A Causative Role for Redox Cycling of Myoglobin and Its Inhibition by Alkalization in the Pathogenesis and Treatment of Rhabdomyolysis-induced Renal Failure*

Kevin P. Moore‡§, Steve G. Holt‡, Rakesh P. Patel¶, Dimitri A. Svistunenko§, William Zackert**, David Goodier‡, Brandon J. Reeder, Martine Clozel‡‡, Radhi Anand‡, Christopher E. Cooper†, Jason D. Morrow**, Michael T. Wilson†, Victor Darley-Usmar‡, and L. Jackson Roberts II**

From the ‡Joint Department of Medicine, Royal Free and University College Medical School, London NW3 2QG, United Kingdom, the §Center for Free Radical Biology and Department of Pathology, Molecular and Cellular Division, University of Alabama, Birmingham, Alabama 35243, the ¶Departments of Pharmacology and Medicine, Vanderbilt Biomedical Center, Nashville, Tennessee 37232; †Preclinical Research, Hoffmann-La Roche, Basel 4070, Switzerland, and the §§Department of Biological Sciences, University of Essex, Colchester, Essex CO4 3SQ, United Kingdom

Muscle injury (rhabdomyolysis) and subsequent deposition of myoglobin in the kidney causes renal vasoconstriction and renal failure. We tested the hypothesis that myoglobin induces oxidant injury to the kidney and the formation of F2-isoprostanes, potent renal vasoconstrictors formed during lipid peroxidation. In low density lipoprotein (LDL), myoglobin induced a 30-fold increase in the formation of F2-isoprostanes by a mechanism involving redox cycling between ferric and ferryl forms of myoglobin. In an animal model of rhabdomyolysis, urinary excretion of F2-isoprostanes increased by 7.3-fold compared with controls. Administration of alkali, a treatment for rhabdomyolysis, improved renal function and significantly reduced the urinary excretion of F2-isoprostanes by ~80%. EPR and UV spectroscopy demonstrated that myoglobin was deposited in the kidneys as the redox competent ferric myoglobin and that its concentration was not decreased by alkalization. Kinetic studies demonstrated the reactivity of ferryl myoglobin, which is responsible for inducing lipid peroxidation, is markedly attenuated at alkaline pH. This was further supported by demonstrating that myoglobin-induced oxidation of LDL was inhibited at alkaline pH. These data strongly support a causative role for oxidative injury in the renal failure of rhabdomyolysis and suggest that the protective effect of alkalization may be attributed to inhibition of myoglobin-induced lipid peroxidation.

In 1941 Bywaters and Beall (1) described four patients who developed acute renal failure, associated with dark brown urinary granular casts following crush injury. This syndrome has subsequently been termed rhabdomyolysis, since it occurs as a consequence of massive breakdown of muscle. It is usually associated with trauma, but may occur in a variety of clinical settings such as hyperthermia, seizures, muscle ischemia, or exposure to toxins such as alcohol or drug overdose. The associated muscle breakdown releases myoglobin (Mb)1 into the circulation, which then becomes deposited in the kidney causing renal failure in 30% of cases. Rhabdomyolysis accounts for 7% of cases of acute renal failure in the United States (2, 3).

The mechanism of the renal failure in rhabdomyolysis has been attributed to both intense renal vasoconstriction and renal tubular necrosis (4–6). Recent studies have suggested a potential role for free radicals in the pathogenesis of myoglobinuria-induced renal failure based on the findings that in rats with rhabdomyolysis, levels of malondialdehyde in the kidney are increased (2–3-fold). There is an induction of antioxidant enzymes and consumption of antioxidants, and administration of antioxidants partially protects against the renal failure (7–11). The modest increases of malondialdehyde levels are difficult to interpret, since it is well recognized that measurements of aldehyde lack specificity as a marker of lipid peroxidation, and others have failed to replicate these findings (12). To explain how myoglobinuria could induce an oxidant injury to the kidney, it has been suggested that Mb may release free ferrous (Fe2+) iron, which could then induce lipid peroxidation as a result of the generation of hydroxyl radicals via the Fenton reaction (13, 14). However, it has been difficult to demonstrate hydroxyl radical production in the kidneys of animals with myoglobinuria and administration of desferrioxamine at doses that effectively chelate all free iron in the urine is only partially effective in preventing renal failure (15).

