The Journal of Microbiology, June 2001, p.142-145 Vol. 39, No. 2


## NOTE

# Alterations in the Activities of Antioxidant Enzymes of Human Dermal Microvascular Endothelial Cells Infected with *Orientia tsutsugamushi*

**Young-Sang Koh***

*Department of Microbiology, Cheju National University Medical School, Cheju 690-756, Korea*

(Received May 28, 2001 / Accepted June 8, 2001)

**Changes in the activities of several antioxidant enzymes in transformed human dermal microvascular endothelial cells (HMEC-1) by infection with the obligate intracellular bacterium *Orientia tsutsugamushi*, the causative agent of scrub typhus, were investigated. The activities of glucose-6-phosphate dehydrogenase, catalase, and glutathione peroxidase were significantly decreased in HMEC-1 cells infected with *O. tsutsugamushi*. However, the level of superoxide dismutase increased slightly. Furthermore, increased levels of intracellular peroxide was observed in HMEC-1 during infection. These results support the hypothesis that cells infected by this intracellular bacterium experience oxidant-mediated injury that may eventually contribute to cell death.**

***Key words***: *Orientia tsutsugamushi*, HMEC-1, antioxidant enzymes, intracellular peroxide, oxidative injury

*Orientia tsutsugamushi*, an obligate intracellular bacterium, is the causative agent of scrub typhus (tsutsugamushi disease), which is one of the most prevalent febrile illnesses in South Korea (6, 12). This bacterium infects a variety of host cells *in vitro* and *in vivo*, including macrophages, polymorphonuclear leukocytes (PMN), lymphocytes, and endothelial cells, where it replicates in the cytoplasm without being surrounded by a phagolysosomal membrane (12, 16, 17, 21). The disease is characterized by fever, rash, eschar, pneumonitis, meningitis, and disseminated intravascular coagulation, which leads to severe multiorgan failure in untreated cases (2, 7, 27). Although the extent of the pathological changes of vasculature is less severe than those of other rickettsial diseases (2), it has been reported that vascular endothelial cells are one of the major target cells of *O. tsutsugamushi* infection (14, 20, 29). Although the precise mechanism of vascular damage caused by *O. tsutsugamushi* infections remains unclear, the primary cause of the pathophysiological consequences might be the destruction of endothelial cells lining small blood vessels and the accompanying inflammatory responses (2, 15). At the ultrastructural level, *O. tsutsugamushi* infection of human endothelial cells is evidenced by distinctive changes in host cell organelles; including a reduction in ribosome-coated endoplasmic reticulum and Golgi activity, swelling of mitochondria, and an increase in vacuolation within the cytoplasm (17). These changes might be due to oxidant-mediated cell injury during intracellular multiplication of orientiae (9, 24) and is possibly mediated by accumulation of oxygen radicals, which may cause peroxidation of internal membrane lipids (22). To date, however, the relationship between the status of the host cell antioxidant system and of free radicals and vascular endothelial damage by *O. tsutsugamushi* infection has not been elucidated. For this reason, it is important to investigate alterations in host antioxidant systems during infection by *O. tsutsugamushi*.

Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide radical. Peroxides produced by the dismutase reaction are primarily scavenged by catalase and glutathione peroxidase, which simultaneously catalyze the oxidation of reduced glutathione and the conversion of toxic peroxides to innocuous by-products. Under conditions of normal cellular metabolism, balanced interactions of these three enzymes are necessary to protect the cellular environment against oxidative injury. Glucose-6-phosphate dehydrogenase (G-6-PD) plays a key role in the reduction of glutathione through NADPH produced by the hexose monophosphate shunt.

In the present study, we have selected antioxidant enzymes

* To whom correspondence should be addressed.
(Tel) 82-64-754-3851; (Fax) 82-64-725-2593
(E-mail) yskoh7@cheju.cheju.ac.kr

considered to be important in normal cellular defenses against reactive oxygen species to determine how infection of human microvascular endothelial cells (HMEC-1) by *O. tsutsugamushi* affects their endogenous levels and the amount of intracellular peroxide.

