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MYOCARDIAL FLAVIN REDUCTASE AND RIBOFLAVIN: A POTENTIAL ROLE IN DECREASING REOXYGENATION INJURY

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SUMMARY: Ferrylmyoglobin has been implicated in cardiac reoxygenation damage. Flavin reductase, an enzyme previously isolated from erythrocytes, can reduce ferrylmyoglobin in the presence of sufficient flavin concentrations. Flavin reductase mRNA signals were detected in rabbit heart, lung, liver, kidney, and isolated cardiomyocytes. It was hypothesized that increasing flavin reductase catalysis by administering flavins exogenously could decrease cardiac reoxygenation damage in isolated rabbit hearts. Riboflavin (150 μM) inhibited reoxygenation-induced lactate dehydrogenase release by 57%, an effect prevented by hematoporphyrin, a flavin reductase inhibitor. The results suggest that riboflavin supplementation has cardioprotective effects during reoxygenation and that these effects are mediated by flavin reductase.

Considerable evidence supports the involvement of the reactive oxygen species, superoxide and hydrogen peroxide (H_2O_2), in the pathophysiology of cardiac damage caused by reoxygenation after hypoxia or ischemia (1-4). Superoxide and H_2O_2 have limited reactivity and probably do not cause oxidative damage themselves. It is more likely that cell damage occurs when these substances interact with free iron or iron contained in hemeproteins. Myoglobin, an abundant myocardial protein (5), reacts with H_2O_2 and other peroxides to form a ferryl derivative that is unstable, very reactive, and has been shown to cause lipid peroxidation in several *in vitro* preparations (6-11). It has been suggested that myoglobin oxidation may be an important process in free radical-mediated myocardial reoxygenation injury (7). Arduini and colleagues have detected ferrylmyoglobin in ischemic rat hearts (12).

An NADPH-dependent reductase detected originally in erythrocytes (13, 14) and subsequently in heart (15) can catalyze the reduction of ferrylmyoglobin. The enzyme, flavin reductase, catalyzes the reduction of flavins to dihydroflavins. Reduced flavins then reduce oxidized myoglobin in a two-electron transfer. Flavin reductase-dependent ferrylmyoglobin would be expected to proceed very slowly under physiologic conditions

ABBREVIATIONS USED: SSC, saline sodium citrate; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecylsulfate; BPSS, bicarbonate-buffered physiologic saline solution; LDH, lactate dehydrogenase; Mb-Fe(II), ferromyoglobin; Mb-Fe(IV)O, ferrylmyoglobin.

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because free flavin concentrations are relatively low in comparison to the K_m of flavin reductase for flavins. However, increasing flavin concentrations by exogenous addition, stimulates flavin reductase-mediated ferrylmoglobin reduction (16). In the context of reoxygenation injury, increased reduction of reactive oxidized hemeproteins may be an approach in the treatment of iron-mediated injury. In this study we tested the cardioprotective effects of riboflavin in an isolated rabbit heart model of reoxygenation. We also used Northern Blot analysis to provide further support for the presence of flavin reductase in the rabbit heart.

MATERIALS AND METHODS

Northern Blot Analysis

New Zealand White rabbits were sacrificed and tissue samples from heart, liver, kidney, and lung were removed and immediately put on ice. Isolated cardiomyocytes were prepared using collagenase perfusion (1 mg/mL; Worthington Biochemical, Freehold, NJ) (17). Total cellular RNA was prepared using Ultraspec (Biotecx Laboratories Inc, Houston, TX) according to the manufacturer's recommendations. Tissue samples and cell pellets were lysed directly in Ultraspec. Total cellular RNA was quantified by absorbance at 260 nm. RNA (10 μg/lane) was loaded on a 1% agarose gel and separated electrophoretically. RNA was then transferred to a 0.45 μm magna nylon transfer membrane (Micron Separations Inc., Westboro, MA) in 10x saline sodium citrate (SSC). After baking in a vacuum oven, the membrane was prehybridized in hybridization buffer [7% (w/v) SDS, 1% (w/v) bovine serum albumin (fraction V), 0.5 M NaH_2PO_4, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4] for 1 hr at 65°C. Membranes were then hybridized overnight at 65°C with a 381-bp cDNA probe specific for bovine flavin reductase. The probe was labeled with [^{32}P]-labeled CTP (>3000 Ci/mmol, Amersham) using a random primer kit (BRL, Gaithersburg, MD). After hybridization, the membranes were washed in 1x SSC with 0.1% (w/v) SDS at 52°C for 20 min, followed by three washes in 0.1x SSC with 0.1% (w/v) SDS at 52°C. The blots were then exposed to XOMAT-AR film (Kodak, Rochester, NY) for 5 days at -70°C.

