Rickettsia rickettsii Induces Superoxide Radical and Superoxide Dismutase in Human Endothelial Cells

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Human endothelial cells infected with Rickettsia rickettsii, the etiological agent of Rocky Mountain spotted fever, undergo striking morphological changes to the endoplasmic reticulum outer nuclear envelope complex. These changes are accompanied by concurrent accumulation of intracellular peroxides. Both of these findings are consistent with the notion that cells undergo some form of oxidative stress. Since oxidant injury is often initiated or mediated through oxygen radicals, we examined superoxide radical generation when endothelial cells were exposed to R. rickettsii. We also examined the levels of superoxide dismutase, an enzyme induced in response to increased superoxide formation. The levels of both superoxide and superoxide dismutase increased when endothelial cells were exposed to R. rickettsii. These results, together with our previous findings, support our hypothesis that cells infected by this intracellular bacterium experience oxidant-mediated injury that may eventually contribute to cell death.

Previous studies in our laboratory have provided both morphological (19) and biochemical (21) evidence to suggest that cells infected by Rickettsia rickettsii, the etiological agent of Rocky Mountain spotted fever, injure human umbilical vein endothelial cells (HUVEC) by a mechanism that may be unique to an obligate intracellular bacterial parasite and, perhaps, to bacteria in general. Studies done in our laboratory by using transmission electron microscopy (19, 23) have shown striking changes to the organizational arrangement of internal membranes of cells infected with this organism. These changes, which are obvious in most cells by 48 h after infection with R. rickettsii, consist of widespread dilatation of the rough-surfaced endoplasmic reticulum outer nuclear envelope complex, resulting in formation of large intracellular cisternae. Recent studies in our laboratory also have shown that these morphological changes are accompanied by increased accumulation of intracellular peroxides (21). Both the morphological changes and increased peroxide levels are consistent with cell injury potentially due to oxidative stress. Furthermore, cellular thiol levels which play an important role in defense against free-radical-induced cell injury are significantly decreased in cells infected with R. rickettsii (22). As a result of these findings, we have proposed that endothelial cells infected with this organism may be injured by peroxidation of membrane lipids and that this may be an important pathogenic mechanism in Rocky Mountain spotted fever (21, 22). Several indicators which measure the potential for oxidative stress-related injury may be examined both in tissue and in cell culture systems. In the study reported here, which used human umbilical vein endothelial cells, we examined the kinetics of induction of superoxide dismutase (SOD) and generation of the superoxide radical (O2−) in cells exposed to R. rickettsii. SOD catalyzes dismutation of the superoxide radical by the following reaction:

\[
\text{SOD} \quad 2\text{O}_2^- + 2\text{H}^+ \longrightarrow \text{O}_2 + \text{H}_2\text{O}_2
\]  

This enzyme is present in cells to protect them from the toxic effects of superoxide (6) which is generated during normal cellular metabolism via either enzymatic reactions or autoxidation of endogenous compounds (5) and either directly or indirectly may initiate peroxidation of membrane lipids. Some cell lines normally respond to increased superoxide radical levels by producing larger quantities of superoxide dismutase to neutralize potentially injurious levels of the radical and thus prevent oxidative injury (6, 12).

While the deleterious effects of the superoxide radical itself have been questioned (1), this anion can be protonated to HO2 (perhydroxyl radical), which is a strong oxidant and can directly attack polyunsaturated fatty acids (3). In addition, oxidative injury can be initiated through dismutation of the superoxide radical with formation of hydrogen peroxide, which itself is a potent oxidant and is also capable of reacting with additional superoxide radicals to form the highly reactive hydroxyl radical (\(\cdot\text{OH}\)), a potent initiator of membrane lipid peroxidation (10):

\[
\text{Fe} \quad \text{O}_2^- + \text{H}_2\text{O}_2 \longrightarrow \text{OH} + \text{OH}^- + \text{O}_2
\]  

Here we report that superoxide dismutase and the superoxide radical levels increase following exposure of human endothelial cells to R. rickettsii. These data contribute further evidence in support of our hypothesis that cell injury caused by R. rickettsii may be mediated by oxidative stress.

