Hydroxyl Radical Participation in the In Vitro Effects of Gram-Negative Endotoxin on Cardiac Sarcolemmal Na,K-ATPase Activity

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ABSTRACT—The effect of in vitro exposure of sarcolemmal membrane (SL) vesicles to Gram-negative endotoxin lipopolysaccharides (LPS) was studied. LPS decreased the Na,K-ATPase activity of SL vesicles; this effect was inhibited by hydroxyl radical (·OH) scavengers such as dimethylthiourea and dimethyl sulfoxide, but not by superoxide dismutase, a scavenger of superoxide anion radicals or by the hydrogen peroxide scavenger catalase. ESR spin-trapping with 5,5-dimethyl-l-pyrroline N-oxide verified the generation of ·OH from LPS itself under the conditions used; ·OH generated from LPS was not affected by deferoxamine, a powerful iron chelator. The Na,K-ATPase activity was reduced by an ·OH radical generating system consisting of dihydroxyfumarate and Fe³⁺-ADP. Furthermore, exposure of SL vesicles to LPS caused an increase in malondialdehyde formation. It can be concluded that LPS damages cardiac SL by an oxygen free radical mechanism by the generation of ·OH, due to inhibition of Na,K-ATPase activity and peroxidation of lipids, and that the effect of LPS is not dependent on the presence of contaminating iron.

Endotoxins are lipopolysaccharides located in the outer membrane of Gram-negative bacteria. Much of the morbidity and mortality associated with Gram-negative infections is thought to be due to release of endotoxin (1). Injection of microgram amounts of endotoxin into susceptible experimental animals produces a number of biological effects. These include multiple hematologic events such as complement activation, disseminated intravascular coagulation, and intestinal bleeding. Injection of larger doses of endotoxin frequently results in shock and death (2).

A current hypothesis for the mechanism underlying the cardiovascular collapse of Gram-negative septic or endotoxin shock begins with the observation of Jacob et al. (3) that endotoxin within the vascular space activates complement and leads to the production of complement components; most importantly, C₅a. C₅a activates polymorphonuclear leukocytes (4) which become adherent to endothelial surfaces and other leukocytes and subsequently release oxygen free radicals, lysosomal hydrolases, and arachidonic acid derivatives. These oxygen free radicals (superoxide anion, ·O₂⁻; hydroxyl radical, ·OH; singlet oxygen, ·O₂; and hydrogen peroxide, H₂O₂) are capable of extensive endothelial cell (5) and cardiac cell damage (6–9) and could potentially produce the loss of contractility in vascular smooth muscle and cardiac muscle which is

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observed in septic shock.

In other tissues, oxygen free radicals produce a similar pattern of injury to that present in the cardiovascular system during Gram-negative sepsis. This pattern includes endothelial cell damage (5), lysis of phospholipid membranes (10), damaged mitochondria (11), lysosomal disruption (12), and increased vascular permeability (13).

Whether the injury is a direct effect of endotoxin on endothelial cells and cardiac cells or whether complement activation and granulocyte sequestration are also involved is not clear. For example, experiments in granulocyte-depleted animals suggest that the increase in vascular permeability in response to endotoxin is attenuated (14), whereas in leukopenic animals (15, 16), it has been shown that endothelial damage still occurs. Additionally, in the animal given a single infusion of complement-activated plasma, only a modest increase in vascular permeability occurs, and the accompanying endothelial damage is transient (17). Thus, neither granulocyte sequestration nor complement activation seems to result in the severe vascular changes seen during endotoxemia, which suggests that endotoxin can cause damage directly, but the effect of endotoxin is species- and site-dependent (18).

The Na,K-adenosine triphosphatase (ATPase) plays a major role in myocardial excitation-contraction coupling. Furthermore, it has been reported that cardiac muscle sarcolemma may be an important target organelle for the oxygen free radical damage (19, 20). We, therefore, designed the following study to gain further insight into the direct effect of purified endotoxin lipopolysaccharides (LPS) on the sarcolemmal Na,K-ATPase activity of cardiac muscle. Attempts were also made to test the hypothesis that an oxygen free radical mechanism contributes to the effect of endotoxin. In this paper, for the first time, we report that the Na,K-ATPase activity is inhibited directly by endotoxin due to oxygen free radical generation from endotoxin itself.