Isolated Heart Perfusion Protocol

Hearts were isolated from healthy New Zealand White rabbits (ca. 1.5 kg each). The protocols for heart isolation, perfusion, and instrumentation were the same as those described previously (4). In brief, all hearts were immediately perfused retrogradely via the aorta with bicarbonate-buffered physiologic saline solution (BPSS) that had been equilibrated at 37°C by direct, vigorous bubbling with 95% O_2 + 5% CO_2 (P_{O_2} > 600 mm Hg). The composition of the BPSS was (mM): NaCl, 118; KCl, 4; $NaHCO_3$, 25; $MgCl_2$, 1.2; $CaCl_2$, 2.4; D-glucose, 11. During initial perfusion, perfusion pressure was maintained at 75 mm Hg by adjusting inflow from a calibrated nonpulsatile roller pump. Once oxygenated BPSS perfusion was started, the pulmonary artery was cannulated and other vessels were ligated to facilitate perfusate outflow and collection. A saline-filled balloon connected to a force transducer was placed in the left ventricle through a left atriotomy. Left ventricular end-diastolic pressure was set to 5 mm Hg by adjusting balloon volume. Hearts were paced with a Grass SD9 stimulator using square-wave pulses at 3.3 Hz (4 msec duration, nominally 5.0 V). Once heart rate stabilized in response to pacing, end-diastolic pressure was readjusted (if needed) to 5 mm Hg and left unchanged thereafter. The hearts were equilibrated under these conditions for an additional 30 min. They were then made hypoxic for 60 min by switching to BPSS that had been equilibrated with 95% N_2 + 5% CO_2 ($P_{O_2} \leq 7.5$ mm Hg). Hearts were reoxygenated for 30 min by abruptly switching to the original oxygenated BPSS solution.

Hearts in the control group were perfused with BPSS only. In treated groups, 150 μM riboflavin was added to the BPSS 5 min before reoxygenation. Hematoporphyrin (20 μM), a flavin reductase inhibitor, was added to some hearts treated with riboflavin; some hearts received only 20 μM hematoporphyrin. These additives remained throughout

reoxygenation. Because riboflavin is light-sensitive, the perfusion system was covered with foil and the room lights were turned off during riboflavin perfusion. After each experiment, a sample of the left ventricular free wall was removed, blotted to remove surface moisture, weighed, and then dried to constant weight.

During perfusion, pulmonary artery effluent and myocardial lymph drainage were collected continuously, in 5 or 10 min periods during equilibration, hypoxia, and reoxygenation, and kept on ice. The volume of each aliquot was measured to compute the flow per min over each collection interval. The lactate dehydrogenase (LDH) activity of each aliquot was measured spectrophotometrically (18). Enzyme activity was normalized per g of ventricle dry weight, and expressed in total LDH units released during equilibration, hypoxia, and reoxygenation. Commercially available LDH standards (Sigma, St. Louis) were assayed in the presence and absence of riboflavin and hematoporphyrin (at concentrations used in the BPSS) to demonstrate that these substances did not affect the LDH assay.

Statistical Analyses

Data are expressed as arithmetic means (± 1 S.E.M.). Between-group data for a given variable were analyzed for statistically significant differences using analysis of variance (ANOVA) and Scheffe's test when data from more than two groups were compared. Within-group data were analyzed using paired *t*-tests. Statistical significance was defined as a p value less than or equal to 0.05.

