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ICAM-1 EXPRESSION AND CELLULAR INJURY IN CULTURED ENDOTHELIAL CELLS UNDER HYPOXIA/REOXYGENATION

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INDEXING WORDS

ICAM-1; HUVEC; superoxide; cell injury; SOD

SYNOPSIS

Intercellular adhesion molecule 1(ICAM-1) expression and cellular changes in human umbilical vein endothelial cells(HUVEC) and ECV304, an established cultured line derived from HUVEC, under hypoxia and hypoxia(H)/reoxygenation(R) were investigated by immunological, cytochemical, and morphological methods. ICAM-1 expression in HUVEC decreased slightly under hypoxia(92%) and was up-regulated under reoxygenation(114%), but this up-regulation was diminished by superoxide dismutase(SOD). The up-regulation of ICAM-1 expression by interleukin-1β(IL-1β) was detected at almost equal levels under hypoxia, and H/R. Using lucigenin-chemiluminescence, we demonstrated superoxide generation from HUVEC

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under H/R. The Ca^{2+} influx under hypoxia, and the Ca^{2+} release from the cells under H/R reduced by SOD were detected cytochemically. Vacuole formation as cell injury under hypoxia and H/R was detected by electron microscopy. The present findings provide evidence that superoxide generated from HUVEC is responsible for the up-regulation of ICAM-1 expression under H/R, and the cause of endothelial cellular injury.

INTRODUCTION

The vascular endothelium is very sensitive to oxidative damage mediated by superoxide released from the endothelium itself or from inflammatory cells.[18] During H/R, adhesion molecules such as intercellular adhesion molecule-1(ICAM-1), E- and P-selectins in the endothelium,[4,8] and adhesion molecules on leukocytes have been reported to be induced.[17,26] ICAM-1 is constitutively expressed in endothelial cells and is up-regulated when exposed to cytokines(IL-1β,tumor necrosis factor(TNF)) and endotoxins.[3,13] A recent study has indicated that ICAM-1 expression was up-regulated by IL-1 produced during hypoxia,[20] but whether this up-regulation is related to superoxide generated during H/R has not been clearly evaluated.

The other response of the endothelium to H/R is injury in the endothelial cells themselves.[16] The most likely to be responsible for this injury is superoxide generated during reperfusion.[15] Several kinds of cytotoxicity assays using ^{51}Cr, fura-2 or others have been used to assess endothelial cell injury,[25] but no clear morphological studies of superoxide-induced injury has been yet reported.

In this study, we investigated ICAM-1 expression and cellular injury under hypoxia and H/R using human umbilical vein endothelial cells (HUVEC) to determine the role of superoxide and its generation from endothelial cells.

MATERIALS AND METHODS

Endothelial Cell Cultures and Hypoxia or Hypoxia/Reoxygenation(H/R) Treatment.

Human umbilical vein endothelial cells(HUVEC) were isolated from an umbilical cord by treatment with 0.1% collagenase(Worthington Biochemical Corporation, Freehold, NJ. USA) as described previously.[12] Primary isolates were cultured and further subcultured in 0.2% gelatin-coated dishes in RPMI1640 medium supplemented with 15% heat-inactivated fetal calf serum(FCS), streptomycin(100mg/ml), penicillinG(100U/ml), endothelial cell growth supplement(ECGS)(50mg/ml)(Becton Dickinson Labware, Franklin Lakes, NJ. USA), and heparin(5ml/ml)(Mochida Pharmaceutical Co., Ltd., Tokyo, Japan).[10] Several strains were passaged(1:3 split ratios) and were serially type-selected for experimental use at the third passage .

ECV304, a cell line established from HUVEC kindly provided by Dr. K.Takahashi(Department of Biochemistry, Japan National Defence Medical College), was cultured and futher subcultured in 0.2% gelatin-coated dishes in RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum(FCS).[23]

In vitro models of hypoxia and hypoxia/reoxygenation(H/R) were treated as described.[17,20] Endothelial cell monolayers were exposed to 30 min. hypoxia at 37°C in a 94%N_2/5%CO_2/1%O_2 atmosphere. Reoxygenation was subsequently attained by placing the cells under normoxic conditions(95% air/5% CO_2) for 1 hour. When necessary, IL-1β(10U/ml)(Genzyme Corporation, Cambridge, MA. USA) was applied for a total of 4 hours; SOD(200μg/ml)(UBE industries Ltd, Tokyo, Japan) was added just before hypoxia.[2,7]

Immunological detection of ICAM-1 Expression in HUVEC.

