Chapter 8

General Discussion

8.1 Summary of results

In this thesis I aimed to answer the following questions. How is intracellular metabolism affected by the reduction of extracellular oxidants? Are membrane redox effects in RBCs animal-species specific? And do electrons/reducing potential import mechanisms exist in the plasma membrane of RBCs? To answer these questions a variety of techniques and methods were used. The two main techniques used in this work were NMR spectroscopy and enzyme-based spectrophotometric assays.

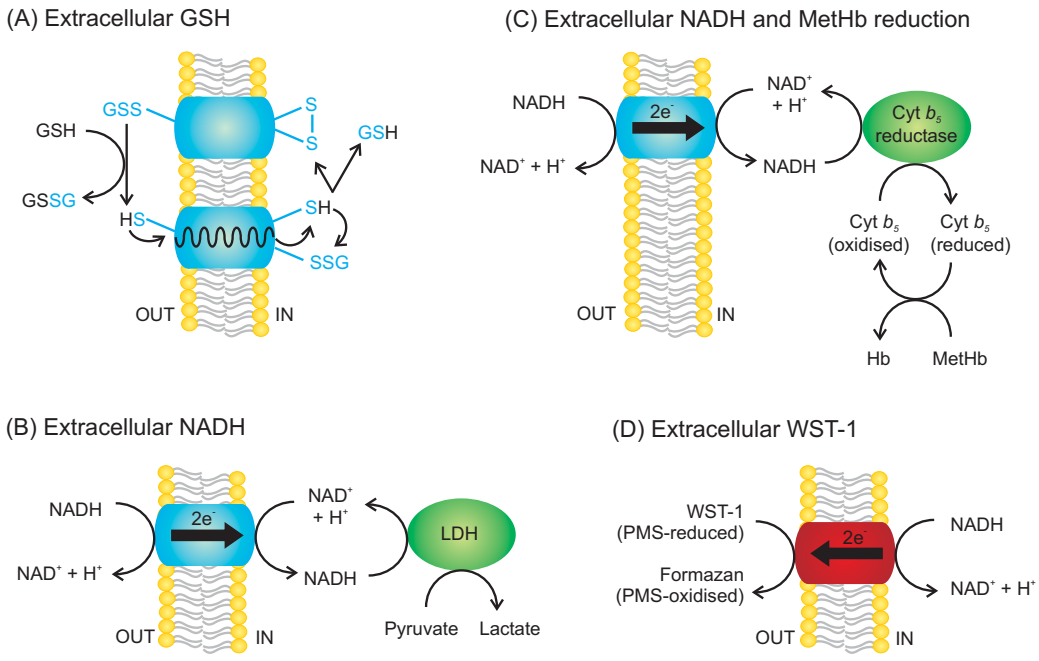

Figure 8.1: Hypothesized mechanisms for the effect of (A) extracellular GSH on the intracellular GSH concentration, (B) extracellular NADH on the intracellular lactate-to-pyruvate ratio, (C) extracellular NADH on the intracellular metHb reduction reaction, and (D) extracellular WST-1 on the intracellular NADH-to-NAD^+ ratio.

In Chapter 3 extracellular GSH was investigated as a potential electron donor/reducing equivalent for the reduction (release) of intracellular membrane-bound GSH (Figure 8.1A). It was found that extracellular GSH had no effect on the intracellular GSH levels of glucose-starved RBCs regardless of whether these cells had been exposed to tBHP and that extracellular GSH was not oxidised. This result was in variance with the findings of another (less direct) ^{1}H NMR-based study (Ciriolo, *et al.*, 1993). The rates of change of the GSH-to-GSSG and the lactate-to-pyruvate ratios were the same as those observed in the absence of extracellular GSH. The use of [*glycyl*-2-^{13}C]GSH and ^{1}H and ^{13}C NMR allowed changes in intra- and extracellular GSH to be monitored over the same time period without disrupting the system. This was the first probe of this putative reaction that employed an isotopomer of glutathione.