RESULTS

Northern Blot Analysis

Figure 1 shows the results of Northern blot analysis on rabbit heart, lung, liver, kidney, and isolated cardiomyocytes. RNA message for flavin reductase was detected in all lanes. The signal from isolated cardiomyocytes was slightly greater than that from whole heart.

Cardioprotection Studies

The rate of LDH release at the end of equilibration was slight and not significantly different among the various groups. During hypoxia, LDH release in all groups remained relatively constant and after 60 min was not significantly different from that measured during equilibration. In the control group, reoxygenation caused an increase in the rate of LDH release from an average of 0.12 ± 0.09 U/min/g dry weight during hypoxia, to 0.94 ± 0.17 U/min/g after 30 min of reoxygenation. The total amounts of LDH released during drug treatment are summarized in Figure 2. Adding 150 μM riboflavin, 20 μM hematoporphyrin, or both after 55 min of hypoxia did not have significant effects on the amount of LDH released during the remaining 5 min of hypoxia. During reoxygenation, however, riboflavin caused a 57% ($p<0.05$) drop in LDH loss, an effect that was significantly prevented by hematoporphyrin ($p<0.05$). Hematoporphyrin had no effect of its own on LDH release.

DISCUSSION

The oxidation of myoglobin to ferrylmyoglobin by H_2O_2 may be important during myocardial reperfusion injury (reactions 1 and 2).

(1) Mb-Fe(II) + H_2O_2 ——————> Mb-Fe(IV)O + H_2O

(2) Mb-Fe(IV)O + Lipids and Proteins ——————> Oxidized Lipids and Proteins

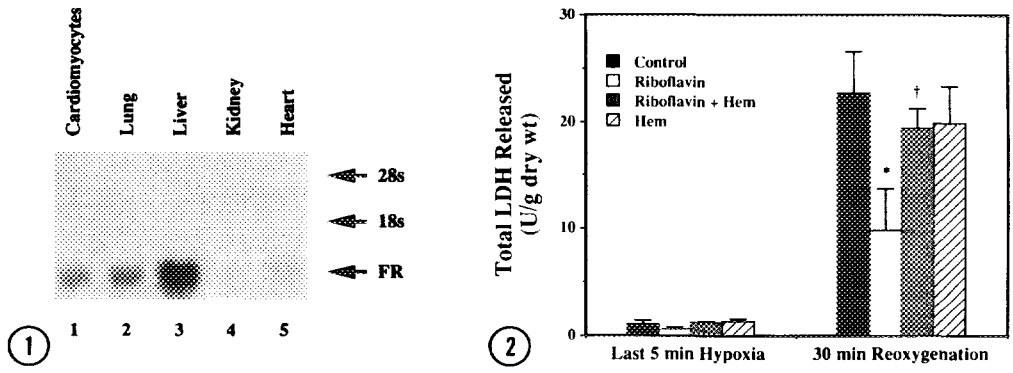

Figure 1. Northern blot analysis of flavin reductase in rabbit tissues. RNA (10 μg/lane) was loaded on a 1% agarose gel, separated electrophoretically, transferred to nylon membrane, and hybridized overnight with a [^{32}P]-labeled cDNA probe specific for flavin reductase (FR). RNA was isolated from isolated cardiomyocytes (lane 1), lung (lane 2), liver (lane 3), kidney (lane 4), and heart (lane 5). For size comparison, the positions of the 28s and 18s ribosomal fragments are also labeled. The data shown are representative of three experiments.

Figure 2. Effects of riboflavin on LDH release. The total amounts of LDH in the combined samples of coronary effluent and lymph fluid were measured during drug treatment which included the last 5 min of hypoxia and 30 min of reoxygenation. 150 μM riboflavin attenuated the reoxygenation-induced increase in LDH release. This effect was inhibited by 20 μM hematoporphyrin (Hem). Values are means ± 1 S.E.M. *Significantly different ($p<0.05$) from control value during the same period. †Significantly different from riboflavin-treated group.

Flavin reductase, flavins, and NADPH can mediate the reduction of oxidized myoglobin through reactions 3 and 4 (16).