Materials and Methods

Preparation of sarcolemmal membrane (SL)

The SL was isolated by the method of Jones (21). Briefly, healthy, adult, filaria-free dogs were anesthetized with sodium pentobarbital (25 mg/kg, i.v.), and the heart was excised rapidly and placed in ice-cold 0.9% NaCl. The free wall of the left ventricle was washed, minced, and then homogenized in 10 mM histidine, 0.75 M NaCl with a polytron PT-20 (5 sec, setting 5). The resulting homogenate was centrifuged at 14,000 x g for 20 min, and the supernatant was discarded. The pellet was resuspended in 10 mM histidine and 0.75 M NaCl. The sample was homogenized and centrifuged a second time as described above. The supernatant was again discarded, and the pellet was resuspended in 10 mM NaHCO₃ and 5 mM histidine. The sample was homogenized and centrifuged a third time as described above, and the supernatant was again discarded. The pellet obtained from the third centrifugation was resuspended in 10 mM NaHCO₃ and 5 mM histidine and then homogenized three times for 30 sec with the polytron PT-20 (setting 5). The sample was next sedimented at 14,000 x g for 20 min, and the supernatant was centrifuged for 30 min at 44,000 x g. The resultant pellet was resuspended in ice-cold deionized water, and 2.0 M sucrose solution containing 300 mM NaCl, 50 mM tetrasodium pyrophosphate and 100 mM Tris-HCl was next added. The solution containing 0.6 M sucrose, 50 mM tetrasodium pyrophosphate and 100 mM Tris-HCl was layered over the membrane suspension, and then 0.25 M sucrose solution was layered on top of the 0.6 M sucrose. After centrifugation at 370,000 x g for 60 min, the band at the 0.25 M/0.6 M sucrose interface was taken and diluted with three volumes of ice-cold deionized water. A final centrifugation at 250,000 x g for 30 min resulted in a pellet rich in SL. This pellet was resuspended in 0.25 M sucrose and 10 mM histidine and then stored at −80°C until used. Protein was determined by the method of Lowry et al. (22).
**Determination of Na,K-ATPase activity**

Ouabain sensitive Na,K-ATPase activity was monitored in duplicate reaction baths containing 5 mM MgCl₂, 100 mM NaCl, 10 mM KCl, 25 mM Tris-HCl (pH 7.4) and 2.5 mM Tris-ATP with or without 1 mM ouabain (Merck) using a coupled enzyme system consisting of phosphoenolpyruvate (Sigma), pyruvate kinase (Sigma), L-lactic dehydrogenase (Sigma) and reduced nicotinamide adenine dinucleotide (NADH, Sigma), which was previously described by Schwartz et al. (23). The ATPase activity was calculated from the rate of decrease of absorbance at 340 nm. The activity in the presence of 1 mM ouabain was 7.32 ± 0.46 μmol Pi/mg-hr (mean ± S.E.M., n = 12) in our system.

**Endotoxin LPS preparations**

*Escherichia coli* 0127:B8 prepared by the Westphal method was obtained from Difco Laboratories, Detroit. LPS preparations were maintained at 4°C.

**Spin trapping and electron spin resonance (ESR) spectrometry**

The spin trapping agent that has been most often used to detect oxygen-centered free radicals has been 5,5-dimethyl-1-pyrroline N-oxide (DMPO) due in part to both the relative efficiency with which DMPO is capable of trapping ′O₂ and ′OH as well as the stability of the resulting spin adducts (24–26). Therefore, the ESR spin trapping studies were performed using DMPO (Mitsui Toatsu Chemicals, 99–100% pure) as the spin trap. The desired reaction mixtures (0.2 ml) were prepared in glass tubes and transferred to a flat quartz ESR cuvette (0.3 mm thickness), which was in turn fixed to the cavity of the ESR spectrometer (JEOL JES FE-1X with 100-kHz field modulation, X-band). Sequential ESR scans were started 45 sec after the addition of endotoxin to DMPO-containing reaction mixtures. The ESR spectra of DMPO-OH, the spin-trapped adduct of ′OH, was identified from the hyperfine parameters. ESR spectrometer settings were modulation amplitude, 0.1 mT (100 kHz); recording range, 10 mT; recording time, 2 min; time constant, 0.1 sec; microwave power, 8 mW; magnetic field, 334.9 ± 5 mT; at room temperature.