HMEC-1, derived from human dermal microvascular endothelial cells (1), was kindly provided by the Center for Disease Control and Prevention (Atlanta, GA). The cells were propagated in MCDB 131 medium (Life Technologies, Grand Island, NY) supplemented with 15% fetal bovine serum (Life Technologies), hydrocortisone (1 μg/ml; Sigma Chemical Co., St. Louis, MO), epidermal growth factor (10 ng/ml; Life Technologies), penicillin (100 U/ml; Life Technologies), and streptomycin (100 μg/ml; Life Technologies). Endothelial cells were seeded onto 100-mm-diameter dishes (Becton Dickinson Labware, Franklin Lakes, NJ) for the preparation of cell extracts.

*O. tsutsugamushi* Karp (American Type Culture Collection, Manassas, VA) was propagated in monolayers of L929 cells (8). The titer of infectivity of the inoculum was determined as described previously (8, 25). An *O. tsutsugamushi* infected-cell counting unit (25) of $1.26\times10^7$ was used to infect endothelial cells. Uninfected cells served as controls. The degree of infection was monitored by an indirect immunofluorescent-antibody technique (11). At the end of the experimental period, cells were trypsinized from the culture dishes, washed once by centrifugation to remove trypsin, and resuspended in 1 ml of potassium phosphate buffer (0.05 M, pH 7.8). Cell extracts were prepared by sonicating the cells for 1.2 min by using four 30-s bursts in a model UP 50 H Ultrasonic Processor (Dr. Hielscher GmbH, Germany) at a constant pulse and 80% amplitude setting. The protein content of the endothelial cell extracts for all of the experiments was determined by the method of Smith *et al.*(23), using a bicinchoninic acid protein assay kit (Sigma, St. Louis, MO).

The catalase assay is based upon the spectrophotometric measurement, at 240 nm, of the decomposition of $H_2O_2$ (3). Briefly, 0.55 ml of a 10 mM solution of $H_2O_2$ in 50 mM phosphate buffer, pH 7.0, was placed into a cuvette, and the reaction was started by the addition of 0.05 ml of the endothelial cell extract. The decrease in optical density was monitored for 5 min at 1-min intervals. Enzyme activity was calculated by using the molar extinction coefficient of $H_2O_2$ (43.6 $M^{-1}$ $cm^{-1}$). The results are expressed as milliunits of enzyme per milligram of cell protein . One milliunit of catalase is that amount of enzyme that decomposes 1 nmol of $H_2O_2$ per min.

Glutathione peroxidase was measured according to the method of Gunzler *et al.* (10). The reaction mixture in each cuvette initially contained 0.54 ml of 50 mM phosphate buffer (pH 7.0) with 0.5 mM diethylene-triaminepentaacetic acid (DETAPAC), 60 μl of either buffer (blank) or cell extract, 12 μl of glutathione (0.1 M ), and 12 μl of glutathione reductase (50 U/ml) (Sigma). They were allowed to equilibrate for 10 min before addition of 12 μl of NADPH (4 mM) and 12 μl of *t*-butyl hydroperoxide (3 mM) to start the reaction. The decrease in $A_{340}$ was recorded for 6 min at 1-min intervals. Enzyme activity was calculated by using the millimolar extinction coefficient of NADPH (6.22). The results are expressed as milliunits of enzyme per milligram of cell protein. One milliunit of glutathione peroxidase oxidizes 1 nmol of glutathione per min.

G-6-PD activity was determined according to the method described in the *Worthington Manual* (28). The reaction mixture was placed in a 1 ml cuvette and consisted of 0.54 ml 55 mM Tris-HCl buffer with 33 mM $MgCl_2$ (pH 7.8), 0.02 ml of 6 mM $NADP^+$, and 0.02 ml of 0.1 M glucose 6-phosphate (Sigma). The reaction was initiated by the addition of 0.02 ml of endothelial cell extract. The increase in optical density at 340 nm due to the reduction of $NADP^+$ was measured spectrophotometrically for 4 min at 1-min intervals. Enzyme activity was calculated by using the millimolar extinction coefficient of NADPH. The results are expressed as milliunits of enzyme per milligram of cell protein. One milliunit of G-6-PD is defined as that amount which is capable of reducing 1 nmol of $NADP^+$ per min.