An alternative mechanism could involve the heme group in Mb itself, which can redox cycle between different oxidation states and promote lipid peroxidation reactions as a consequence of its ability to decompose lipid hydroperoxides to peroxyl and alkoxyl radicals (16). This is achieved catalytically by a redox cycle between the ferric (Fe3+) and ferryl ([Fe=O]2+) Mb oxidation states, the latter of which can directly initiate lipid oxidation (17, 18) (Fig. 1). In muscle cells, the iron in Mb is maintained predominantly in the reduced ferrous (Fe2+) state, which is unable to participate in such reactions. However, the redox state of Mb in renal tubules has not been determined. We hypothesized that Mb in the extracellular milieu, i.e. in renal tubules, would undergo autooxidation to the ferric form (MetMb), which is then catalytically competent to

1 The abbreviations used are: Mb, myoglobin; MetMb, metmyoglobin; IsoP, isoprostane; PG, prostaglandin; 13-HPODE, 13(9E,11Z)-octadecadienoic acid; LDL, low density lipoprotein; DTPA, diethylenetriaminepentaacetic acid; REM, relative electrophoretic mobility.
Lipid Peroxidation: IsoP formation.

**Fig. 1. The mechanism for Mb-induced lipid peroxidation and IsoP formation.**

...promote lipid peroxidation reactions. Importantly, unlike the Haber-Weiss/Fenton reactions involving free iron, this reaction is not dependent on the generation of superoxide or hydrogen peroxide; it can be driven solely by endogenous lipid hydroperoxides (17-20). This hypothesis may also explain the partial efficacy of desferoxamine to protect against renal failure in the rat model of rhabdomyolysis, since desferoxamine is capable of reducing ferryl-Mb and its associated globin radical to the ferric form (21, 22). Interestingly, nitric oxide also partially protects against the renal failure (23). In accord with our hypothesis, this might be explained by an effect of nitric oxide to inhibit heme-dependent lipid peroxidation (24).

Mb-induced lipid peroxidation could explain the occurrence of renal tubular necrosis in myoglobinuria, but how this could be causally linked to the reduction in renal blood flow that also characterizes the nephropathy of rhabdomyolysis is not immediately obvious. One hypothesis that could be advanced is that vasoactive products of lipid peroxidation are generated that cause renal vasoconstriction. In this regard, one of the most attractive candidates would be the isoprostanes (IsoPs), which we previously identified as a group of prostaglandin (PG)-like compounds that are formed nonenzymatically in vivo as products of free radical-induced peroxidation of arachidonic acid (25). PGF₂α, E₂, and D₂-like compounds are formed in vivo by this process (25, 26). Importantly, two IsoPs formed in abundance, namely 15-F₂IsoP (27) (8-iso-PGF₂α) and 15-E₂IsoP (8-ISO-PGE₂), are very potent renal vasoconstrictors that appear to interact with a unique receptor (25, 28, 29).

An intriguing question then arises as to whether an effect on oxidant injury could be mechanistically linked with the observation that alkalization of the urine significantly attenuates the renal dysfunction (2, 12). A plausible and accepted explanation is that alkalization enhances the solubility of Mb and thus its elimination from the kidney (12). However, we considered an alternative explanation based on the recent demonstration that Mb-induced lipid peroxidation reactions are accelerated at acidic pH (30).

In this study, therefore, we examined the role of oxidative injury to the kidney in the pathogenesis of the renal failure associated with rhabdomyolysis in a rat model by measuring the specific marker of lipid peroxidation, F₂IsoPs, which has emerged as one of the most reliable approaches to assess oxidative stress status in vivo (31). For this purpose, F₂IsoPs were measured both in urine and in renal lipids. A novel aspect of the formation of IsoPs is that they are initially formed in situ esterified to tissue lipids and subsequently released (32). Since IsoPs generated in renal lipids would, in part, be directly released into the urine, measurement of urinary F₂IsoPs can provide an index of lipid peroxidation that occurs in the kidney (31). More direct evidence localizing oxidant injury to the kidney can also be obtained by measurement of levels of F₂IsoPs esterified in renal lipids. Furthermore, we explored the hypothesis that the protective effect of alkalization operates by stabilizing ferryl-Mb, thus reducing its potential to induce lipid peroxidation.