ELISA for detecting ICAM-1 expression in HUVEC was carried out in 96-well plates as reported previously.[26] After incubation for a definite period, the cell monolayers were washed with RPMI1640 containing FCS, fixed with 100%

methylalchohol for 15 min, blocked with 0.2% gelatin containing 0.1% BSA for 30 min, and washed. An anti-ICAM-1 antibody (1mg/ml)(Cosmo-Bio, Tokyo, Japan) was added for 60 min at 37°C, cells washed 3 times and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin(13mg/ml)(Cappel, Durham, NC. USA) were added for 60 min at 37°C and washed again four times. The substrate used was orthophenylenediamine in phosphate-citrate buffer, pH 5.0. Color development was terminated after approximately 15 min of incubation by the addition of 2 M H_2SO_4. Absorbance at 492 nm was determined using an ELISA reader(Biorad, Tokyo, Japan). To eliminate the nonspecific reaction to immunogloblin and to clarify the differences in the findings due to O_2 concentration, the data obtained under normoxic conditions were converted to 0.2. ELISA data are given as the mean of five determinations.

Northern Blot Analysis of ICAM-1 expression in HUVEC.

After incubation for a definite period, total RNA was isolated from HUVEC using the acid guanidinium thiocyanate/phenol/chloroform extraction method.[5] Total RNA(10μg) was electrophoresed through a 1% agarose/0.8% formaldehyde gel. RNA was visualized by ethidium bromide staining/ultraviolet photography to assess the integrity and loading and then transferred on to a nylon membrane for hybridization. Blots were then hybridized with an ICAM-1 probe(Cosmo-Bio, Tokyo, Japan).

Measurement of O_2^- Formation by HUVEC.

The lucigenin-dependent chemiluminescence response was used as the measure of superoxide release.[12,21] After incubation for a definite period, cells were gathered with a cellscraper and suspended in cuvettes containing Krebs-Ringer's Phosphate Buffer with glucose(KRPG) and 0.1% bovine serum albumin(BSA). After adding 20 mM lucigenin (Sigma Chemical Co, St. Louis, MO, USA), the cuvettes were placed in a liquid scintillation analyzer(Beckman, LS 5000TA)(Beckman Instruments, Inc. Somerset, NI, USA) with a single photon monitor, and chemiluminescence counts were recorded.

Cytotoxicity Assay of HUVEC.

The cytotoxicity of HUVEC was measured using fura-2-AM(Wako Pure Chemicals,Ltd., Osaka, Japan), as described previously.[1] Briefly, to load with fura-2, confluent endothelial cells were incubated with 2 μM fura-2-AM in RPMI1640 containing 15% FCS for 30 minutes, and washed twice with RPMI1640 containing 15% FBS to remove unloaded fura-2-AM. The cells were then incubated again with RPMI1640 containing 15% FBS and washed twice to remove spontaneous release of fura-2-AM. After changing the culture medium to RPMI1640 without FBS, the cells were incubated for a definite period. The fluorescent intensities of the culture media of the cells were measured at an excitation wavelength of 350 nm and 500nm emission, where fura-2 binding to calcium would show the most intensive fluorescence on a spectrofluorophotometer(HITACHI F4010)(HITACHI, Tokyo, Japan).[1,16] Cell injury was assessed as follows:

Index(in medium)=(Test-O_2 20%)/(TritonX-O_2 20%)

Calcium concentrated in the cell as compared as that under normoxia was assessed as follows:

Index(in cell)=(Test-TritonX)/(Normoxia-TritonX)

Test, TritonX, Normoxia in the formula substitute for measured value of fura-2. The index in the medium and the cell were recognized as cell injury and Ca^{2+} arrested by HUVEC, and plotted on the positive and negative sides of the figure, respectively (Fig.4).

Electron Microscopy(E.M.).