**Measurement of lipid peroxidation**

The incubation mixture contained 0.1 mg of SL vesicles per ml, 0–0.5 mg/ml of endotoxin, 0.12 M KCl, 0.05 M sucrose and 0.01 M potassium phosphate (pH 7.4, at 22°C). Reactions were initiated by the final addition of endotoxin at 37°C.

The rates of lipid peroxidation were measured by formation of thiobarbituric acid-reactive material (27) for 45 min. After incubation, the reaction was stopped by the addition of 0.1 ml of 5% (w/v) trichloroacetic acid. One milliliter of 0.5% (w/v) 2-thiobarbituric acid was then added, and the mixture was heated at 80°C for 30 min. After cooling, 1 ml of 70% (w/v) trichloroacetic acid followed by 3 ml of chloroform were added to each sample tube. The samples were vortexed to disperse lipids and centrifuged. The accumulation of thiobarbituric acid-reactive products was estimated at 535 nm. The absorbance was standardized against the acid hydrolyzed malondialdehyde (dimethylacetyl) over the range of 0.5 to 20 nmol to give malondialdehyde equivalence in a similar reaction mixture. The assay is nonspecific and gives a general indication of thiobarbituric acid-reactive products.

**Determination of superoxide dismutase (SOD)-like activity of SL vesicles**

′O₂ was generated by the xanthine oxidase (XOD, from cow milk, 1.0 U/mg protein, Boehringer Mannheim) reaction acting on hypoxanthine (HPX, Sigma) as a substrate. It has been shown that XOD generates ′O₂ according to the reaction (28):  

\[
\text{HPX} \xrightarrow{\text{XOD}} \text{xanthine} + \prime\text{O}_2 \quad \xrightarrow{\text{XOD}} \text{urate} + \prime\text{O}_2
\]
\( \cdot \text{O}_2^\cdot \) produces \( \text{H}_2\text{O}_2 \) according to the dismutation reaction:

\[
\cdot \text{O}_2^\cdot + \cdot \text{O}_2^\cdot + 2\text{H}^+ \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 + \text{O}_2
\]

This reaction can proceed spontaneously or it can be catalyzed by SOD.

The sampling procedure was as follows: One hundred millimolar sodium phosphate buffer solution (pH 7.8) was used as the solvent. A solution of 2.0 mM HPX, 5.5 mM diethylenetriamine-N,N',N",N'-pentaaacetic acid (DETAPAC, Wako Pure Chemical), a chelator for trace metal impurities, desired concentrations of SL vesicles, and 0.33 U/ml XOD was prepared before use. The XOD solution was stored in an ice bath so as to prevent any inactivation of enzyme. Fifty microliters of HPX, 35 \( \mu l \) of DETAPAC, various concentrations of standard SOD (from bovine erythrocyte, Boehringer Mannheim), 50 \( \mu l \) of prepared SL vesicles of solvent, and 15 \( \mu l \) of DMPO were put into a test tube. To the mixed solution, 50 \( \mu l \) of XOD was added. After quick stirring, 200 \( \mu l \) of the mixture was taken into a flat cell. The mixture contained 0.5 mM HPX, 0.96 mM DETAPAC, 0.69 M DMPO, 0.083 U/ml XOD and an adequate concentration of the SL protein.

A quantitative analysis of DMPO-OOH, the spin-trapped adduct of \( \cdot \text{O}_2^\cdot \), by ESR spectrometry was performed under the following conditions for obtaining high reproducibility of the spin adduct yield: Recording for the ESR spectrum was started 40 sec after the addition of XOD. The recording rate was 5 mT/min. After recording, the signal intensity of the lowest field peak of the spectrum (about 85 sec after the addition of XOD) was normalized as a relative height against the standard signal intensity of the manganese oxide marker (MnO). An absolute concentration of DMPO-OOH was finally determined by double-integration of the ESR spectrum according to the previously reported method (29, 30).