**MATERIALS AND METHODS**

**Preparation and Oxidation of Human LDL—**Human LDL was isolated by differential centrifugation (33). Horse heart Mb (determined to be MetMb) was purchased from Sigma, Poole, Dorset, United Kingdom. MetMb (0-100 µM final concentration) was added to LDL (50 or 100 µM LDL in Hanks' buffered saline solutions in which the pH was adjusted as indicated and incubated at 37 °C for the time specified. Ferryl-Mb was prepared immediately before use by mixing 15 µM hydrogen peroxide with 10 µM MetMb. Control experiments involved incubation in phosphate-buffered saline alone or together with hydrogen peroxide (15 µM). The reaction was terminated by addition of butylated hydroxytoluene (100 µM). All experiments were performed in the presence of the iron chelator, DTPA (diethylenetriaminepentacetic acid) (10 µM), to exclude free iron-mediated oxidation. Electrophoretic mobility of LDL was determined using a lipoprotein electrophoresis system (Beckman Co). Gels were fixed, stained with Sudan black B, and results related to mobility of unoxidized LDL (33).

**Experimental—**The rat model of glycerol-induced rhabdomyolysis used has been described previously (12). Animals were divided into three groups designated as controls, rhabdo, or rhabdo-alk. Control animals (n = 8) were untreated. Animals in the rhabdo group (n = 6) were injected with glycerol (10 ml/kg of a 50% solution in saline), which produces a nonlethal form of renal failure associated with high myoglobinuria. The third group (rhabdo-alk, n = 6) was retreated for 24 h with water containing potassium bicarbonate (150 mM) and injected with potassium bicarbonate (2.5 ml/kg of 150 mM potassium bicarbonate) 1 h prior to injection with glycerol. Urine was collected from 0 to 24 h after injection of glycerol, after which the animals were sacrificed and kidneys snap-frozen in liquid N₂ for measurement of esterified F₂IsoPs, or frozen in isopentane (at −56 °C), and stored at −80 °C. For the purpose of measuring renal esterified F₂IsoPs a further three animals in the rhabdo group and a further four animals in the rhabdo-alk group were also studied. Kidney frozen in isopentane was embedded in OCT medium (Tissue-Tek, Miles Inc., IN) and 10-µm frozen sections cut onto glass slides for spectrophotometric analysis.

**Extraction and Measurement of F₂IsoPs—**Esterified F₂IsoPs in LDL and kidney and free F₂IsoPs in urine were extracted and quantified by stable isotope dilution mass spectrometric assay as described, except [³H]8-iso-prostaglandin F₂α (Cayman Chemical Co., Ann Arbor, MI) was used as the internal standard (34). Urinary excretion of F₂IsoPs was normalized to creatinine clearance to correct for the widely differing degrees of renal function, i.e. excretion rate/creatinine clearance. This is calculated as shown below and results expressed as pg/ml Cr.Cl. (pg of F₂IsoPs/ml of creatinine clearance )/[urinary F₂IsoPs (pg/ml) × [plasma creatinine (µM)/urine creatinine (µM)].

**Optical Spectroscopy Analysis of Mb in the Kidney—**Examination, under a microscope of thin (10 µm) sections of rhabdomyolytic kidney showed, in contrast to normal kidney sections, a red/brown pigment to be located within the tubules. This pigment could easily be obtained from the mounted section by washing the 10-µm sections with 200 µl of sodium phosphate buffer, pH 7.4, containing 10 µM DTPA. This extract was diluted to 1 ml with the same buffer. The spectrum of the extract from the kidney was compared with purified MetMb. Sodium dithionite (1 mM) was added to produce the deoxy form for comparison with purified deoxy-Mb. Optical spectroscopy was performed using a Varian Cary 5E UV-visible spectrophotometer.