HUVEC were harvested on gelatin-coated Lab-Tec-Chamber(Nunc, Naperville, IL, USA), and grown to confluency. After incubating for a definite period, electron micrographs were prepared using a 1200EX E.M.(JEOL Ltd, Tokyo, Japan)[6] as described, and the number of vacuoles considered to be caused by injury was calculated.[16]

Statistical Analysis.

All data are expressed as the mean + S.E.M. Group comparison of data was made using Student's unpaired t-test. Differences were considered significant when $P < 0.05$.

RESULTS

Increased Expression of ICAM-1 on HUVEC under H/R.

Surface levels of ICAM-1 expression in HUVEC were determined by ELISA to define the effect of hypoxia and H/R with or without IL-1β(Fig.1). Without IL-1β the expression decreased slightly under hypoxia(92%) and was up-regulated under reoxygenation(114%) compared with that in normoxic controls, and this up-regulation was diminished by SOD(200μg/ml)(103%). Preliminary studies indicated that IL-1β treatment at least up to 100U/ml induced a dose-dependent increase in ICAM-1 expression in HUVEC under normoxic conditions(data not shown). With IL-1β, the expression of ICAM-1 was up-regulated under normoxic conditions(128%), hypoxia(137%), and H/R(121%) as compared with that without IL-1β under each condition as shown in Fig.1. Thus, ICAM-1 expression was up-regulated by IL-1β almost equally under normoxic, hypoxic and H/R conditions. With IL-1β, ICAM-1 expression did not change under hypoxia(100%), but was up-reglated under H/R(117%) compared with that under normoxia with IL-1β. Thus ICAM-1 expression was up-regulated under H/R with or without IL-1β, but this up-regulation was not as significant as that induced by IL-1β(10U/ml) treatment. ICAM-1 expression in ECV304 under hypoxic or H/R conditions was shown to have the same tendency as in HUVEC, but a much smaller up- or down-regulation was observed (data not shown).

Marked induction of ICAM-1 mRNA was detected after IL-1β treatment(Fig.2), but no signifficant changes were detected under H/R (data not shown). This result may imply that up-regulation under H/R was very slight compared with that after IL-1β treatment.

Superoxide may contribute to this up-regulation under H/R because SOD diminished this effect to almost control level.

Superoxide Generation by HUVEC under H/R.

Lucigenin-dependent chemiluminescence was used to detect O_2^- formation directly in HUVEC(Fig.3). A significantly greater lucigenin-dependent chemiluminescence(CL), which substituted for superoxide generation, was observed in HUVEC under H/R with the mean CL peak of 829000cpm, compared with CL under normoxic control conditions(base line)($p<0.05$). SOD reduced CL under H/R to 85000cpm, which corresponded to almost the base line level. CL of ECV304 showed the same tendency, although the peak of CL under H/R was smaller(data not shown). Thus, O_2^- formation in HUVEC under H/R was confirmed.

Cytotoxicity Assay of HUVEC under Hypoxia and H/R detected by Fura-2.

HUVEC injury caused by hypoxia or H/R was detected. Fura-2, a fluorescent calcium indicator, has been used to detect HUVEC injury by measuring specific release[14]. We measured not only specific release of fura-2 to detect HUVEC injury but also fura-2 in the cell to detect calcium concentrated in the cytosol(Fig.4). The index(in medium), which indicated cell injury, was significantly higher under H/R(Index=0.18) than that under normoxia($p<0.05$), but the index under hypoxia was almost equal to that under normoxic conditions. Under H/R, SOD diminished the index(in medium) significantly(index=0.03) compared with that without SOD. The index(in cell), which indicated Ca^{2+} ratio concentrated in the cell, increased slightly under hypoxia(index=1.09), and diminished significantly under H/R(index=0.62)($p<0.05$). Because of the SOD diminishing index(in medium) under H/R, free radicals appeared to be greatly involved in cell injury. It could be that the slight increase in index(in cell) under hypoxic conditions made HUVEC vulnerable to superoxide generated under H/R. ECV304 revealed almost the same tendency, although the change in index was smaller(data not shown).

Observation of HUVEC damage under Hypoxia and H/R by Electron Microscopy.