In Chapter 4 the possibility that extracellular NADH could be an electron donor for a transmembrane import PMOR was investigated (Figure 8.1B). The addition of NADH to a suspension of glucose-starved RBCs resulted in an increase in the rate of lactate production and a change in the lactate-to-pyruvate ratio over time. The NADH-induced changes were enhanced by the addition of pyruvate and were inhibited weakly by atebrin and pCMBS, and strongly by αCHC, oxalate and oxamate. The NADH oxidase inhibitor capsaicin inhibited the NADH-induced effect in the absence of pyruvate and it stimulated the NADH-induced effect in the presence of pyruvate. The effects of the LDH inhibitors oxalate and oxamate and the monocarboxylate inhibitor αCHC suggested the involvement of LDH, in both the intra- and extracellular compartments.

The location of the NADH-induced effects and the involvement of LDH were investigated in more detail in Chapter 5. MetHb is confined to the intracellular compartment, so that changes in its rate of reduction reflect an increase in the availability of intracellular NADH (Figure 8.1C). Extracellular NADH was found to increase the rate of metHb reduction in both the presence and absence of glucose, indicating an increase in intracellular NADH concentrations. MetHb reduction rates in the presence of NADH varied between RBCs of the animal species tested (cattle,

horse, human, grey kangaroo, pig, sheep). Low rates of metHb reduction in the presence of NADH could be enhanced by the addition of extracellular LDH. It was found that the LDH activity released by RBCs through haemolysis was sufficient to produce the observed NADH oxidation rates, and that the activity correlated with the rates of metHb reduction in the presence of extracellular NADH for RBCs from all of the animal species tested.

In Chapter 6, the reduction of WST-1 at the RBC plasma membrane was investigated (Figure 8.1D). RBCs were found to reduce WST-1 in both the presence of the intermediate electron acceptor PMS. The rate of WST-1 reduction by whole RBCs was substantially lower than that observed in other cell lines (Berridge and Tan, 2000a, 2000b). The rate of reduction of WST-1 was concomitant with an increase in the rate of pyruvate production, implying a need for intracellular NADH for the reduction to take place. The rate of WST-1 reduction was also observed to vary between RBCs from a variety of mammalian species.

In Chapter 7 attempts to purify ferricyanide and WST-1 reductase activities from RBC plasma membranes are reported. The two redox activities co-purified with the main protein peak in the majority of the fractionation techniques employed. After purification by three chromatographic techniques, five proteins were associated with the activity. The possible identity of these proteins was determined by LC-MS on peptide fragments; however the sizes of the proposed proteins were inconsistent with the sizes estimated from SDS-PAGE.

The main findings from the work performed in this thesis and described above are summarised in Figure 8.2. I could find no evidence for a transmembrane electron/reducing equivalent import system in the plasma membrane of RBCs using either GSH or NADH as the electron donor. The NADH-induced effects on RBC metabolism could be explained by the presence of LDH in the extracellular environment (released from RBCs by haemolysis). In cells with reduced Hb, the NADH was oxidised by reaction with pyruvate and the rate of oxidation was increased by supplementing the extracellular compartment with additional pyruvate. In RBCs containing metHb, the increased rate of production of lactate, and

equilibration of lactate and pyruvate across the plasma membrane, drove the formation of NADH inside the cells and hence the reduction of metHb.

Although no evidence was found to support the concept of electron-import PMORs, the work in this thesis has identified a new electron-export PMOR in the RBC plasma membrane, a PMS-mediated WST-1 reductase. As observed with other cell lines, this reductase activity requires electrons from intracellular NADH and can be inhibited by iodoacetate and atebrin. The attempted purification of this activity, together with a ferricyanide reductase activity, from RBC plasma membranes, did not provide sufficient evidence to show that these two oxidants are reduced by the same protein or allow the identification of the proteins responsible.