MATERIALS AND METHODS

Endothelial cells. HUVEC were isolated by the method of Gimbrone (8) as previously described (21). The cells were removed from the umbilical vein by using a 0.1% type A collagenase solution (Boehringer Mannheim Corp., Indianapolis, Ind.) prepared in phosphate-buffered saline. Pooled HUVEC were grown at 37°C in 60-mm-diameter Nunclon culture dishes (Nunc, Kamstrup, Denmark) in McCoy’s 5A medium (GIBCO, Grand Island, N.Y.) containing 20% heat-inactivated fetal bovine serum (Upstate Biotechnology, Inc., Saranac Lake, N.Y.), 30 μg of H-Neurext endothelial cell growth supplement (Upstate Biotechnology), and 50 μg of

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sodium heparin (Sigma Chemical Co., St. Louis, Mo.) per ml. The cells were cultured at 37°C in a humidified atmosphere of 5% CO2-95% air. First- or second-passage cells were used in all experiments.

*Rickettsia.* A plaque-purified isolate of the Sheila Smith strain of *R. rickettsii,* propagated in C1008 Vero cells (American Type Culture Collection, Rockville, Md.), was used to infect confluent monolayers of endothelial cells. Dilutions of the original seed stock which contained 10^7 PFU of the organism per ml were used with approximate infecting doses ranging from 0.25 to 6 rickettsiae per endothelial cell. The rickettsial inoculum was added to the endothelial cells in McCoy’s medium, incubated for 1 or 2 h, and removed, and fresh culture medium was added to the cells. To monitor the infection of endothelial cells, coverslips were added to some of the 60-mm dishes, removed at the appropriate intervals, and stained by the method of Gimenez (9). At least 250 cells were counted for each time and condition.

**Measurement of SOD.** SOD activity in HUVEC was determined by the method of Oberley and Spitz (14). Reaction buffer contained final concentrations of 0.1 mM xanthine (prepared fresh weekly), 5.6 x 10^-5 M nitroblue tetrazolium (stored in a brown bottle), 1 mM diethylenetriamine-pentaacetic acid, and 1 U of catalase per ml, all prepared in 0.05 M phosphate buffer (pH 7.8). Blanks (minimum of five) contained 900 µl of reaction buffer and 100 µl of 0.05 M phosphate buffer. One-hundred-microliter quantities of several dilutions of xanthine oxidase, 1.2 U/mg of protein (in phosphate buffer containing 1.33 mM diethylenetriamine-pentaacetic acid), were added to the blanks to determine the concentrations that caused a change in the absorbance rate of between 0.015 and 0.025 optical density unit per min at 560 nm. The rate of change in absorbance was recorded at 1-min intervals for 10 min on a Beckman DU 64 spectrophotometer. Once a satisfactory concentration of xanthine oxidase was determined, this dilution of xanthine oxidase was used for all of the standards and samples in the assay. To prepare the samples for SOD determination, the cells were trypsinnized from the culture dishes, washed once by centrifugation to remove trypsin, and suspended in 0.5 ml of potassium phosphate buffer (0.05 M, pH 7.8). Cell extracts were prepared by freeze-thawing the cells twice on dry ice or by sonicating the cells for 1 min by using four 15-s bursts in a model 450 Sonifier (Branson Instruments, Danbury, Conn.) and then centrifuging the lysates for 10 min at 11,000 x g in an Eppendorf 5413 microcentrifuge (Brinkmann Instruments, Westbury, N.Y.).

A standard curve of SOD activity was prepared by using SOD (1 to 1,000 ng/100 µl) from bovine kidney at 10,000 U/mg (Sigma). All chemicals for the SOD assay were purchased from Sigma. Protein determinations were made on an aliquot of each lysate by the method of Smith et al. (24). SOD activity was expressed as percent inhibition versus protein concentration, with percent inhibition determined as follows: ( [slope without SOD - slope with SOD]/slope without SOD) x 100%. One unit of SOD activity is defined as that amount of protein which gives half-maximal inhibition.

