated buffer, only 15% of the O_2 fluxing through XOD is reported to be univalently reduced to O_2^- [Fridovich, J. Biol. Chem. 245: 4053-4057, 1970]. To verify low O_2^- flux, we used O_2^--mediated nitroblue tetrazolium (NBT) reduction and sulfite oxidation as indicators to match rates of O_2^- production from known activities of commercial XOD (Sigma Chemical) to rates of O_2^- production from riboflavin photo-oxidation under constant illumination. Using either NBT reduction or sulfite oxidation as O_2^- indicators, photo-oxidation of 1.7 nM riboflavin produces O_2^- at rates comparable to 1.4 mU of XOD, suggesting that O_2^- flux from the observed activities of XOD can not account for the ~12 mM instantaneous free radical concentrations reported in isolated reperfused mammalian myocardium [Zweier, Flaherty and Weisteldt, PNAS 84: 1404-14015, 1987]; there must be alternative intracellular sources of radicals.

One possible alternative source of radicals is autoxidation of mitochondrial electron transport NADH-dehydrogenase and ubiquinone. Accordingly, we examined respiration and oxy-radical production in intact mitochondria isolated from gill tissue of the subtidal scallop Placopecten magellanicus, a stenoxic species that succumbs to O_2 denial-resupply, and from the intertidal mussel Mytilus edulis, a euryoxic species that tolerates these conditions.

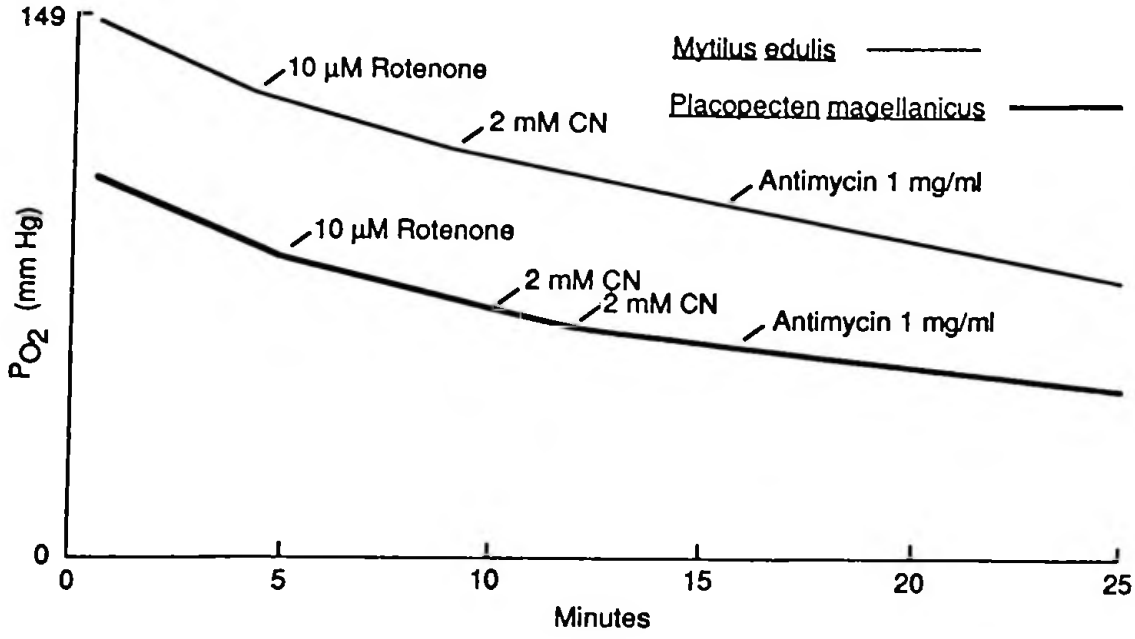

Figure 1. Cyanide-insensitive oxygen utilization by mitochondria isolated from gill tissue of the mussel Mytilus edulis (light line) and the scallop Placopecten magellanicus (heavy line). Respiration was monitored in the presence of 200 μM ADP, 2.5 mM succinate, 2.5 mM malate, 0.1 mM NADH, 30 mM PO_4, 50 μM $MgCl_2$, 15°C. Mitochondria were isolated with Teckmar Ultra-Turrax or teflon-glass Potter-Elvehjem homogenizers in ice-cold 12.5 mM Tris-HCl buffer, pH 7.2, 850 mOsm, containing 0.2M sucrose, 75mM KCl, 75mM NaCl, 5mM EGTA, plus 1% defatted bovine serum albumin.