**Oxygen free radical generating system**

To compare with the effect of endotoxin, an in vitro oxygen free radical generating system consisting of 0.66 mM dihydroxyfumarate (DHF) and \( \text{Fe}^{3+} \)-adenosine 5'-diphosphate (ADP) (2.2 \( \mu M \) FeCl$_3$ plus 80 \( \mu M \) ADP) was chosen as a chemical source of \( \cdot \text{O}_2^\cdot \), which then form additional reactive intermediates such as \( \text{H}_2\text{O}_2 \), \( \cdot \text{OH} \), or \( \cdot \text{O}_2 \) by the Haber-Weiss or Fenton reactions. Furthermore, our previous study (31) demonstrated that the ESR spectrum of DHF in the presence \( \text{Fe}^{3+} \)-ADP reveals an \( \cdot \text{OH} \) signal which is quenched by \( \cdot \text{OH} \) radical scavengers. Therefore, our DHF plus \( \text{Fe}^{3+} \)-ADP system is a valid means for assessing the effect of \( \cdot \text{OH} \). The timed sequence of reagent addition is described under “Results”. All materials were reagent-grade unless otherwise indicated.

**Radical scavengers used**

Superoxide dismutase (from bovine blood, 2,800 U/mg protein), except that used for determining SOD-like activity of SL vesicles, was obtained from Sigma Chemicals; catalase (from bovine liver, 3,400 U/mg protein) was also obtained from Sigma Chemicals. We used dimethylthiourea (Aldrich) and dimethyl sulfoxide (Wako Pure Chemical) to scavenge \( \cdot \text{OH} \). Deferoxamine (Desferal mesylate) was a product of Ciba-Geigy.

**Statistical analyses**

The statistical tests of significance used were one-way analysis of variance and the Dunnett's multiple range test. A significance level of \( P > 0.05 \) was used to reject the null hypothesis.

**RESULTS**

Figure 1 demonstrates the concentration dependency of LPS inhibition of the Na,K-ATPase activity of the SL vesicles. Increasing the concentration of LPS from zero to 0.25 mg/ml caused a linear depression of the
Fig. 1. Concentration-response effect of LPS on Na,K-ATPase activity of cardiac sarcolemma. Sarcolemmal vesicles were incubated at 37°C with LPS for 1.5 min before initiation of the reaction by the addition of substrate (ATP). Na,K-ATPase activity was calculated as the difference between the ATPase activity in the presence and the absence of 1 mM ouabain. Each point represents the mean (n = 4-7), and vertical lines show S.E.M. *Significantly (P < 0.05) different from the control.

ATPase activity. At LPS concentrations ranging from 0.25 to 1.0 mg/ml, a significant decrease in the ATPase activity was observed. The data obtained between 0.25 and 1.0 mg/ml indicated no further changes in the ATPase activity.

It is well-known that biological membranes contain relatively large amounts of polyunsaturated fatty acids (PUFA’s), which are vulnerable to oxidative attack. With regard to this, there is the possibility that LPS may directly modify the Na,K-ATPase activity of SL membrane vesicles through a non-cellular, oxygen free radical mechanism by initiating lipid peroxidation (32). To determine the relationship between LPS inhibition of the ATPase activity and lipid peroxidation of SL vesicles, we investigated the effect of LPS on lipid peroxidation. The results of this experiment are shown in Fig. 2. LPS produced an increase in malondialdehyde formation, in a concentration-dependent manner. The inhibition of the ATPase activity and the stimulation of lipid peroxidation caused by LPS, therefore, did correlate well in our experimental system.

It appeared from the results described above that LPS depressed the Na,K-ATPase activity of SL vesicles in part by a mechanism dependent on oxygen free radicals. To describe further the effect of LPS, the ATPase activity was measured in the presence or absence of radical scavengers. Table 1 shows the results of this study. SOD, a scavenger of O₂, slightly, but not significantly, inhibited LPS-induced decrease in Na,K-ATPase activity; the decreased ATPase activity induced by LPS was not affected by the H₂O₂ scavenger.