In some experiments to determine whether SOD was activated by infection or synthesized de novo, cycloheximide (Sigma) was added to the cell cultures at a concentration of 10 µg/ml 1 h prior to infection by *R. rickettsii,* allowed to remain for the 2-h period of incubation of the organisms with the cells, and added again to the fresh medium after the inoculum was removed. *R. rickettsii* growth studies were done in the presence of cycloheximide to determine whether it had any inhibitory effects on replication of the organism.

To determine whether SOD activity might have been contributed by disrupted intracellular rickettsiae, *R. rickettsii* organisms purified on Renografin density gradients (11) were sonicated in 0.5 ml of 0.05 M phosphate buffer under the same conditions as infected HUVEC. SOD and protein determinations were performed on these samples as described above.

**Superoxide radical measurement.** Superoxide radical concentrations were measured in the supernatant fractions of uninfected and *R. rickettsii*-infected HUVEC by the method of Winterbourn and Stern (26) by monitoring the reduction of ferricytochrome c to ferrocytochrome c spectrophotometrically at 550 nm in a Beckman DU 64 spectrophotometer. Superoxide formation was calculated by measuring the rate of change in absorbance at 1-min intervals by using an extinction coefficient for ferrocytochrome c of 27.7 x 10^3 M^-1 cm^-1 (2). To determine the amount of superoxide produced at the end of 1 h after exposure to rickettsiae, 200-µl samples of culture medium were removed from each dish and centrifuged at 11,000 x g for 3 min to remove cell debris. These supernatants were combined with 600 µl of 75 µM ferricytochrome c (Sigma) and brought to a total volume of 1 ml with 200 µl of phosphate-buffered saline. To evaluate the amount of inhibition of oxidation of ferrocytochrome c by SOD, an additional 200 µl of culture medium was removed from each dish and 100 µl of SOD (5.3 x 10^5 U) was added prior to addition of ferricytochrome c. After addition of ferricytochrome c, the reaction was monitored spectrophotometrically for 15 min. The amount of superoxide in the rickettsial seed was determined by adding 0.5 ml of the seed material (the number of *R. rickettsii* organisms used to achieve a ratio of six microorganisms per cell) to 1.5 ml of culture medium, incubating it for 1 h at 37°C, and testing 200-µl aliquots for the superoxide radical. Superoxide from the culture medium itself was determined by testing aliquots under the conditions described above but without exposure to any cells or rickettsiae. Following measurement of superoxide levels, the cells were trypsinnized from the surface of the culture dishes and total cell counts per dish were determined with a hemacytometer.

In some experiments, either cycloheximide (1 µg/ml) or cytochalasin D (0.5 µg/ml) was used to pretreat the endothelial cells prior to infection with *R. rickettsii* to examine their effects on superoxide radical generation. These treatments were done for 30 min at 37°C.

**RESULTS**

Endothelial cells infected with *R. rickettsii* showed a significant increase in SOD activity compared with uninfected controls (Fig. 1). An increase in enzyme activity in infected cells was evident as early as 2 h after exposure of the cells to the organism (time zero on the graph). Six hours later, SOD activity in the infected cell population was 45% higher than in the uninfected cells. The percent increase in activity in infected cells plateaued at 6 h and remained elevated through 48 h, when the experiments were terminated. To determine whether SOD was activated by the infection process or synthesized de novo, cycloheximide was introduced into the system and SOD activity was monitored for 48 h after infection. The results of these experiments (Fig. 2) demonstrate that cycloheximide is effective in preventing de novo synthesis of the enzyme. At 24 h in uninfected HUVEC, the levels of SOD were rela-
FIG. 1. Percent increase in SOD activity in human endothelial cells following infection with *R. rickettsii*. Enzyme activity was measured at 0, 6, 24, and 48 h postinfection and is expressed as the percent increase in SOD activity in infected cells compared with that in uninfected controls. The data presented represent mean values ± the standard errors of at least four separate experiments at each time point.

