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Effects of hypoxia/reoxygenation and cytokines on adhesion of leukocytes to cerebral microvascular endothelial cells[1]

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KEY WORDS tumor necrosis factor; interleukin-1; adhesions; vascular endothelium; vascular smooth muscle; anoxia; neutrophils; monocytes; flow cytometry

ABSTRACT

AIM: To study the influence of hypoxia/reoxygenation (H/R) on the adhesion of neutrophils (Neu) and monocytes (Mon) to cultured bovine cerebral microvascular cells induced by tumor necrosis factor α (TNF-α) or interleukin 1-α (IL-1α). **METHODS**: Bovine cerebral microvascular endothelial cells (CMEC) and smooth muscle cells (CMSMC) were exposed to normoxia or H/R in the presence or absence of TNF-α (IL-1α). The number of adhesions of Mon and Neu to CMEC and CMSMC was measured by flow cytometry. **RESULTS**: CMEC were subjected to hypoxia for 2 h followed by reoxygenation with TNF-α (2 $\mu g \cdot L^{-1}$) for 2 h, the adhesion rate of CMEC for Mon and Neu were increased to 35.0 % ± 0.9 % and 36.0 % ± 0.6 % (the TNF-α treated group was 28.9 % ± 1.1 % and 28.8 % ± 1.3 %), respectively. CMSMC were treated as above, the adhesion rate of Mon and Neu was enhanced to 49.9 % ± 0.4 % and 43.9 % ± 1.4 % (the TNF-α treated group was 34.0 % ± 1.9 % and 34.0 % ± 1.3 %), respectively. CMEC and CMSMC were treated by IL-1α, similar results were obtained. **CONCLUSION**: H/R enhanced the adhesion of Mon and Neu to CMEC and CMSMC induced by TNF-α or IL-1α.

INTRODUCTION

Ischemia and ischemia-reperfusion are likely to play an important role in the pathogenesis of a wide variety of clinical conditions such as peripheral vascular disease, stroke, myocardial infraction, etc. Reperfusion injury is associated with leukocyte accumulation and adhesion involved in this progress. Activation of multiple adhesion mechanisms during ischemia-reperfusion may be the result of indirect activation pathways, perhaps secondary to the release of inflammatory mediators such as IL-1, TNF, and IL-6 after cellular damage[1]. The purpose of the present work was not only to study the effects of H/R on adhesion of cerebral microvascular cells, but also to examine cooperative effects of H/R and TNF-α or IL-1α on the adhesion of Neu and Mon to cultured CMEC and CMSMC to mimic cerebral ischemia.

MATERIALS AND METHODS

Cell cultures Primary cultures of bovine CMEC and CMSMC were prepared from the neonatal bovine as previously described[2]. Cells were cultured in Eagle's minimum essential medium supplemented with 20 % bovine serum, benzylpenicillin 100 $kU \cdot L^{-1}$ and streptomycin 100 $mg \cdot L^{-1}$ at 37 ℃ in humidified 95 % O_2 + 5 % CO_2. Primary CMEC and CMSMC were obtained after 13 - 15 d, and passaged every 3 - 5 d. All experiments were performed on cells from 4 - 6 passage.

Preparation of Mon and Neu Blood was

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collected from adult Sprague-Dawley rats in heparin-saline. Mon and Neu were isolated and resuspended in PBS containing 0.1 % bovine serum albumin (BSA)[3]. The Mon and Neu concentrations were adjusted to $5 \times 10^9 \cdot L^{-1}$.

Hypoxia and reoxygenation Hypoxia was achieved by using an anaerobic jar containing 95 % N_2 and 5 % CO_2. Cultured CMEC or CMSMC were subjected to a hypoxic condition for 2 h by immediately replacing the medium with the hypoxic medium in the anaerobic jar. The medium was deoxygenated by bubbling the mixture gas (95 % N_2 and 5 % CO_2) for 45 min. Cells were reoxygenated for 2 h by immediately replacing the hypoxic medium with a normoxic serum-free medium.