**Electron Paramagnetic Resonance (EPR) Spectroscopy Analysis of Mb in the Kidney—**Rat kidney was cut into pieces of similar size and frozen in liquid nitrogen. One EPR sample, always made of one kidney, consisted of 20-30 such pieces (~0.3 g). When placed directly into a Bruker finger Dewar (inner diameter = 4.5 mm) without using an EPR tube, such a sample occupied the volume of the Dewar, the length of which was always greater than the working zone of the cavity, 22 mm. This ensured that an EPR spectrum was always taken of the same volume (the cavity working zone) fully filled with the sample, the packing of this volume with tissue pieces being the only variable factor. This packing factor, however, was not a problem, since an EPR spectrum taken of the same sample after reloading into the Dewar (thus providing a different packing) differed by less than 5% in signal intensity. Furthermore EPR spectra of samples made from different parts of the same kidney differed by less than 10% in signal intensity. The tissue...
Mechanism of Rhabdomyolysis-induced Renal Failure

TABLE I
Mb-induced formation of IsoPs in LDL and changes in relative electrophoretic mobility (REM)

| Preparation | Base line | 24-h Mb | n-Fold Increase | REM |
|-------------|-----------|---------|----------------|-----|
| F2-IsoPs    | ng/mg LDL |         |                |     |
| 1           | 0.72 ± 0.05 | 1       |                |     |
| 2           | 2.15 ± 0.05 | 3.15 ± 0.05 | 3.17, Fig. 2) | 1.53 ± 0.07 |
| 3           | 4.07 ± 0.05 | 6.07 ± 0.05 | 6.09, Fig. 3 | 3.01 ± 0.01 |
| 4           | 6.07 ± 0.05 | 9.07 ± 0.05 | 9.09, Fig. 3 | 6.01 ± 0.04 |
| Mean ± S.E. | 0.6 ± 0.1  | 29 ± 7  | 50 ± 14        | 3.8 ± 0.3 |

TABLE II
Ferryl-Mb dependent oxidative modification of LDL

| LDL (control) | ng/mg/LDL | F2-isoprostanes | REM |
|---------------|-----------|----------------|-----|
| LDL + MetMb   | 0.72 ± 0.05 | 1               |     |
| LDL + MetMb + H2O2 | 2.15 ± 0.05 | 3.15 ± 0.05 | 3.17, Fig. 2) | 1.53 ± 0.07 |
| LDL + H2O2   | 4.07 ± 0.05 | 6.07 ± 0.05 | 6.09, Fig. 3 | 3.01 ± 0.01 |
| LDL + H2O2   | 6.07 ± 0.05 | 9.07 ± 0.05 | 9.09, Fig. 3 | 6.01 ± 0.04 |
| Mean ± S.E.  | 0.6 ± 0.1  | 29 ± 7  | 50 ± 14        | 3.8 ± 0.3 |

pieces were retained in the finger of the Dewar with a quartz rod to avoid movement of the pieces caused by liquid nitrogen boiling. Since sufficient kidney tissue was used to extend the sample beyond the working zone, the rod did not cause a problem with either frequency tuning or induce any background signals. All EPR measurements were performed at 77 K. The EPR spectra were measured on a Bruker EMX spectrometer with an ER 041XG microwave bridge (X-band). A 4103TM cavity was used. The experimental conditions comprised: microwave frequency, 100 kHz; modulation amplitude, 7 G; time constant, 0.041 s; sweep time, 54 s; receive gain, 104; number of scans, 4; data points, 2048/scan.

Where integration of the EPR signals was possible, the concentrations are quoted in μM by comparing the double integrals to that of known standards separately measured in an EPR tube (35). Due to uncertainties in comparing signal intensities from different types of EPR sample (tissue slice placed directly in the Dewar versus frozen standard in EPR tube), we estimate that the systematic error of these absolute measurements (in terms of μM spins) could be as high as 50%, but the relative intensities of the signals in controls versus rhabdomyolytic kidneys are accurate to within 5%. When integration of the EPR signal was not straightforward due to overlapping signals, the signal intensity was normalized to the average intensity seen in the control rat. It is important to note that it is not the peak intensity, but pure line shape second integral, that is proportional to concentration. The low spin ferric signals are much wider than the rhombic signal at g = 4.3; therefore, at equal concentrations of the two centers, the peak intensity of the low spin signal will be smaller. In addition the EPR absorbance “efficiency” of the paramagnetic centers (EPR signal second intensity) depends on the respective g factors.