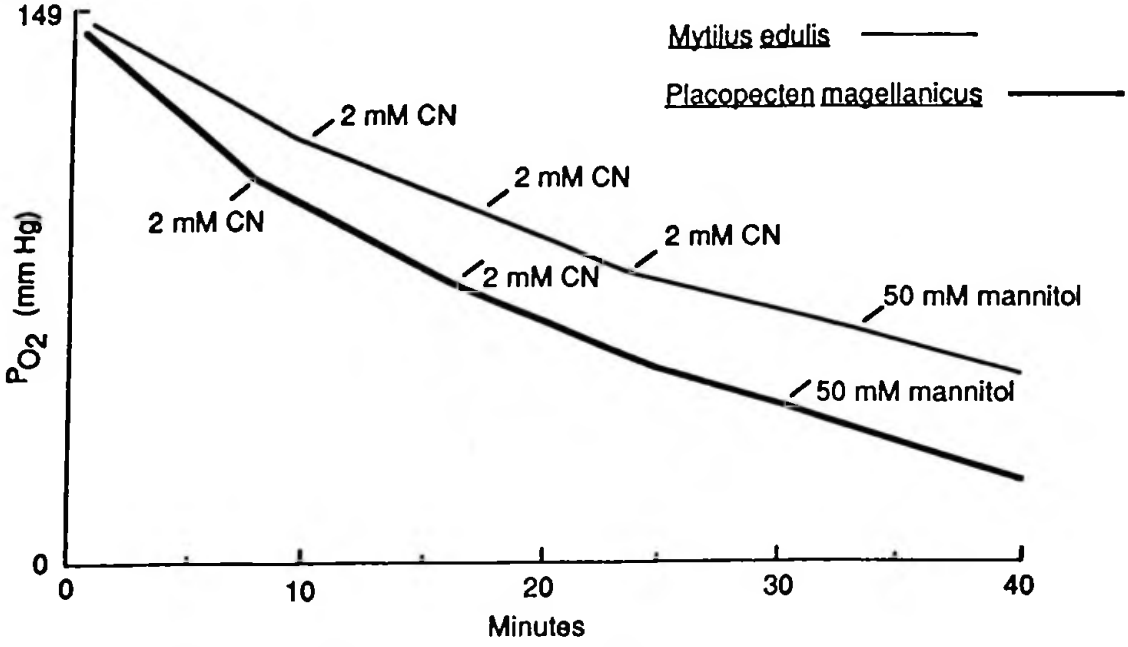

Figure 2. Oxygen utilization by mitochondria isolated from gill tissue of the mussel Mytilus edulis (light line) and the scallop Placopecten magellanicus (heavy line) is reduced, but continues, in the presence of rotenone, cyanide and antimycin. Mannitol has no effect on inhibitor-insensitive respiration. Conditions as Figure 1.

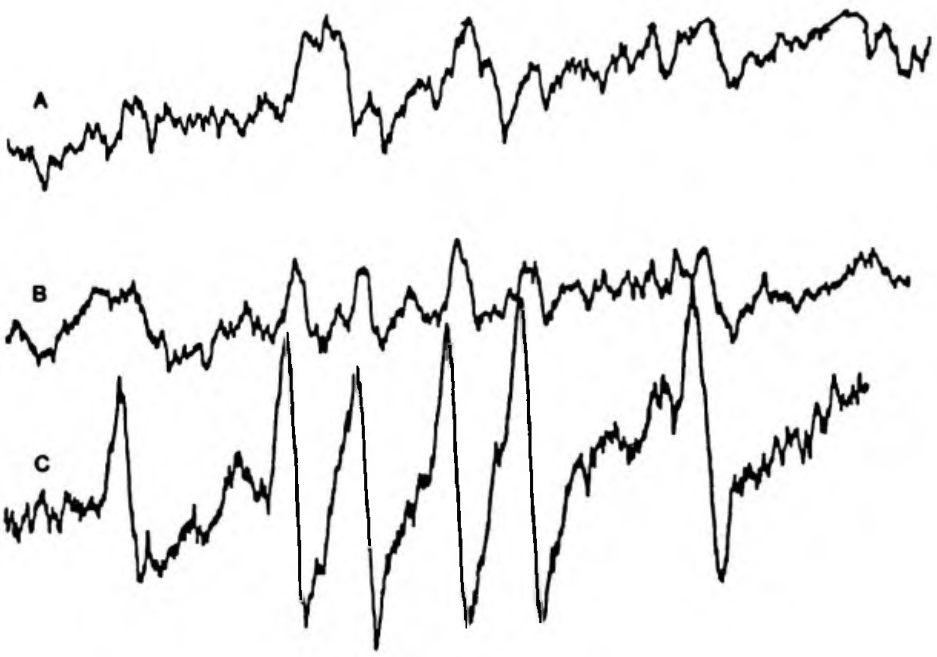

Figure 3. Electron spin resonance spectra showing free radical production by mitochondria isolated in 0.4 M sucrose from Placopecten magellanicus gill. (Traces are microwave absorption spectra by radicals in a known magnetic field; spectra are diagnostic for specific radicals.) Spectrum A was obtained during the first 8 minutes the mitochondria were in a closed flat cell; B, the ensuing 8 minutes; C, the ensuing 8 minutes. After 16 minutes the mitochondria are certainly hypoxic, and the characteristic 6 peak pattern of a DMPO-sucrose radical adduct begins to appear. ESR was done in a Varian E-4 spectrometer at the University of Iowa Medical Center. Spin Trap dimethyl 5,5-dimethylpyrroline-N-oxide (DMPO) was present at 100mM final concentration. Buffers and additions as in Figure 1.