SOD activity was determined by the method of Oberley and Spits (18). Reaction buffer contained a final concentration of 0.1 mM xanthine, $5.6\times10^{-5}$ M nitroblue tetrazolium, 1 mM DETAPAC, 1 U of catalase per ml, an 60 μl of xanthine oxidase (0.235 U/mg of protein). The rate of change in absorbance at 540 nm was recorded at 1-min intervals for 5 min on a Shimadzu UV-1601 spectrophotometer. A standard curve of SOD activity was prepared by using several dilutions of bovine erythrocyte SOD (2500 U/mg). All chemicals for the SOD assay were purchased from Sigma. 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]×100%. One unit of SOD activity is defined as that amount of protein which gives half-maximal inhibition.

Endothelial cells were assayed for intracellular peroxide by a modification of the method of Cathcart *et al.* (5). Two milliliters of PBS containing 1 mM 5 (and 6)-carboxy-2,7-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes, Eugene, OR) was added to each 60-mm dish, and the plates were incubated for 10 min at room temperature. The cells were then rinsed three times with 2 ml of PBS. After the final rinse, the PBS was aspirated and 2 ml of 0.05% Nonidet P-40, in distilled water was added to lyse the cells. The lysed cells were removed with a rubber policeman, and the contents were assayed in a Spectra Fluor A-5082 fluorescence spectrophotometer (Techan, Australia) with an emission wavelength of 530 nm and an excitation wavelength of 485 nm. The background fluorescence, determined in the absence of added cell sam-

**Table 1.** Activities of catalase, glutathione peroxidase, G-6-PD, and SOD in HMEC-1 infected with *O. tsutsugamushi*

| Time (h) | Level (mU/mg protein)[a] of: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Catalase in: | | Glutathione peroxidase in: | | G-6-PD in: | | SOD in: | |
| | Uninfected cells | Infected cells | Uninfected cells | Infected cells | Uninfected cells | Infected cells | Uninfected cells | Infected cells |
| 0 | 2897±220 | ND | 299±14 | ND | 99.5±8.24 | ND | 2081±157 | ND |
| 24 | 3570±254 | 4414±272 | 254±24 | 257±27 | 94.0±5.66 | 81.6±5.34 | 1450±258 | 1504±167 |
| 48 | 4379±325 | 2856±126 | 185±31 | 210±23 | 91.5±6.25 | 80.1±4.78 | 2125±176 | 2193±135 |
| 72 | 3449±146 | 2047±120 | 268±21 | 194±17 | 89.4±5.47 | 87.3±6.23 | 2376±230 | 3380±310 |
| 96 | 3934±245 | 2045±135 | 291±25 | 178±13 | 116.3±17.2 | 71.7±3.35 | 2957±320 | 3004±267 |

[a]Each value represents the mean ± standard errors from three individual experiments. ND, not determined.

ples, was subtracted. Peroxide levels were expressed as fluorescence units per milligram of protein.

The activities of catalase, glutathione peroxidase, and G-6-PD, three key enzymes in cellular defence against oxygen-mediated injury, were all significantly decreased in HMEC-1 following infection with *O. tsutsugamushi* (Table 1). The activity of catalase remained fairly stable during the first 24 h after infection. By 96 h, however, the activity of this enzyme also decreased significantly to 51% of the value in corresponding uninfected cells. The activity of glutathione peroxidase did not change during the first 48 h following infection. By 72 h postinfection, this enzyme level decreased by 28% to 194 mU/mg of protein; by 96 h it decreased by 39% to 178 mU/mg of protein. The activity of G-6-PD decreased by 14% to 81.6 mU during the first 24 h following infection. By 96 h postinfection, the level of this enzyme decreased significantly to 61% of those of uninfected cells. Endothelial cells infected with *O. tsutsugamushi* showed a slight increase in SOD activity (42% higher than in the uninfected cells) at 72 h after infection. However, this increase was not maintained at 96 h postinfection.