![Figure 2: Lipid peroxidation of sarcolemmal vesicles by LPS. Sarcolemmal vesicles (0.1 mg of protein per ml) were incubated with LPS in phosphate buffer containing 0.12 M KCl, 0.05 M sucrose at 37°C for 45 min. Malondialdehyde was measured immediately after incubation as described under “Methods.” The data points are the mean of triplicate determinations.](image)

Table 1. Effect of LPS on Na,K-ATPase activity of cardiac sarcolemma and effects of radical scavengers

| Agent used | Na,K-ATPase activity (µmol Pi/mg-hr) |
|------------|-------------------------------------|
| None       | 20.07 ± 1.72                        |
| LPS        | 13.99 ± 1.75*                       |
| SOD        | 21.33 ± 1.60                        |
| CAT        | 19.75 ± 0.82                        |
| SOD plus LPS | 15.87 ± 1.22                        |
| CAT plus LPS | 14.21 ± 0.97                        |

Sarcolemmal vesicles were incubated at 37°C with or without LPS (0.5 mg/ml) for 1.0 min before initiation of the reaction by the addition of substrate (ATP). Superoxide dismutase (SOD, 20 µg/ml) or catalase (CAT, 10 µg/ml) was added before the addition of sarcolemmal vesicles. All values are means ± S.E.M. (n = 6). *Significantly (P < 0.05) different from none-control.
catalase, suggesting that $\cdot O_2$ or $H_2O_2$ is not directly involved in the effect of LPS.

The identification of the oxygen free radicals that are responsible for the effect of LPS rests entirely on the use of radical scavengers. Therefore, the spin-trapped adduct(s) produced by LPS under the conditions in the presence or absence of SL vesicles were examined, by using a highly sensitive ESR spectroscopy and the spin-trap DMPO. The 1:2:2:1 quartet (the hyperfine splittings were $A_N = A_H = 1.49$ mT), characteristic of DMPO-OH (33), was observed under the conditions where 1 mg/ml LPS alone was incubated (Fig. 3, A), and its intensity decreased by increasing SL proteins added (Fig. 3, B–F).

A strong possibility exists for the formation of $\cdot OH$ via the Fenton and Haber-Weiss reactions, due to contaminating iron salts as shown below:

\[
\begin{align*}
\cdot O_2 + Fe^{3+} &\rightarrow O_2 + Fe^{2+} \\
Fe^{2+} + H_2O_2 &\rightarrow Fe^{3+} + \cdot OH
\end{align*}
\]

If contaminant iron-dependent $\cdot OH$ radical formation were involved in LPS-induced production of the DMPO-OH signal, then it should be altered by the iron chelator deferoxamine. We examined the effect of deferoxamine. Deferoxamine at concentrations of 0.1 and 1.0 mM had a very little protective effect on this system (Fig. 4).

The data of Fig. 5 document further the effect of LPS on the Na,K-ATPase activity of SL vesicles in the presence of $\cdot OH$ radical scavengers. $\cdot OH$ radical scavengers such as dimethylthiourea (DMTU) and dimethyl sulfoxide (DMSO) effectively protected against the decrease in Na,K-ATPase activity caused by LPS.

Having established that LPS may be able to depress the Na,K-ATPase activity of SL vesicles at least in part by an oxygen free radical mechanism by contaminant iron-independent generation of $\cdot OH$, we next turned our attention to the effect of an in vitro $\cdot OH$ radical generating system consisting of DHF and $Fe^{3+}$-ADP (31) on the Na,K-ATPase activity of SL vesicles. Figure 6 shows the comparable experiment to the effect of LPS. When SL was incubated with either DHF or $Fe^{3+}$-ADP, the ATPase activity was slightly, but not significantly, decreased. The combination of DHF
Fig. 5. Effect of *OH radical scavengers on the LPS-induced changes in Na,K-ATPase activity of cardiac sarcolemma. Sarcolemmal vesicles were incubated at 37°C with or without LPS (0.5 mg/ml) for 1.0 min before initiation of the reaction by the addition of substrate (ATP). The timed sequence of addition of dimethylthiourea (DMTU, 30 mM) or dimethyl sulfoxide (DMSO, 150 mM) was the same as that of SOD or CAT described in Table 1. Each column represents the mean (n = 3-5) and the error bar, ± S.E.M. *Significantly (P < 0.05) different from the corresponding value for the none-control; tsignificantly (P < 0.05) different from LPS alone.

plus Fe³⁺-ADP produced a large significant inhibition of the ATPase activity; this inhibition was protected against by SOD or SOD plus catalase. Catalase alone had little effect. However, deferoxamine, a powerful iron chelator, protected against the effect elicited by the combination of DHF plus Fe³⁺-ADP. This set of observations suggests that *OH radicals formed via the Fenton and Haber-Weiss reactions can depress the Na,K-ATPase activity of SL vesicles, and that *O₂⁻, which can subsequently result in the production of H₂O₂ and *OH, can be regulated by SOD treatment, thereby decreasing the production of the *OH in our experimental system.