Assessment of the pH Dependence of Lipid Hydroperoxide Consumption by Mb and Decay of Ferryl-Mb—A solution of MetMb (10 μM) was prepared and the reaction initiated by addition of 40 μM 3(S)-hydroperoxy-9( Z)-octadecadienoic acid (3-HPODE) (Cascade Biochem, Reading, United Kingdom). Consumption of 3-HPODE was followed by monitoring the loss of the conjugated diene chromophore at 234 nm, which occurs as 3-HPODE is converted to an epoxy peroxyl radical (Fig. 1). The single exponential progress curves were fitted by a regression analysis. The pH dependence of the decay of ferryl-Mb was determined by mixing 10 μM MetMb with 20 μM hydrogen peroxide to form ferryl-Mb, and the decay of ferryl-Mb was followed by monitoring the increase in absorbance at 406 nm due to reformation of MetMb.

Statistics—Urinary F2-ISOp excretion in animals and esterified F2-IsoPs in LDL were compared between groups using the nonparametric Mann Whitney U test and were considered significant when p < 0.05. Where mean values are reported, the S.E. is also indicated. For changes in creatinine clearance, the unpaired t test was used.

RESULTS

Formation of F2-ISOPs during Oxidation of LDL by MetMb—We initially examined the capacity of Mb to induce the formation of F2-ISOps using LDL as a model lipid containing system. MetMb (10 μM) caused marked lipid peroxidation of LDL resulting in a ~50-fold increase over baseline in esterified levels of F2-IsoPs (p < 0.01). This was accompanied by a corresponding increase in the relative electrophoretic mobility of the LDL, indicative of significant oxidative modification of ApoB-100 (Table I). In a separate experiment ferryl-Mb (10 μM), MetMb (10 μM), or hydrogen peroxide (15 μM) were incu-
The ferric oxidation state of Mb (Fe$^{3+}$), but not the ferrous or ferryl forms, is detectable by EPR spectroscopy. Thus quantitative changes in the MetMb concentration can be measured using this technique. Significant changes were seen in EPR spectra from the kidneys of rhabdomyolytic rats (Fig. 5B) compared with controls (Fig. 5A). These data have been quantified and the results reported in Table III. Specifically, there was a large increase in the $g = 6$ high spin ferric heme signal, corresponding to metmyoglobin, and a low spin heme signal at $g_x = 2.58$; $g_y = 2.28$ (Table III). At the same time a new rhombic iron signal with the same $g = 4.3$ but unknown ligands appears in the spectrum from the rhabdomyolytic kidneys, suggestive of an increase in low molecular weight ferric pools in the rhabdomyolytic kidneys.

Decreases are seen in the other cellular EPR signals, e.g. cytochrome P450 and iron-sulfur enzymes, which may be explained as a result of general cellular damage and associated edema in the kidney. The low spin heme signal evident in the spectra B and C has different characteristics to cytochrome P450 with a different shape and slightly different $g_2$. Since the high spin form of MetMb is very much enhanced in spectra B and C, it is likely to be the source of this new signal. Alkalization had no significant effect on any of the components of the EPR spectra in the rhabdomyolytic kidneys, including the size of the new iron signal at $g = 4.3$ (Fig. 5, C and B, Table II). In particular there was no change in the concentration of MetMb following alkalization (Table III).

**Effect of Alkalization on LDL Oxidation**—The hypothesis that the oxidative potential of Mb to induce lipid peroxidation is pH-dependent was explored using MetMb-induced oxidation of LDL in vitro. LDL (three preparations) were incubated with 5 μM MetMb at a pH range of 5.5–8.0. The results obtained indicate that Mb-induced formation of F$_2$-IsoPs is highly pH-dependent, being markedly suppressed at alkaline pH (Fig. 6).

**Effect of Alkalization on the Stabilization of Ferryl-Mb and Consumption of Lipid Hydroperoxides**—Oxidation of lipids by Mb is a lipid hydroperoxide-dependent process (17). To further probe the mechanism for the pH dependence of Mb-induced lipid oxidation, consumption of the lipid hydroperoxide, 13-HPODE, by Mb was assessed as a function of pH. Calculated rate constants for consumption of 13-HPODE by MetMb at different pH values is shown in Table IV. The pH profile shows that consumption of 13-HPODE is low at pH values above 7 but increased 35-fold with a reduction in pH from 8.0 to 5.0. This profile is consistent with the pH dependence for the formation of F$_2$-IsoPs in LDL (Fig. 6). To determine whether alkalization stabilized the decay of ferryl-Mb, ferryl-Mb was incubated as above and the decay rate of ferryl-Mb followed spectrophotometrically as a function of pH. The decay rate of ferryl-Mb...
was markedly reduced by 450-fold at pH 8.0 compared with that at pH 5.0 (Table IV).