Initially low and there was no significant difference between cells that were treated with cycloheximide and those that were not treated. At 48 h in uninfected HUVEC, SOD activity was elevated but this increase could be suppressed by addition of cycloheximide. At 24 and 48 h after infection by *R. rickettsii*, SOD levels rose about threefold compared with uninfected HUVEC controls. These increases, however, could be prevented by addition of cycloheximide. When cycloheximide was added to HUVEC cultures 1 h prior to infection and then again to the fresh culture medium after the inoculum was removed, the levels of SOD at 24 and 48 h postinfection were significantly reduced and similar to those measured at 48 h in uninfected, untreated cells.

A growth curve of *R. rickettsii* in HUVEC was determined concurrently with the SOD assays to monitor the infection and correlate the number of rickettsiae with the levels of SOD measured in the infected cell population (Fig. 3). Growth studies were also done to determine the effects of cycloheximide on replication of rickettsiae. The graph shows that the number of rickettsiae increased with time, as expected, and that cycloheximide had no effect on the growth of *R. rickettsii*.

To ensure that the SOD originated from infected host cells and not rickettsiae, the enzyme activity from Renografin-purified, sonicated and unsonicated suspensions of *R. rickettsii* was tested. The SOD activity measured in 10⁸ sonicated rickettsiae was 0.56 U/mg of protein, and that in unsonicated rickettsiae was 0.42 U/mg of protein. Comparison of these data with the activity in infected cells (Fig. 2), where the lowest SOD activity was about 5 U/mg of protein, shows that the contribution of enzyme activity from the rickettsiae was negligible. The SOD units measured for 10⁸ rickettsiae probably even represent a 100-fold higher activity than could actually be contributed in the experimental samples of infected cells. Since each 60-mm dish of approximately 10⁶ endothelial cells contained an average of 13 rickettsiae per infected cell at 48 h postinfection, the actual number of rickettsiae in each sample tested was estimated to be about 1.3 x 10⁷. We report the values for 10⁸ organisms because there was no detectable SOD activity at a purified rickettsial concentration of 10⁹ PFU/ml.

Since SOD induction in cells is due primarily to stimulation caused by increases in superoxide radical levels, we measured the levels of this one-electron reduction product of molecular oxygen when HUVEC were exposed to *R. rickettsii*. The results of these experiments are shown in Fig. 4. Superoxide radical levels were measured when the equivalents of both one and six infecting rickettsia per HUVEC were added to culture dishes. Cells to which no rickettsiae were added served as a control. The amount of superoxide produced when six rickettsiae per cell were added was about...
threefold higher than that produced when one or no rickettsiae per cell was added. Addition of SOD to parallel samples reduced the amount of detectable superoxide radical to zero and confirmed the specificity of the reaction. Elevated levels of superoxide were detected soon after exposure of rickettsiae to endothelial cells, suggesting that superoxide is probably generated during the attachment or entry phase of the infection cycle. To clarify whether attachment or internalization was responsible for this burst of superoxide, cytochalasin D, which inhibits internalization of rickettsiae, was used to pretreat the cells prior to infection. The results of these studies showed a significant reduction in the amount of superoxide produced compared with that in infected, untreated endothelial cells (Fig. 5). That the generation of large amounts of superoxide was due to the initial exposure of endothelial cells to a relatively large number of rickettsiae is attested to by the fact that at 24 h postinfection, when there were much larger numbers of rickettsiae per infected endothelial cell, the amount of superoxide measured in the cell supernatants was not detectably different from that in uninfected endothelial cells. Purified R. rickettsii at the same concentration as that used to achieve a ratio of six rickettsiae per HUVEC in the above experiments had no detectable superoxide when measured by the same technique.