Morphological damage in HUVEC was examined by electron microscopy(Fig.5). Under hypoxia, small vacuoles containing a myelin-like substance appeared in the cytoplasm of HUVEC, and the cell organelles became slightly warped. The number of vacuoles increased significantly under hypoxia, and still more under H/R as compared with that under normoxia($p < 0.01$), and SOD(200μg/ml) decreased the number significantly under H/R($p < 0.05$). Irregularities in the contours of the cell and cytoplasmic organelles was observed mostly under H/R which was almost normalized by SOD(200μg/ml)(Table1). By E.M., very little vacuole formation was observed in ECV, and the contours of the cells and cytoplasmic organelles remained almost normal even under H/R(data not shown).

DISCUSSIONS

Reperfusion injury has been widely studied. PMN which attach to the endothelium has been reported to play a role in injury, and in a series of experiments, superoxide generated from the endothelium or PMN were reported to mediate adhesion of PMN to the endothelium.[10,22] Some experiments even suggested that P- and E-selectins were involved in H/R.[9] In our study, we designed in vitro models to investigate the mechanism of H/R and evaluated ICAM-1 expression and cellular changes of the endothelium under hypoxia and H/R. Our results showed that ICAM-1 expression decreased slightly under hypoxia and was up-regulated under reoxygenation. This up-regulation was reduced by SOD, demonstrating that at least superoxide was involved in this increased ICAM-1 expression. Moreover, IL-1β upregulated ICAM-1 expression under normoxia, hypoxia and H/R almost equally. Regarding ICAM-1 expression under hypoxia and H/R, Shreeniwas et al. reported that IL-1 was produced by HUVEC under hypoxia, and that ICAM-1 expression was up-regulated during reoxygenation, but that ICAM-1 expression in response to IL-1 was very slight under hypoxia compared to that under H/R. We considered this discrepancy between these previous and our present results

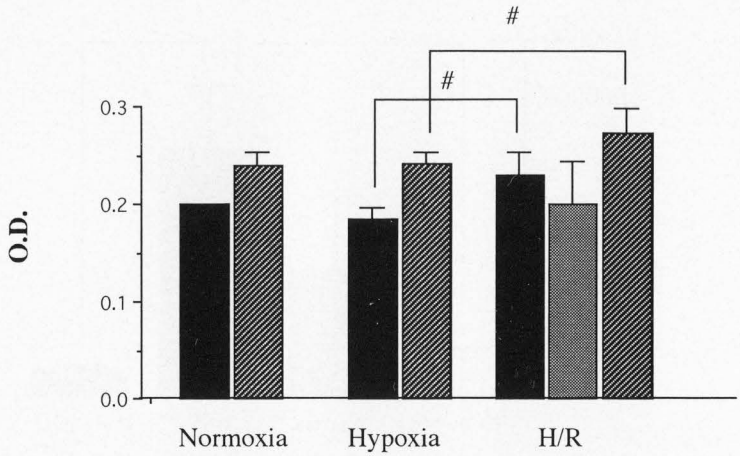

Fig.1
Effect of hypoxia or H/R, and effect of IL-1β added to medium under normoxia, hypoxia, H/R condition and effect of SOD added to medium under H/R condition on surface level of ICAM-1 in HUVEC. Confluent HUVEC were either placed in nornoxia, hypoxia, or H/R. IL1β(10U/ml) was added and incubated for four hours in total. SOD(200μg/ml) was added just before H/R. Values represent the mean ±S.E.M. of all samples. (#) statistical significance of $p < 0.05$. (■) no reagent; (▨) IL1β(10U/ml); (▩) SOD(200μg/ml)

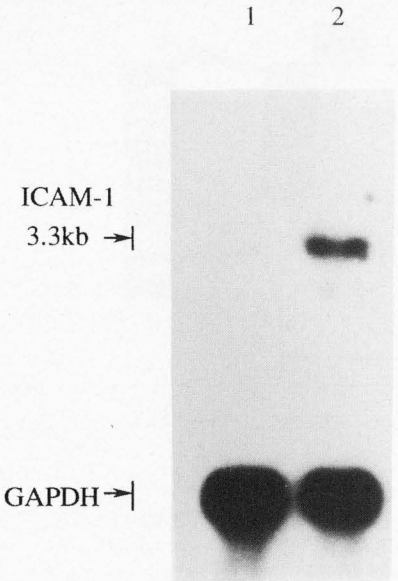

Fig.2
Northern blot analysis of ICAM-1 mRNA signal in HUVEC without(lane1) or with(lane2) IL1β challenge under normoxia. Total RNAs(10μg) from HUVEC were analysed with ICAM-1 probe as described in Materials and Methods. IL1β(10U/ml) was added and incubated for four hours in total. The arrow designates the position of mRNA for ICAM-1 and GAPDH. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