(3) NADPH + Flavin $\xrightarrow{\text{Flavin Reductase}}$ $NADP^+$ + Dihydroflavin

(4) Mb-Fe(IV)O + Dihydroflavin ——————> Mb-Fe(II) + H_2O + Flavin

Previous studies from our laboratories have immunologically detected flavin reductase in liver and heart (15, 19) and detected a flavin reductase transcript in human tissues (20). The results of Northern analysis reported here provide excellent corroborative evidence for the presence of flavin reductase not only in the rabbit heart but in rabbit liver, lung and kidney as well. In the cardioprotection experiments, we attempted to increase flavin reductase catalysis (reaction 3) during reperfusion by increasing riboflavin concentrations. The resulting increase in dihydroriboflavin should favor the elimination of oxidized myoglobin through reaction 4 and thus limit oxidative damage caused by reaction 2.

Riboflavin administration 5 min before reoxygenation significantly reduced the rate of reoxygenation-induced LDH release as well as the total amount of LDH released during reoxygenation. A number of findings suggest that the effects of riboflavin are somewhat specific and mediated by flavin reductase. First, riboflavin had no effect on any measured hemodynamic or functional parameter, making a protective effect due to a change in oxygen supply or demand unlikely. Second, cardioprotection was seen using a fairly low riboflavin concentration (150 μM). In contrast, many other cardioprotective agents must be

present in much higher concentrations (e.g., mM), making it difficult to eliminate the possibility of nonspecific effects. Third, the effects of riboflavin were attenuated by the flavin reductase inhibitor, hematoporphyrin. Fourth, previous studies have shown that the "nonflavin" substrate, pyrroloquinoline quinone, was also cardioprotective (15). This suggests that the cardioprotective effects we found do not involve riboflavin's role as a coenzyme in cellular redox reactions. Others have also reported that riboflavin protects against various forms of tissue damage (21, 22).

The proposed mechanism for cardioprotection in this model relies on flavin reduction by flavin reductase and the subsequent reduction of oxidized myoglobin by reduced riboflavin. The cardioprotective effects of reducing compounds have been known for some time. Reduced glutathione, methionine, and ergothionine have all been shown to protect the ischemic or hypoxic myocardium against several manifestations of reoxygenation injury (7, 23, 24). These compounds have also been shown either to prevent the formation, or to enhance the dissipation, of ferrylmyoglobin in both *in vitro* and isolated heart preparations (9, 24, 25). Therefore, the protective effects of reduced riboflavin and other reducing compounds may be similar and dependent upon hemeprotein reduction.

Other possible mechanisms for tissue protection by riboflavin may also be operating. Dihydroriboflavin may be acting on other reactive oxygen species, or riboflavin may be acting directly as an oxygen radical scavenger or antioxidant. To address these possibilities, several free radical generating systems were exposed to flavins. Riboflavin had no effect on the superoxide-mediated reduction of ferricytochrome c during the xanthine oxidase-catalyzed conversion of hypoxanthine to uric acid. In addition, flavins had no detectable effects on the aromatic hydroxylation assay (26), designed to measure the formation of hydroxyl radicals in the presence of superoxide and an iron chelate. Xu and Hultquist have shown that dihydroriboflavin can reduce H_2O_2, but only at a very slow rate (16). However, there is some evidence that reduced flavins, similar to other reducing compounds, may react directly with hydroxyl radicals (16, 27).

In conclusion, riboflavin had protective effects on the rabbit myocardium during reoxygenation. The mechanism may involve flavin reductase and the reduction by dihydroriboflavin of highly oxidized heme species. Although further experiments are needed, these results suggest that riboflavin, which is virtually nontoxic, may be an effective therapy in situations where tissue injury is caused by reactive oxygen species and/or oxidized cellular constituents.