When endothelial cells were pretreated with cycloheximide for 30 min prior to infection by R. rickettsii to inhibit SOD induction, the superoxide levels at 1 h postinfection were increased significantly (Fig. 5).

**DISCUSSION**

Rickettsiae, which are obligate intracellular bacterial parasites, enter nonphagocytic cells by a process termed induced phagocytosis that requires participation by both the host cell and the microorganism (4, 25). After internalization, these organisms replicate by binary fission, and one species, R. rickettsii, causes unique changes in the arrangement of intracellular membranes within 48 h as seen by transmission electron microscopy (19, 23). In previous experiments, we have shown that cultured human endothelial cells infected by this organism also produce or retain increased amounts of peroxides (21). These findings have led us to hypothesize that cells infected by R. rickettsii may sustain injury by an oxidative stress-related mechanism, possibly through oxygen radicals (21, 22). Lipid hydroperoxides are generated through direct attack by the hydroxyl radical (formed from the superoxide radical and hydrogen peroxide by the iron-catalyzed Haber-Weiss reaction) on polyunsaturated fatty acids (10), or following the attack of protonated superoxide (HO$_2^-$), a radical species also capable of directly attacking polyunsaturated fatty acids (3). Superoxide is generated intracellularly during normal cellular metabolism, either by enzymatic reactions, such as oxidation of xanthine or hypoxanthine, or by nonenzymatic reactions that involve autooxidation of cellular components by molecular oxygen (5). In addition, superoxide has been shown to be generated during ingestion of bacteria (salmonellae) by nonprofessional phagocytes, such as endothelial cells, with release of this oxygen radical into the extracellular medium (15).

The results of the experiments presented here, which used human endothelial cells infected by R. rickettsii, show that significant levels of the superoxide radical are generated and released into the culture medium after exposure of endothelial cells to this organism. Superoxide generation appears to occur within a relatively short time after exposure of endothelial cells to rickettsiae (probably during the internalization stage), and the superoxide amounts produced appear to be

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**FIG. 4.** Superoxide radical measurement in supernatant fluids of human endothelial cells infected with R. rickettsii. Superoxide was measured by monitoring ferricytochrome c reduction spectrophotometrically at 550 nm. In the infected populations, endothelial cells were exposed to an average of either one rickettsia per cell or six rickettsiae per cell for 1 h. SOD was added to parallel supernatants to ensure the specificity of the reaction. The data represent mean values ± the standard errors of eight determinations at each time point. Symbols: [ ], six R. rickettsii organisms per cell; ◆, one R. rickettsii organism per cell; ■, no R. rickettsii; ○, purified R. rickettsii; ■, six R. rickettsii organisms per cell plus SOD; ■, one R. rickettsii organism per cell plus SOD; ▲, SOD without rickettsiae.

**FIG. 5.** Superoxide radical measurement in supernatant fluids of human endothelial cells that were pretreated with either cycloheximide or cytochalasin D for 30 min prior to infection with R. rickettsii. In these experiments, endothelial cells were exposed to an average of six rickettsiae per cell for 1 h. The data represent mean values ± the standard errors of at least 10 determinations at each time point. Symbols: [ ], no rickettsiae; ◆, R. rickettsii; ■, 1 µg of cycloheximide per ml plus R. rickettsii; ○, 0.5 µg of cytochalasin D per ml plus R. rickettsii.
dependent upon the size of the inoculum added to the cells. This phenomenon was detected only during the initial infection period when a large enough number of PFU (six) was added to the cells. At 24 h postinfection, for example, even though the number of intracellular rickettsiae was significantly higher than at 1 h, the superoxide levels in the supernatant fractions were not different from those in uninfected control cells. *R. rickettsii* does have the capacity for bidirectional movement through the plasma membrane of cells, unlike most other rickettsiae (27), and theoretically, therefore, has the potential for continuous superoxide generation. However, the number of simultaneous rickettsial re-entry events that occur at 24 h probably is not sufficient to generate the same superoxide quantities as during the initial cell infection. The superoxide burst probably is related to a phagocytosis-like mechanism, as has been shown for salmonellae (15), since cytochalasin D, at levels not toxic to the endothelial cells, significantly reduced the superoxide amounts in the supernatants of infected cells. The superoxide levels produced during internalization apparently are sufficient to induce increased de novo synthesis of cellular SOD. This increase in SOD was detectable early after infection, and SOD remained elevated for at least 48 h, when the experiments were terminated. A 10-μg/ml dose of cycloheximide added to the endothelial cells 1 h prior to infection by *R. rickettsii* and then another dose (same concentration) added after the inoculum was removed effectively inhibited this increase. The effectiveness of cycloheximide in preventing de novo synthesis of SOD indicated that the increased activity of this enzyme following infection by *R. rickettsii* originated from the endothelial cells and not from the bacteria themselves.