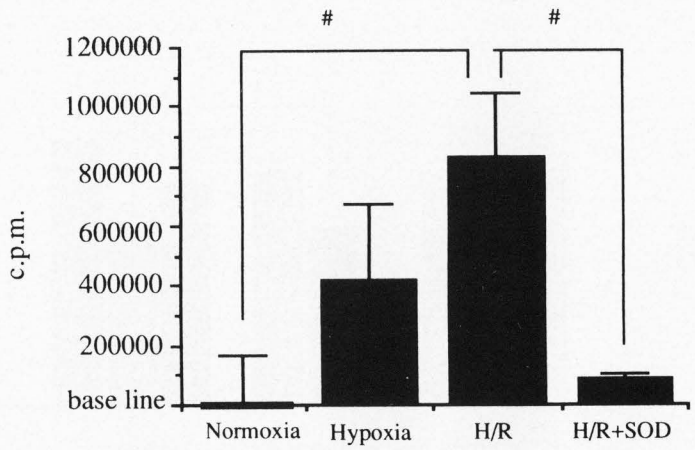

Fig.3
Superoxide generation from HUVEC detected by lucigenin-chemiluminescence. HUVEC was exposed to nornoxia, hypoxia, H/R or H/R in addition to SOD(200μg/ml)(SOD+H/R). SOD was added just before H/R. Superoxide was generated during H/R which was blocked by SOD. Values represent the mean ±S.E.M. of all samples. (#) statistical significance of $p < 0.05$.

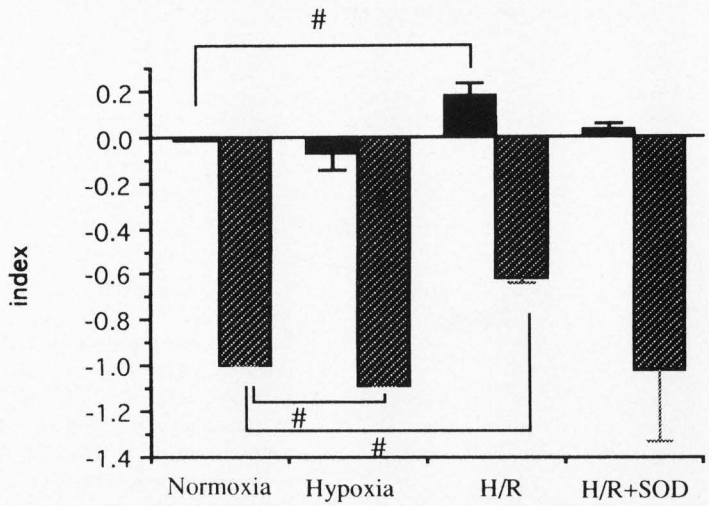

Fig.4
Effect of hypoxia or H/R on injury of HUVEC, and calcium concentration in the cytosol. HUVEC was exposed to nornoxia, hypoxia, H/R or H/R in addition to SOD(200μg/ml)(SOD+H/R). SOD was added just before H/R. Values represent the mean ±S.E.M. of all samples. (#) statistical significance of $p < 0.05$. (■) Index in medium; (▨) Index in cell. The formula, by which we calculated these Index, were descreibed in Materials and Methods.