Reduction in the activity of all three enzymes following infection was accompanied by an increase in the number of intracellular orientiae. At 24 h after infection, there were 2 to 4 orientiae per infected cell, with about 20% of the cells infected; at 96 h, there were 15 to 22 orientiae per infected cell, with more than 95% of the cells infected (data not shown). There was no difference in the protein content between infected and uninfected cells.

It is likely that the decreased levels of catalase and glutathione peroxidase in infected endothelial cells lead to the elevated intracellular peroxide levels. To test this possibility, intracellular peroxide levels were determined using a fluorescent probe, DCFH-DA (Fig. 1). Although this probe does not necessarily distinguish between organic lipid peroxides and hydrogen peroxide, it does indicate whether there are increased levels of total intracellular peroxide in infected cells. Endothelial cells infected by *O. tsutsugamushi* have statistically significant higher levels of intracellular peroxides than do uninfected control cells at 72 and 96 h postinfection. At present, I have no explanation for the decrease in catalase and glutathione peroxidase activities in infected endothelial cells; however, it may be due to inhibition by superoxide radicals (4, 13) or to inhibition of protein synthesis.

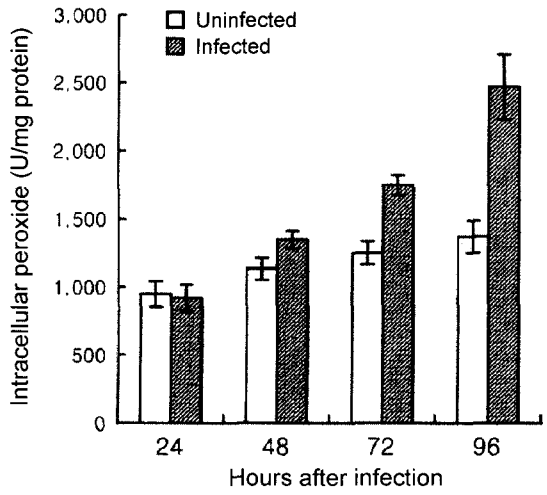


**Fig. 1.** Intracellular peroxide levels in HMEC-1 infected with *O. tsutsugamushi*. The data represent the mean values ± standard errors of at least three determinations per experimental variable.

Walker *et al.* (26) reported that the epidemiological association between human G-6-PD deficiency and Rocky Mountain spotted fever, which is caused by *Rickettsia rickettsii*. Although there is no epidemiological study which shows the relationship between human deficiency of this enzyme and scrub typhus, the above results predict the importance of human G-6-PD in the pathogenesis of oriential infection. The decrease in activity of G-6-PD in infected cells probably causes the decrease in glutathione levels. This decrease in G-6-PD levels may be due to an inhibition in its synthesis or to some oxidative modification in enzyme structure, such as carbonyl formation (19).

Whatever the mechanism may be, the decrease of these three enzymes may cause the depletion of reduced glutathione and the increase in intracellular peroxides observed in this study. Further studies involving the measurement of the ratio of reduced glutathione to oxidized form (GSH/GSSG), the enzymes of glutathione synthesis, and glutathione reductase may further clarify the role of oxidative

damage and antioxidant defenses in the pathogenesis of endothelial cell injury by *O. tsutsugamushi*. Furthermore, if this hypothesis can be verified in a relevant animal model system, adequate antioxidant therapy may become warranted in the treatment of scrub typhus.

This work was supported by Korea Research Foundation Grant (KRF-99-015-DP0307) to Y.-S. Koh. I thank Dr. Ik-Sang Kim in the Department of Microbiology and Immunology, Seoul National University College of Medicine for sending *O. tsutsugamushi* strains and anti-*O. tsutsugamushi* antibodies.

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