Cell adhesion assays The assays for leukocyte adhesion to CMEC and CMSMC were carried out according to the method described previously[4]. CMEC and CMSMC monolayers cultured in 96-well plates were made quiescent for 24 h and treated by H/R and stimulated with TNF-α and IL-1α (a generous gift from Dainippon Pharmaceutical Co, Japan) as described below. Prior to the addition of Mon and Neu to monolayer, the CMEC and CMSMC monolayers were washed with PBS (containing 0.1 % BSA) three times. Mon or Neu (5×10^5 cells/well) were incubated with the monolayer of CMEC or CMSMC at 37 ℃ for 60 min. Nonadherent cells were removed by washing twice with Hanks' solution. The Mon or Neu adhering to the monolayer were detached in the presence of PBS containing edetic acid 10 mmol · L^{-1} and their numbers were counted by flow cytometry (FACS Ⅲ, BD, USA). Mon or Neu adhesion to CMEC or CMSMC was expressed as percent of total cell added.

Experimental protocol Cultured confluent CMEC and CMSMC were washed twice with PBS containing 0.1 % BSA. CMEC and CMSMC monolayers were subjected to 2 h of hypoxia (95 % N_2 and 5 % CO_2) followed by 2 h of reoxygenation, control being normoxia (95 % O_2 and 5 % CO_2). The confluent monolayers were either exposed to 2 h of hypoxia and followed by 2 h of reoxygenation with TNF-α ($2\ \mu g \cdot L^{-1}$) and IL-1α ($200\ kU \cdot L^{-1}$) or treated by TNF-α ($2\ \mu g \cdot L^{-1}$) and IL-1α ($200\ kU \cdot L^{-1}$) for 2 h.

Statistics Data were expressed as $\bar{x} \pm s$ and compared with t-test.

RESULTS

Effects of H/R and TNF-α (IL-1α) on adhesion of Mon and Neu to CMEC Adhesions of CMEC induced by TNF-α ($2\ \mu g \cdot L^{-1}$) and by IL-1α ($200\ kU \cdot L^{-1}$) to Mon and Neu were all shown to be significantly higher than control, and reached about 2.0-fold and 1.8-fold, respectively at 2 h. The adhesion rates of CMEC after treated by 2 h of hypoxia and 2 h of reoxygenation for Mon and Neu were increased by 20 % and 25 %, respectively ($P < 0.01$, *vs* control $n = 4$). CMEC was exposed to hypoxia for 2 h followed by reoxygenation and stimulation with TNF-α ($2\ \mu g \cdot L^{-1}$) or with IL-1α ($200\ kU \cdot L^{-1}$) for 2 h, the Mon and Neu adhesion rates were increased about by 20 % and 24 %, respectively, as compared with TNF-α treated group or IL-1α treated group ($P < 0.01$, Tab 1).

Effects of H/R and TNF-α (IL-1α) on adhesion of Mon and Neu to CMSMC Adhesion of CMSMC stimulated by TNF-α ($2\ \mu g \cdot L^{-1}$) and IL-1α ($200\ kU \cdot L^{-1}$) for 2 h to Mon and Neu increased by about 3.0-fold and 3.6-fold, respectively, as compared with control. CMSMC were subjected to 2 h of hypoxia and 2 h of reoxygenation, the adhesion of Mon and Neu was enhanced 22 % and 51 %, respectively ($P < 0.01$). If CMSMC were exposed to H (2 h)/R (2 h) and TNF-α ($2\ \mu g \cdot L^{-1}$), simultaneously, the adhesion of Mon and Neu to CMSMC was increased by 47 % and 29 %, respectively, as compared with TNF-α treated group. CMSMC were treated by H (2 h)/R

Tab 1. Effects of H/R and TNF-α (IL-1α) on adhesion of Mon and Neu to CMEC and CMSMC. $n = 4$ experiments, $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* TNF-α. $^iP < 0.01$ *vs* IL-1α.

Treatment	Mon adhesion rate/%	Neu adhesion rate/%
CMEC		
Control	13.7 ± 0.7	14.9 ± 0.3
H/R	16.5 ± 0.9^c	18.6 ± 0.9^c
TNF-α (2 μg·L^{-1})	28.9 ± 1.1^i	28.8 ± 1.3^c
IL-1α (200 kU·L^{-1})	27.8 ± 1.9^i	27.6 ± 2.8^c
H/R + TNF-α (2 μg·L^{-1})	35.0 ± 0.9^i	36.0 ± 0.6^f
H/R + IL-1α (200 kU·L^{-1})	33.4 ± 1.7^i	34.2 ± 2.2^i
CMSMC		
Control	10.7 ± 1.25	9.0 ± 1.0
H/R	13.0 ± 3.0^c	13.7 ± 0.6^i
TNF-α (2 μg·L^{-1})	34.0 ± 1.9^c	34.0 ± 1.3^c
IL-1α (200 kU·L^{-1})	32.1 ± 1.5^i	32.1 ± 1.4^c
H/R + TNF-α (2 μg·L^{-1})	49.9 ± 0.4^i	43.9 ± 1.4^f
H/R + IL-1α (200 kU·L^{-1})	47.7 ± 1.2^i	43.9 ± 1.0^i