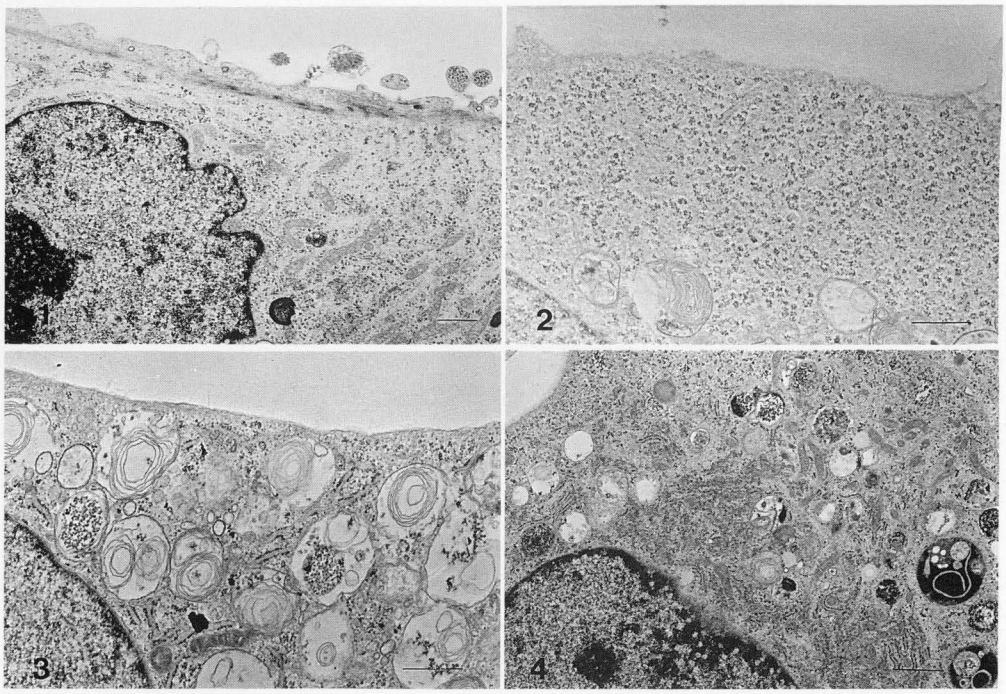

Fig.5
Damage caused by hypoxia or H/R which was detected by E.M. 1:nornoxia 2:hypoxia 3:H/R 4:H/R in addition to SOD(200μg/ml). SOD was added just before H/R. Bar=5μ

Table 1. Morphometric Analysis

Incubation conditions	Number of vacuoles	Irregularity in the [a] contour of the cell	Abnormality in the [b] cytoplasmic organelles
normoxia	2± 1	—	—
hypoxia	36 ± 12#	±	±
H/R	165± 38.5 #	++	++
H/R+SOD	97 ± 41##	+	±

Values are expressed as mean± SE. Normoxia, HUVEC incubated under normoxic condition before fixation. Hypoxia, HUVEC incubated under hypoxic condition for 30 min. before fixation. H/R, HUVEC incubated under hypoxic condition for 30 min. and then reoxygenated for 60 min. before fixation. H/R+SOD, SOD(200μg/ml) was added just before H/R.
#P<0.01 compared to control(normoxia);
##P<0.05 compared to H/R.

a :Irregularity of contour was assessed from — to ++ by ultrastructual observation.

b : Abnormal shapes of cytoplasmic organelles such as mitochondria swelling and dilatation of rough endoplasmic reticula were assessed from -to ++ by ultrastructual observation.

regarding the response to IL-1 to be mainly due to a difference in the duration of hypoxia. By northern blot analysis, mRNA level of ICAM-1 expression was greatly increased by IL-1β, but no significant change was detected under H/R conditions, the reason possibly being that up-regulation of ICAM-1 expression under H/R was very slight as compared to that by IL-1β, at both mRNA and protein levels(Fig.1 and 2).