The combination of DHF plus Fe³⁺-ADP or LPS alone was without effect on the ATPase activity in the presence of 1 mM ouabain (data not shown), suggesting that the Na,K-ATPase of the SL vesicles is sensitive to oxygen free radicals or LPS.

One concern raised is whether SOD-like activity is present in isolated SL vesicles, thus inhibiting the *OH radical formation (Fig. 3) due to prevention of *O₂⁻-dependent reduc-

Fig. 6. Oxygen free radical-mediated inhibition of the Na,K-ATPase activity of cardiac sarcolemma and effects of radical scavengers and iron chelator. Reaction conditions were as described under "Methods"; and when added, 0.66 mM dihydroxyfumarate (DHF), 2.2 μM FeCl₃ plus 80 μM ADP (Fe³⁺-ADP), 15 μg/ml superoxide dismutase (SOD), 15 μg/ml catalase (CAT), 1.0 mM deferoxamine (DFX). The timed sequence of additions was designed to ensure exposure of sarcolemmal vesicles to oxygen free radicals generated from the complete system (DHF plus Fe³⁺-ADP) for 10 min before initiation of the reaction by the addition of substrate (ATP). The radical scavengers and iron chelator used were added before the exposure of sarcolemmal vesicles to the oxygen free radical generating system. Each column represents the mean (n = 4-6) and the error bar, ± S.E.M. *Significantly (P < 0.01) different from DHF alone; tsignificantly (P < 0.01) different from DHF plus Fe³⁺-ADP.

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DISCUSSION

Membrane components of cells consist of phospholipid bilayers and intercalated proteins. They play a pivotal role not only in the physiological regulation of cell function but also in the pathogenesis of a number of disease states (36). Katz and Messineo (36) indicated that the functioning of the cell is
affected profoundly by alterations in the lipid microenvironment of the cell membranes. Furthermore, the polyunsaturated fatty acid moiety of membrane lipids is readily oxidized by oxygen free radicals. Therefore, it is possible that oxygen free radicals affect the following: 1) membrane lipids, the peroxidation of which may secondarily modify the membrane protein (37); 2) exclusively the lipids, which alter the microenvironment of the enzyme protein (36); or 3) both enzyme protein and membrane lipids independently and concurrently (38).

The effects of Gram-negative endotoxin LPS on myocardial function can be divided into two subcategories: one dealing with a holistic effect on the heart and its subcellular components and the other, dealing with the direct effects of LPS on the myocardium. In order to understand the direct effects of LPS better, we have directed our attention to cellular derangements which may occur as a result of in vitro exposure to LPS. Toward this objective, we have chosen a model membrane preparation containing Na,K-ATPase because 1) the enzyme protein is the predominant component of the membrane fraction prepared from the myocardium (21); 2) the enzyme requires the lipid domain for its functioning (39), which makes it suitable for the study of interactions between the enzyme protein and membrane phospholipids; 3) its characteristics are relatively well-known (39); and 4) cardiac SL is more vulnerable to oxygen free radical attack (19), due to its substantially higher content of unsaturated fatty acids in comparison to the microsomal system.

The present study demonstrates that LPS, due to its ability to generate 'OH or a closely related species of oxygen free radicals, can depress the Na,K-ATPase activity of SL membrane vesicles, and that the mechanism by which LPS produces oxygen free radicals is not contaminant iron-dependent. This postulation is inferred from the following significant observations: 1) LPS decreased Na,K-ATPase activity (Fig. 1), and this effect was inhibited by 'OH radical scavengers (Fig. 5), but not by SOD or catalase alone (Table 1); 2) using spin trapping ESR spectrometry, the species of oxygen free radicals generated from LPS was identified as 'OH (Fig. 3), and the ESR spectra of DMPO-OH, the spin-trapped adduct of 'OH produced by LPS was not affected by the iron chelator deferoxamine (Fig. 4); and 3) the Na,K-ATPase activity was reduced by an in vitro 'OH radical generating system (Fig. 6). Evaluation of the role of 'OH in tissue injury has been hindered by the fact that there are no known enzymatic scavengers of this radical, and the chemical scavengers available (e.g., DMSO, mannitol, ethanol) may lack potency or specificity (40). Recently, Fox et al. (40, 41) have described the 'OH-scavenging properties of DMTU, a compound that is more effective than traditional 'OH scavengers and is highly cell-permeable. We therefore used DMTU as well as DMSO as a probe to explore the role of the 'OH in the experiment shown in Fig. 5, in which DMTU effectively protected against the decrease in Na,K-ATPase activity caused by LPS. It is reported that DMTU failed to affect O2 production by neutrophils and reacted only very slowly with H2O2 (estimated rate constant $\leq 0.1 \text{M}^{-1}\text{sec}^{-1}$) (40). DMTU has also been reported to react with H2O2 in another system (42), but because of the extremely low rate constant, it is unlikely that removal of H2O2 is a major mechanism for the protective effects of this agent. Taken together with these observations, our data are consistent with the view that the ability of LPS to reduce the Na,K-ATPase activity is not primarily dependent on 'O2 and the Fenton reaction.