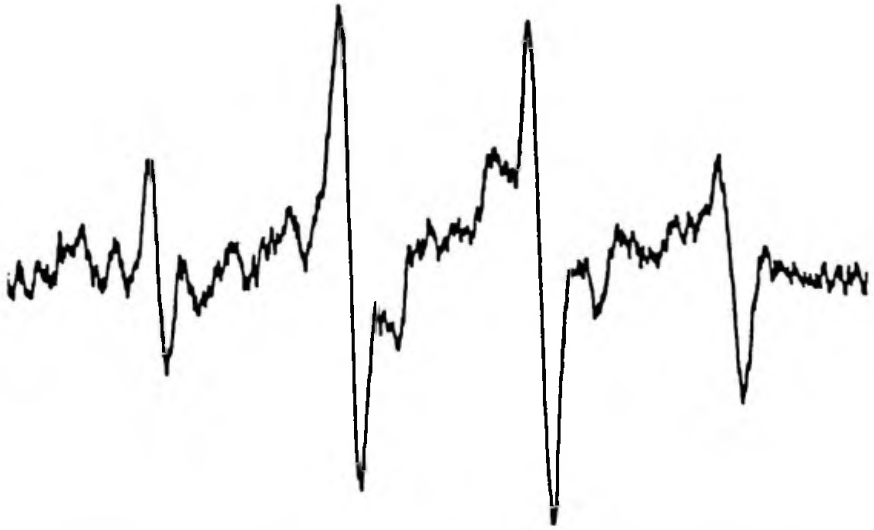

Figure 4. Electron spin resonance spectra of hydroxyl radical-DMPO adduct produced under hypoxia by mitochondria isolated from Placopecten magellanicus gill tissue in Ca-free, Tris-buffered, isotonic artificial seawater (MBL Formulae) to avoid sucrose radical formation. The 1:2:2:1 pattern and peak distribution is diagnostic for ·OH. Conditions as in Figure 1.

The inhibitors rotenone, antimycin and cyanide, which block electron transport in coupled mammalian mitochondria, have only limited effect on O_2 utilization by molluscan mitochondria; fully 30% of the initial rates of O_2 consumption persist in the presence of all three inhibitors, whether used singly or together (Figs. 1 & 2). Uncoupled mitochondria are less sensitive to these inhibitors, and isolated molluscan mitochondria in our hands showed poor acceptor control. However, the critical issue is not one of coupling characteristics, but rather how O_2 reduction persists despite inhibition of electron transport.

H_2O_2 production is usually invoked as a sink for non-electron transport O_2 uptake [Boveris and Cadenas, pp.15-30 in Superoxide Dismutase Vol.II, ed. Oberley, CRC Press, 1982], but only trace amounts of O_2^- are recovered from molluscan mitochondria upon addition of catalase. This is true even when the mitochondria are incubated in the presence of the catalase inhibitor amino-triazole, indicating that H_2O_2 production does not account for inhibitor-insensitive respiration in bivalve molluscs.

Similar observations of cyanide-insensitive respiration in bivalve mitochondria have also been attributed to mannitol oxidase activity [Burcham, Ritchie, and Bishop, J. Exp. Zool., 229:55-67, 1984]. However, we find that the presence or absence of mannitol has no effect on cyanide-insensitive respiration in mitochondria from the two species examined (Fig. 2). In accord with earlier reports [Vorhaben, Scott, and Campbell, J. Biol. Chem., 255:1950-1955, 1980], we have recently found CN^--insensitive respiration that is dependent on mannitol in the visceral mass of the land snail Helix aspersa. In any event, our data indicate that mannitol oxidase is not a source of inhibitor-insensitive bivalve respiration.

As another potential explanation for this CN^--insensitive respiration, Zaba [Mar. Biol. Lett., 4:59-63, 1983] proposed an "alternative electron transport system" that becomes operational only when O_2 availability declines. However, acclimation of M. edulis to severe hypoxia (initial P_{O2} < 25 mmHg) for 14 days (adequate for protein turn-over) did not increase inhibitor-insensitive respiration, suggesting that if this alternative pathway exists, it is not elevated by hypoxia.

Rather, inhibitor-insensitive respiration may be due to redox cycling of electron transport components that directly yields O_2-centered radicals, not peroxide (although H_2O_2 may well be a subsequent product of these radicals). Indeed, our ESR studies of isolated scallop gill mitochondria reveal radical production that begins only as the mitochondria become hypoxic; when mitochondria are isolated in 0.5 M sucrose, carbon-centered sucrose radicals are detected during hypoxia (Fig. 3), and when isolated in buffered isotonic salt solutions to avoid sucrose radical production, hydroxyl radicals are detected directly (Fig. 4). Moreover, when electron flow to O_2 via cytochrome-a/a_3 is blocked with CN^- during normoxia, cyanide radical-DMPO adducts are detected (not shown); because of the extreme redox potentials, these cyanide radical adducts could only have been derived from $\cdot OH$. Given the abundance of mitochondria in aerobically-poised tissues susceptible to RTD, and considering the paucity of XOD in these same tissues, it seems likely that mitochondrial electron transport autoxidation and redox cycling contribute to the radical flux responsible for RTD.

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