**DISCUSSION**

The mechanistic basis for the pathogenesis of the renal failure of rhabdomyolysis is poorly understood. The results obtained in this study demonstrating striking increases in the urinary excretion of F2-IsoPs in rats with rhabdomyolysis provide compelling evidence that lipid peroxidation is a feature of rhabdomyolysis. A finding of elevated levels of F2-IsoPs in the urine is highly suggestive, albeit not definitive, evidence that this resulted from oxidant injury to the kidney. However, we found that levels of F2-IsoPs esterified in renal lipids of rats with rhabdomyolysis were also markedly increased, which unambiguously localizes the occurrence of oxidant injury in the kidney. Importantly, we also found that alkalinization of the urine, which protects against renal failure, significantly suppresses the urinary excretion of F2-IsoPs, supporting a causative link between oxidant injury and the renal dysfunction.

The molecular mechanisms by which Mb deposited in the kidney induces oxidant injury could occur as a result of the
Mechanism of Rhabdomyolysis-induced Renal Failure

No significant difference was seen in any EPR signal between the rhabdomyolysis kidney and the rhabdomyolysis + alkalinization kidney. The maximum concentration limit of signals that were undetectable in the control kidney was determined by comparing the estimated signal size at different concentrations with the noise in the EPR spectrum. Concentrations were established from the second integrals of pure line shapes of the signals obtained under non-saturating conditions. These second integrals were compared with those of standards at known concentrations.

| Species                         | Control kidney | Rhabdomyolysis kidney | Rhabdomyolysis + alkalinization kidney |
|---------------------------------|----------------|-----------------------|--------------------------------------|
| High spin heme                  | 22 ± 5         | 215 ± 46             | 331 ± 67                              |
| Low spin heme                   | Undetectable   | 60 ± 11              | 71 ± 8a                               |
| Rhombic Fe(III)                 | Undetectable   | (>5)                 |                                       |
| HCO₃⁻-coordinated Fe(III)       | 20 ± 1         | 44 ± 12              | 42 ± 5                                |
| Cytochrome P450                  | 105 ± 1.5      | 74 ± 4a              | 76 ± 11a                              |

*a Value significantly different from the control kidney.

Table III

Effect of pH on the Consumption of 13-HPODE by MetMb and Decay of ferryl-Mb

| pH   | 13-HPODE consumption | k (s⁻¹ × 10⁻³) |
|------|-----------------------|---------------|
| 5.0  | 233 ± 29              | 3200          |
| 6.0  | 87 ± 2                | 310           |
| 6.5  | 20 ± 2                | 100           |
| 7.0  | 9 ± 0.3               | 77            |
| 8.0  | 6 ± 0.5               | 7             |

Table IV

FIG. 6. Effect of pH on MetMb-induced formation of F₂-IsoPs in LDL. Three LDL preparations were incubated with 5 μM MetMb for 24 h at the pH specified. Data are expressed as the n-fold increase over base line.

Release of free iron from Mb or, as suggested in the current study, from a direct pro-oxidant effect of the heme protein itself. In order for Mb to catalyze lipid peroxidation, ferrous Mb must be oxidized to the ferric form, which is competent to induce lipid peroxidation by redox cycling with ferryl-Mb. This is a highly reactive form of Mb, which can potently induce lipid peroxidation (Table I and II). In support of the latter hypothesis, we found that the redox form of Mb deposited in the kidney is MetMb.

Importantly, in addition to explaining the occurrence of renal tubular necrosis, oxidant injury may also provide a plausible explanation for the occurrence of renal vasoconstriction in this disorder, which contributes to the renal dysfunction. The finding that IsoPs are markedly overproduced in myoglobinuria is potential highly relevant in that both 15-F₂t-IsoP and 15-E₂t-IsoP are very potent renal vasoconstrictors (25, 26, 28). Furthermore, 15-F₂t-IsoP has been shown to induce secretion of endothelin-1 (39), which is also a potent renal vasoconstrictor. Perhaps relevant to the latter finding is the recent report that bosentan, a combined Endothelin A and B receptor antagonist, partially protects against renal failure in rhabdomyolysis (40). Thus, although it remains to be proven, it is attractive to speculate that overproduction of IsoPs may be responsible for the intense renal vasoconstriction that occurs in myoglobinuria.