The decreased intensity caused by the addition of SL proteins shown in Fig. 3 might be consistent with the view that 'OH generated from constituents of LPS preparations is trapped by SL membrane lipids for peroxidation, thus reducing the DMPO-OH signal. This is reflected by the increased formation of malondialdehyde (Fig. 2). Why LPS does not produce a concentration-dependent decrease in Na,K-ATPase activity at concentrations rang-
ing from 0.25 to 1.0 mg/ml (Fig. 1) is unclear. One possible explanation is that the low-sensitivity component of Na,K-ATPase may be responsible for the refractoriness to high concentrations of LPS.

In the DHF plus Fe\(^{3+}\)-ADP system, SOD appeared to eliminate \(·\)OH either due to scavenging of \(O_2^1\) or an inhibitory effect on autooxidation of DHF and preventing the reduction of Fe\(^{3+}\)-ADP (ferriic iron) to Fe\(^{2+}\)-ADP (ferrous iron). Therefore, \(·\)OH may be involved in decreased Na,K-ATPase activity induced by DHF plus Fe\(^{3+}\)-ADP (Fig. 6). In contrast to the effect of SOD, catalase alone had no protective effect (Fig. 6), which is probably due to the non-accessibility of catalase to the active site of the Na,K-ATPase enzyme because of its large molecular weight. Because of their much smaller molecular size, DHF and Fe\(^{3+}\)-ADP freely reach the active site, generate \(·\)OH, and thus inhibit the enzyme. In the case of deferoxamine, which is a powerful iron chelator (43), the conclusion seems safe that protection of the observed effect (Fig. 6) can be interpreted as indicative that contaminant iron-dependent formation of \(·\)OH is involved.

Singlet oxygen (\(¹O_2\)) is also formed during the complex degradation reactions of lipid peroxidation and might contribute to the chain reaction by causing further initiation of the peroxidation. It has been shown that \(·\)OH radicals also react with \(¹O_2\) quenchers with a rate constant similar to \(¹O_2\) (44). However, due to limited evidence, it is difficult to interpret the role of \(¹O_2\) in the present studies.

Our findings led us to conclude that LPS damages cardiac SL by an oxygen free radical mechanism by the generation of \(·\)OH due to inhibition of Na,K-ATPase activity and peroxidation of lipids. It is also postulated that both protein and lipid moieties of Na,K-ATPase are affected by \(·\)OH. Although we have been able to demonstrate a correlation between lipid peroxidation and enzyme inhibition, the data do not preclude the possibility of direct oxygen free radical-mediated inactivation of membrane protein (45). Enzymes with aromatic or sulfur-containing amino acids may be susceptible to oxygen free radical-induced amino acid modification or crosslinking. This aspect of oxygen free radical membrane injury induced by LPS requires further documentation.

Clearly, our results raise the question of what is the LPS-mediated molecular biochemical reaction sequence leading to the Haber-Weiss or Fenton reaction-independent generation of \(·\)OH radical. There appears to be no obvious answer, and no consideration has been given to this problem as far as we can tell from the current literature. In the present studies, however, we have provided direct in vitro evidence of the role of \(·\)OH radicals in damaging Na,K-ATPase and lipids of SL membrane vesicles, which could be one of the important mechanisms of LPS-mediated myocardial injury.

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