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REFERENCES

1. Zweier, J. L., Flaherty, J. T., and Weisfeldt, M. L. (1987)*Proc. Natl. Acad. Sci.* **84**, 1404-1407.
2. Burton, K. P. (1985) *Am. J. Physiol.* **248**, H637-H643.
3. Jolly, S. R., Kane, W. J., Bailie, M. B., Abrams, G. D., and Lucchesi, B. R. (1984) *Circ. Res.* **54**, 277-285.
4. Shlafer, M., Kane, P. F., and Kirsh, M. M. (1982) *J. Thorac. Cardiovasc. Surg.* **83**, 830-839.
5. Sylven, C., Jansson, E., and Boo, K. (1984) *Cardiovasc. Res.* **18**, 433.
6. Davies, M. J. (1990) *Free Radic. Res. Commun.* **10**, 361-370.
7. Galaris, D., Eddy, L., Arduini, A., Cadenas, E., and Hochstein, P. (1989) *Biochem. Biophys. Res. Commun.* **160**, 1162-1168.
8. Walters, F. P., Kennedy, F. G., and Jones, D. P. (1983) *FEBS Letts.* **163**, 292-296.
9. Galaris, D., Sevanian, A., Cadenas, E., and Hochstein, P. (1990) *Arch. Biochem. Biophys.* **281**, 163-169.
10. Kanner, J., and Harel S. (1985) *Arch. Biochem. Biophys.* **237**, 314-321.
11. Grisham, M. B. (1985) *J. Free Radic. Biol. Med.* **1**, 227-232.
12. Arduini, A., Eddy, L., and Hochstein, P. (1990) *Free Radic. Biol. Med.* **9**, 511-513.
13. Huennekens, F. M., Caffrey, R. W., Basford, R. E., and Gabrio, B. W. (1957) *J. Biol. Chem.* **227**, 261-272.
14. Yubisui, T., Matsuki, T., Takeshita, M., and Yoneyama, Y. J. (1979) *J. Biochem. (Tokyo)* **85**, 719-728.
15. Xu, F., Mack, C. P., Quandt, K. S., Shlafer, M., Massey, V., and Hultquist, D. E. (1993) *Biochem. Biophys. Res. Commun.* **193**, 434-439.
16. Xu, F., and Hultquist, D. E. (1991) *Biochem. Biophys. Res. Commun.* **181**, 197- 203.
17. Jacobson, S. (1989) In *Isolated Adult Cardiomyocytes* (H. M. Piper and G. Isenberg, Eds.), pp. 43-80, CRC Press, Boca Raton, FL.
18. Amador, E., Dorfman, L. E., and Wacker, W. E. C. (1963) *Clin. Chem.* **9**, 391.
19. Xu, F., Quandt, K. S., and Hultquist, D. E. (1992) *Proc. Nat. Acad. Sci. USA* **89**, 2130-2134.
20. Quandt, K. S. and Hultquist, D. E. (1994) *Proc. Nat. Acad. Sci. USA* **91**, 9322-9326.
21. Hultquist, D. E., Xu, F., Quandt, K. S., Shlafer, M., Mack, C. P., Till, G. O., Seekamp, A., Betz, A. L., and Ennis, S. R. (1993) *Am. J. Hematol.* **42**, 13-18.
22. Kotegawa, M., Sugiyama, M., and Haramaki, N. (1994) *Biochem. Mol. Biol. Int.* **34**, 685-691.
23. Chambers, D. J., Astras, G., Takahashi, A., Manning, A. S., Braimbridge, M.V., and Hearse, D. J. (1989) *Cardiovasc. Res.* **23**, 351-358.
24. Arduini, A., Eddy, L., and Hochstein, P. (1990) *Arch. Biochem. Biophys.* **281**, 41-43.
25. Mitsos, S. E., Kim, D., Lucchesi, B. R., and Fantone, J. C. (1988) *Lab. Invest.* **59**, 824-830.
26. Halliwell, B., and Gutteridge, J. M. C. (1986) In *The Handbook of Oxygen Radical Research* (R. Greenwald, Ed.), pp. 177-180, CRC Press, Boca Raton, FL.
27. Hazzard, J. T., Moench, S. J., Erman, J. E., Satterlee, J. D., and Tollin, G. (1988) *Biochemistry* **27**, 2002-2008.