Our findings also provide novel insights into mechanism by which alkalinization of the urine protects against the renal failure of rhabdomyolysis. The accepted explanation is that alkalinization enhances the solubility of Mb, and thus its excretion from the kidney (12). Clearly, this could explain the finding that alkalinization reduced urinary excretion of F₂-IsoPs. However, EPR analysis failed to detect a reduction in the amount of high spin heme (MetMb) deposited in the kidney following treatment with alkali. Thus, an alternative explanation was considered based on the hypothesis for an effect of alkaline pH to reduce the capacity of Mb to catalyze lipid peroxidation. This hypothesis was supported by the finding that Mb-induced formation of F₂-IsoPs in LDL was increasingly suppressed with increasing pH over the range of 5.5–8.0. Further studies undertaken in an attempt to elucidate the mechanism basis for the pH dependence of the capacity of Mb to induce lipid peroxidation revealed that the rate of the ferryl-Mb to ferric Mb transition is strongly pH-dependent, being very low at pH >7.0, indicating stabilization, i.e. reduced reactivity, of ferryl-Mb at alkaline pH. The reason for this pH dependence may reside in the fact that the protonated form of ferryl heme (FeIV=O⁻H⁻) is the reactive species, which is formally equivalent to a hydroxyl radical coordinated to ferric heme. It is important to note that renal tubular fluid is relatively acidic and the pH of the surrounding interstitium decreases further during low blood flow states (41). Thus, MetMb deposited in the renal tubules is in a milieu that greatly promotes the ability of Mb to induce lipid peroxidation.

In summary, a number of important new insights regarding the mechanism of the pathogenesis of the renal failure of rhabdomyolysis have emerged from these studies. First, compelling evidence was obtained for the occurrence of oxidant injury to the kidney in rhabdomyolysis. Second, the observation that IsoP production is increased may explain the occurrence of intense renal vasoconstriction in this disorder. Third, the identification of MetMb as the principal redox form of Mb deposited in the kidney provides a mechanistic basis to explain how myoglobinuria can cause lipid peroxidation independent of free iron and conventional Fenton reactions. Fourth, data obtained suggest that the mechanism by which alkalinization protects against renal failure is not by increasing solubility of Mb as previously proposed, but is consistent with stabilization of the highly reactive ferryl-Mb. These findings may have wider implications to the general functioning of heme proteins during...
changes of intracellular pH and provide a rationale to further explore whether the administration of antioxidants in conjunction with alkaline therapy might have an additive effect in preventing renal injury as suggested by others (8). This would impact significantly on our ability to preserve renal function in patients with rhabdomyolysis.