We demonstrated that superoxide generation induced endothelial cell injury in HUVEC. O_2^- formation from endothelial cells has been studied by detecting O_2^- generated into the medium monitored by cytochrome C.[16] Lucigenin-dependent chemiluminescence(CL) had been used to detect O_2^- formation directly by PMN or macrophages.[12,21] By lucigenin-chemiluminescence, we demonstrated that HUVEC themselves generate superoxide, and that this generation was inhibited by SOD (200μg/ml). According to some studies, xanthineoxidase activity is implicated in this superoxide generation.[19,24] Superoxide may cause damage to many tissues, so we speculated upon the possibility of injury of HUVEC under hypoxia and H/R both cytotochemically and morphologically.[14] Fura-2, a Ca^{2+} indicator, is generally used to investigate Ca^{2+} distribution, and detecting fura-2 release from the cell into the medium is regarded as representative of cell injury.[1,16] We used these assays to evaluate cell injury,and the results demonstrated that Ca^{2+} was concentrated into the cell under hypoxia and released under reoxygenation, and that the release was significantly diminished by SOD (200μg/ml). Consequently, it was confirmed that superoxide was generated from the endothelium under reoxygenation and that this was involved to a high degree in cell injury. However, further studies are warranted on the possibility that Ca^{2+} concentrated into the cell rendered the endothelium susceptible to damage. We also examined cell injury morphologically by E.M. and observed that some vacuoles had already appeared under hypoxia. These vacuoles increased in number under reoxygenation and this increase was reduced by SOD(200μg/ml). Some of these vacuoles contained myelin-like substances, so at least some of them seemed to be lysosomes. The irregularities in the contours of the cells and in the cytoplastic organelles, observed mostly under H/R, were almost normalized by SOD.

ECV304 was used to diminish individual differences. ICAM-1 expression in ECV304 under hypoxia and H/R revealed the same tendency as HUVEC, although the rate of change was smaller, while IL-1β induced ICAM-1 expression strongly as in HUVEC. Injury in ECV304 detected cytochemically

and morphologically revealed also the same tendency as in HUVEC, although injury was very slight. ECV304 showed the same response to superoxide as HUVEC, although response rate was smaller because ECV cells were less susceptible to damage caused by superoxide.

In conclusion, ICAM-1 expression was up-regulated under H/R, and superoxide is probably related to this up-regulation. Superoxide generated from HUVEC was also confirmed, damage of HUVEC by superoxide was detected cytochemically, and cell injury was also detected morphologically by E.M. as vacuoles in the cytosol.

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REFERENCES

1. Abe,M., Morita,I., and Murota,S. : Prostagl.Leukotr.Essen.Fat.Acid. 1988. 34. 69/74. A new in vitro method using fura-2 for the quantification of endothelial cell injury.
2. Bevilacqua,M.P., Pober,J.S., Wheeler,M.E., Cotran, R.S., and Gimbrone, Jr. M.A. : J. Clin.Invest. 1985. 76. 2003/2011. Interleukin1 acts on cultured human endothelium to increase the adhesion on polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines.
3. Birdsall, H.H. : Am. J. Pathol. 1991. 139. 1341/1350. Induction of ICAM-1 on human neural cells and mechanisms of neutrophil-mediated injury.
4. Bradley, J.R., Johnson, D.R., and Pober, J.S. : Am. J. Path.1993.142. 1598/1609. Endothelial activation by hydrogen peroxide.
5. Chomczynski, P., and Sacchi, N. : Anal. Biochem. 1987. 162. 156/159. Single-step method of RNA isolation by acid guanidinium thiocynate-phenol-chloroform extraction.