The primary cellular defense against the injurious effects of superoxide is provided by SOD, which is able to maintain a steady-state superoxide level (7). However, an alteration of steady-state superoxide levels, either because of increased production of this radical or because of reduced scavenging capacity of the cell, may lead to cell injury and death (7). In general, SOD is induced under conditions known to increase intracellular superoxide flux (12). Indeed, the results of our previous study suggested that cells infected by *R. rickettsii* may experience a shift in the steady state between superoxide and SOD in favor of superoxide since increased levels of peroxides in infected endothelial cells are generally dependent upon superoxide for their formation (21). The experiments reported here extend our previous findings by directly demonstrating a significant increase in both superoxide and SOD.

Despite the increased induction of SOD in infected endothelial cells in our study, they still succumb to infection by *R. rickettsii* quickly (19, 20). Although the relative contribution of an oxidative mechanism toward endothelial cell injury and death in *R. rickettsii* infection is not clear, endothelial cells may be a prime target for this type of injury because they apparently lack catalase (18). If large quantities of superoxide are produced upon contact of rickettsiae with the endothelial cell surface, this could then trigger induction of increased amounts of SOD and, subsequently, large quantities of hydrogen peroxide. Superoxide produced at the cell surface can enter the intracellular space by at least one of two possible mechanisms. It may enter with the organism as it is internalized or through anion channels in the plasma membrane (13). Either one of these routes could lead to subsequent induction of SOD. However, even if superoxide produced at the cell surface does not enter the cells directly (which would make it more difficult to explain the intracellular induction of SOD), extracellular interaction of the superoxide radical with H+ does result in formation of hydrogen peroxide, which readily passes through the plasma membrane. Hydrogen peroxide itself is a strong oxidant, but it may also react with additional superoxide radicals produced from normal cellular oxidations to form the highly toxic hydroxyl radical, which has been implicated in initiation of lipid peroxidation. Although glutathione peroxidase, as well as catalase, is capable of detoxifying hydrogen peroxide in most aerobic mammalian organisms, the lack of catalase in endothelial cells may place an untoward burden upon glutathione peroxidase to detoxify both hydrogen peroxide and the lipid hydroperoxides produced in infected endothelial cells (21) (see equations 1 and 2 above). Thus, endothelial cells, despite their apparently appropriate response to an oxidant stressor such as superoxide, can still be significantly damaged. Alternatively, it has been shown that even though SOD levels increase in response to increases in the superoxide radical, this enzyme can be irreversibly inactivated by a by-product of its own catalysis, hydrogen peroxide (16, 17). Degradation of SOD by hydrogen peroxide may, therefore, also prohibit cells from responding optimally to the challenge of oxidative stress.

In summary, we have found that human endothelial cells infected with *R. rickettsii* produce increased amounts of superoxide radical and superoxide dismutase. These studies lend additional support to our hypothesis that cells infected with this organism may experience oxidative stress mediated by oxygen radicals and that this may lead to subsequent injury through peroxidation of membrane lipids.

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