REFERENCES
1. Bywaters, E. G., and Beall, D. (1941) Br. Med. J. 1, 427–432
2. Better, O. S., and Stein, J. H. (1990) N. Engl. J. Med. 322, 825–829
3. Grossman, R. A., Hamilton, R. W., Morse, B. M., Penn, A. S., and Goldberg, M. (1974) N. Engl. J. Med. 291, 807–811
4. Auer, G., Grandchamp, A., Wyler, T., and Truniger, B. (1971) Circ. Res. 29, 128–135
5. Kurtz, T. W., Maletz, R. M., and Hsu, C. H. (1976) Lab. Invest. 35, 131–145
6. Venkatachalam, M. A., Rennke, H. G., and Sandstrom, D. J. (1976) Circ. Res. 38, 267–279
7. Shah, S. V., and Walker, P. D. (1988) Am. J. Physiol. 255, F438–F443
8. Salahudeen, A. K., Wang, C., Bigler, S. A., Dai, Z., and Tachikawa, H. (1996) Nephrol. Dial. Transplant. 11, 635–642
9. Paller, M. S. (1988) Am. J. Physiol. 255, F539–F544
10. Nath, K. A., Dvergsten, J., Correa-Rotter, R., Hostetter, T. H., Manivel, J. C., and Rosenberg, M. E. (1994) Lab. Invest. 71, 209–218
11. Abu-Ali, S. R., Walker, P. D., and Shah, S. V. (1994) Proc. Natl. Acad. Sci. U. S. A. 88, 9833–9837
12. Zager, R. A. (1989) J. Clin. Invest. 83, 606–612
13. Zager, R. A., and Foerder, C. A. (1992) Kidney Int. 42, 277–287
14. Zager, R. A., and Burkhart, K. (1997) Kidney Int. 51, 728–738
15. Zager, R. A. (1992) J. Clin. Invest. 90, 901–910
16. Patel, R. P., Diczfalusy, U., Dzeletovic, S., Wilson, M. T., and Darley-Usmar, V. M. (1996) J. Lipid Res. 37, 2361–2371
17. Hogg, N., Rice-Evans, C., Darley-Usmar, V., Wilson, M. T., Paganga, G., and Bourne, L. (1994) Arch. Biochem. Biophys. 314, 39–44
18. Patel, R. P., Sestier, R. D., Darley-Usmar, V. M., Symons, M. C., and Wilson, M. T. (1996) Free Radic. Res. 25, 117–123
19. Harel, S., and Kanner, J. (1988) Free Radic. Res. Commun. 3, 21–33
20. Grisham, M. B. (1985) J. Free Radic. Biol. Med. 1, 227–232
21. Turner, J. J., Rice-Evans, C. A., Davies, M. J., and Newman, E. S. (1991) Biochem. J. 277, 833–837
22. Cooper, C. E., Green, E. S., Rice-Evans, C. A., Davies, M. J., and Wrigglesworth, J. M. (1994) Free Radic. Res. 20, 219–227
23. Maree, A., Peer, G., Schwartz, D., Serban, I., Blum, M., Wollman, Y., Cabili, S., and Laina, A. (1994) Nephrol. Dial. Transplant. 9, Suppl. 4, 78–81
24. Dee, G., Rice-Evans, C., Obeyesekera, S., Meraji, S. Jacobs, M., and Bruckdorfer, K. R. (1991) FEBS Lett. 294, 38–42
25. Morrow, J. D., Hill, K. E., Burk, R. F., Namnour, T. M., Badr, K. F., and Roberts, L. J., II (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9383–9387
26. Morrow, J. D., Minton, T. A., Makundan, C. R., Campbell, D. M., Zecket, W. E., Daniel, V. C., Badr, K. F., Blair, I. A., and Roberts, L. J., II (1994) J. Biol. Chem. 269, 4317–4326
27. Taber, D. F., Morrow, J. D., and Roberts, L. J., II (1997) Prostaglandins 53, 63–67
28. Takahashi, K., Namnour, T. M., Fukunaga, M., Ebert, J., Morrow, J. D., Roberts, L. J., II, Hoover, R. L., and Badr, K. F. (1992) J. Clin. Invest. 90, 136–141
29. Fukunaga, M., Makita, N., Roberts, L. J., II, Morrow, J. D., Takahashi, K., and Badr, K. F. (1993) Am. J. Physiol. 264, C1619–C1624
30. Rodrigues-Malaver, A. J., Leake, D. S., and Rice-Evans, C. A. (1997) FEBS Lett. 406, 37–41
31. Roberts, L. J., II, and Morrow, J. D. (1997) Biochim. Biophys. Acta 1353, 121–135
32. Morrow, J. D., Awad, J. A., Bass, H. J., Blair, I. A., and Roberts, L. J., II (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10721–10725
33. Moore, K. P., Darley-Usmar, V., Morrow, J., and Roberts, L. J., II (1995) Circ. Res. 77, 335–341
34. Morrow, J. D., and Roberts, L. J., II (1994) Methods Enzymol. 233, 163–174
35. Cammack, R., and Cooper, C. E. (1992) Methods Enzymol. 227, 353–384
36. Maiorino, M., Ursini, F., and Cadenas, E. (1994) Free Radic. Biol. Med. 16, 661–667
37. Berzofsky, J. A., Peishaj, J., and Blumberg, W. E. (1971) J. Biol. Chem. 246, 3367–3377
38. Catalano, C. E., Choe, Y. S., and Ortiz de Mortellano, P. R. (1989) J. Biol. Chem. 264, 10534–10541
39. Fukunaga, M., Yura, T., and Badr, K. F. (1995) J. Cardiovasc. Pharmacol. 26, Suppl 3, S51–S52
40. Karam, H., Bruneval, P., Clozel, J., and Neuberger, A., eds) pp. 13–52, North-Holland, Amsterdam.