(2 h) and IL-1α (200 kU·L^{-1}), the adhesion of Mon and Neu was increased by 48 % and 37 %, respectively over IL-1α treated group ($P < 0.01$, Tab 1).

DISCUSSION

Hypoxia and reoxygenation are principal components of ischemia/reperfusion and have distinctive effects on the tissue. The experiment above utilized an *in vitro* model of hypoxia and reoxygeration of cultured CMEC and CMSMC to mimic I/R *in vivo* and to elucidate the effects of H/R on the adhesion.

The binding of neutrophils to endothelial cells is a prerequisite for tissue injury. Rapid infiltration of neutrophils into reperfused tissue is known to play a pivotal role in reperfusion injury[5]. Recruitment of monocytes to the endothelial space is an important event in the early stage of atherogenesis[6]. Accumulating evidences have suggested that VSMC not only participate in atherogenesis as a structure component of the lesions, but also actively contribute to local inflammatory and immune reactions[7].

Adhesion of neutrophils to cultured endothelial cells after H/R was increased[8]. Our results showed that H/R enhanced the adhesion of Mon and Neu to CMEC. Bevilacqua *et al* showed that TNF and IL-1 stimulated endothelial cells to become more adhesive for neutrophils[9]. Our data indicated that TNF-α and IL-1α increased the adhesion of Mon and Neu to CMEC, in agreement with Bevilacqua. IL-1β stimulates the adhesion of SMC to monocytes and neutrophils[7]. But, there was no report about the effect of H/R on the adhesion of SMC. Our data indicated that TNF-α and IL-1α significantly increased the adhesion of Mon and Neu to CMSMC. H/R significantly enhanced the adherence of CMSMC for Mon and Neu.

Our data also indicated that H/R significantly increased the adherence of CMEC and CMSMC induced by TNF-α and IL-1α for Mon and Neu. The cooperative effects of H/R and cytokines exacerbated the adhesion and injury of cerebral microvascular cells.

In conclusion, the study suggested that there was basal adhesion of Mon and Neu to CMEC and CMSMC. The cytokines such as TNF-α and IL-1α and H/R increased the adhesion of Mon and Neu. H/R may enhance the adhesion of CMEC and CMSMC induced by cytokines to Neu and Mon.

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缺氧/再给氧和细胞因子对白细胞与脑微血管内皮细胞粘附作用的影响[1]

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关键词 肿瘤坏死因子；白细胞介素 1；粘附；血管内皮；血管平滑肌；缺氧症；中性白细胞；单核细胞；流动血细胞计数

目的：研究缺氧/再给氧（H/R）对中性粒细胞（Neu）和单核细胞（Mon）与诱导的培养牛脑微血管细胞粘附作用的影响。**方法**：牛脑微血管内皮细胞（CMEC）和平滑肌细胞（CMSMC）经 H/R 处理或经 TNF-α 和 IL-1α 刺激，Mon 和 Neu 与 CMEC 和 CMSMC 粘附的细胞数目用流式细胞仪测定。**结果**：CMEC 先缺氧 2 h 再经复氧与 TNF-α（2 μg·L^{-1}）作用 2 h，Mon 和 Neu 与 CMEC 的粘附率分别提高到35.0 % ± 0.9 %和36.0 % ± 0.6 %（TNF-α 处理组分别是 28.9 % ± 1.1 % 和 28.8 % ± 1.3 %）。CMSMC 也按上述处理，Mon 和 Neu 的粘附率分别显著提高为49.9 % ± 0.4 %和43.9 % ± 1.4 %（TNF-α 处理组分别为34.0 % ± 1.9 %和 34.0 % ± 1.3 %）。CMEC 和 CMSMC 经 IL-1α 刺激，可得到类似结果。**结论**：H/R 提高 Mon 和 Neu 与 TNF-α 和 IL-1α 诱导的 CMEC 和 CMSMC 的粘附作用。

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