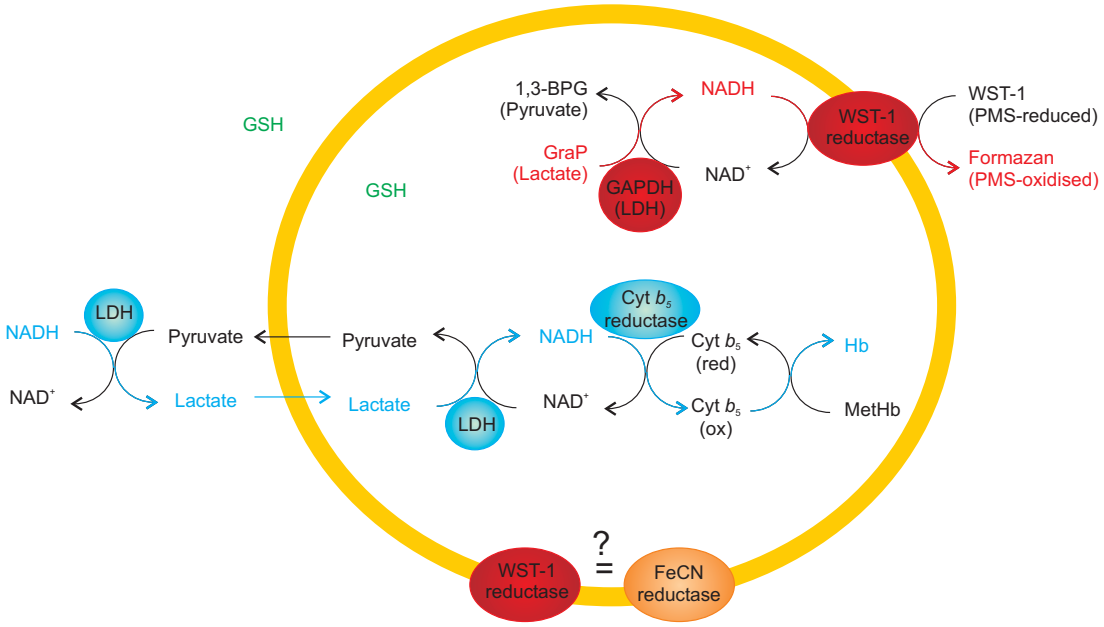

Figure 8.2: Summary of main findings.

8.2 Future Directions

As the results presented in this thesis clearly indicate, there is much still to be learnt about PMORs in RBCs. Further research on the composition and mechanism(s) of action of PMORs is clearly required. A large number of different activities have been observed in plasma membranes, but without isolating the proteins involved it is difficult to distinguish whether a single protein/protein complex reduces multiple

oxidants or whether multiple PMOR complexes are present in the membrane. Hence in the future it is our aim to purify ferricyanide and WST-1 reductase activities from RBC membranes and determine their protein composition. The work presented in Chapter 7 provides a starting point for these purification attempts.

The inability to observe the reduction of WST-1 directly by NMR should be investigated further. The application of different acquisition parameters and/or pulse sequences that counter the effects of constant exchange with complexes in the membrane may enable the observation of WST-1 reduction directly by NMR. New PMOR probes which are suitable for analysis by NMR should also be considered as there is great potential for this technique to be used as an investigative tool for the study of PMORs in other cell types. The ability to observe changes in the intra- and extracellular environments non-invasively, enabling cells to be observed in near *in vivo* conditions, will be of great use in elucidating the mechanisms and effects of these systems on metabolism.

The inclusion of the effects of extracellular oxidants (and reductants) in mathematical models of metabolism will aid in the understanding of how PMORs work, the *in vivo* conditions under which they might be active, and may allow the identification and verification of putative physiological electron acceptors. A comprehensive model of RBC metabolism has been developed in our laboratory (Mulquiney, *et al.*, 1999; Mulquiney and Kuchel, 1999a, 1999b). Inclusion of the experimental results discussed in this thesis and those in the literature at large into the model would enable the verification of their interpretation and may help in the formulation of new approaches to the study of PMORs.