6. Collins, U.P. and Brunk, U.T. : Scanning. 1980. 3. 153/161. An approach to the exploration of scanning electron microscopy in studies of cultures of human cells.
7. Dustin, M.L., Rothlein, R., Bhan, A.K., Dinarello, C.A., and Springer, T.A: J. immunol. 1986. 137. 245/254. Induction by IL 1 and Interferon-γ: Tissue distribution, biochemiostry, and function of a natural adherence molecule(ICAM-1).
8. Fujita, H., Morita, I., and Murota, S. : B.B.R.C. 1991. 177. 664/672. Involvement of adhesion molecules (CD11a -ICAM-1) in vascular endothelial cell injury elicited by PMA-stimulated neutrophils.
9. Gamble, J.R., Skinner, M.P., Berndt, M.C., and Vadas, M.A. : Science. 1990. 249. 414/417. Prevention of activated neurophil adhesion to endothelium by soluble adhesion protein GMP140.
10. Granger, D.N., Benoit, J.N., Suzuki, M., and Grisham, M. B. : Am. J. Physiol. 1989. 257. G683/G688. Leukocyte adherence to venular endothelium during ischemia-reperfusion.
11. Jaffe, E.A., Nachman, R.L., Becker, C.G., and Minick, C.R. : J. Clin. Invest. 1973. 52. 2745/2756. Culture of human endothelial cells derived from umbilical veins.
12. Kowanko, I.C., Ferrante, A.,and Carman, H&K.L. : J. Immunol. 1991.83. 225/230. Granulocyte-macrophage colony-stimulating factor augments neutrophil killing of *Torulopsis glabrata* and stimulates neutrophil respiratory burst and degranulation.
13. Leeuwenberg, J.F.M., Smeets, E.F., Neefjes, J.J., Shaffer, M.A., Cinek, T., Jeunhomme, T.M.A.A., Ahern, T.J., and Buurman, W.A.: Immunol. 1992. 77. 543/549. E-selectin and intercellular adhesion molecule-1 are released by activated human endothelial cells in vitro.
14. Lum, H., Barr, D.A., Shaffer, J.R., Gordon, R.J., Ezrin, A.M., and Malik, A.B. : Circ. Res. 1992. 70. 991/998. Reoxygenation of endothelial cells increases permeability by oxidant-dependent mechanisms.
15. Michiels, C., Arnould, T., Houbion, A., and Remacle, J. : J.Cell.Physiol. 1992. 153. 53/61. Human umbilical vein endothelial cells submitted to hypoxia-reoxygenation in vitro: Implication of free radicals, xanthine oxidase, and energy deficiency.

16.Nishida, K.: J. Jpn. Soc. 1992. 93. 369/376. Measurement of free radical generation from endothelial cells and observation of cell injury exposed to anoxia-reoxygenation.

17.Palluy, O., Morliere, L., Gris, J.C., Bonne, C., and Modat, G. : Free Rad. Biol. Med. 1992. 13. 21/30. Hypoxia/reoxygenation stimulates endothelium to promote neutrophil adhesion.

18.Sacks, T., Moldow, C.F., Craddock, P.R., Bowers, T.K. : J. Clin. Invest. 1978. 1161/1167. Oxygen radicals mediate endothelial cell damage by complement-stimulated granulocytes.

19.Saugstad, O.D. : Ped.Res. 1988. 23. 143/150. Hypoxanthine as an indicator of hypoxia: Its role in health and disease through free radical production.

20.Shreeniwas, R., Koga, S., Karakurum, M., Pinsky, D., Kaiser, E., Brett, J., Wolitzky, B.A., Norton, C., Plocinski, J., Benjamin, W., Burns, D.K., Goldstein, A., and Stern, D. : J. Clin. Invest. 1992. 90. 2333/2339. Hypoxia-mediated induction of endothelial cell Interleukin-1α.

21.Stevens, P., and Hong, D. : Microchem. J. 1984. 30. 135/146. The role of myeloperoxidase and superoxide anion in the luminol-and lucigenin-dependent chemiluminescence of human neutrophils.

22.Suzuki, M., Inauen, W., Kvietys, P.R., Grisham, M.B., Meninger, C., Schelling, M.E., and Granger, D.N. : Am. J. Physiol. 1989. 257. H1740-H1745. Superoxide mediates reperfusion-induced leukocyte-endothelial cell interactions.

23.Takahashi, K., and Sawasaki, Y. : The Tissue Culture. 1992. 18. 40-45. The spontaneously transformed human endothelial cell line.

24.Terada, L.S., Guidot, D.M., Leff, J.A.,Willingham, I.R., Hanley, M.E., Piermattei, D., and Repine, J.E. : Proc. Natl. Acad. Sci. 1992. 89 3362/3366. Hypoxia injures endothelial cells by increasing endogenous xanthine oxidase activity.

25.Whorton, A.R., Mongomery,M.E.,and Kent, R.S. : J. Clin. Invest. 1985. 76 295/302. Effect of hydrogen peroxide on prostaglandin production and cellular integrity in cultured portine aortic endothelial cells.

26.Yoshida, N., Granger, D.N., Anderson, D.C., Rothlein, R., Lane, C., and Kvietys, P.R. : Am. J. Physiol. 1992. 262. H1891/H1898. Anoxia/ reoxygenation-induced neutrophil adherence to cultured endothelial cells.