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Original Research

Pivotal role of glutathione depletion in eNOS uncoupling of LPS-Treated HUVECs

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Abstract: The present study investigated the relationship between uncoupling of endothelial nitric oxide synthase (eNOS) and vascular endothelial cell (VEC) oxidative stress (OS) during sepsis and the role of eNOS glutathionylation in eNOS uncoupling of *septic* VECs. Human umbilical vein endothelial cells (HUVECs) cultured *in vitro* (EA.hy269 cell line) were incubated with Dulbecco's modified Eagle's medium (DMEM) (normal control group), lipopolysaccharide (LPS) (sepsis group), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (glutathionylation group), and LPS+ dithiothreitol (DTT) (deglutathionylation sepsis group). As result, compared with the DMEM group, malondialdehyde (MDA) level and uncoupling eNOS activity significantly increased in the LPS and BCNU groups. However, in the LPS + DTT group, only the NO level increased. Compared with the LPS group, MDA level, NO concentration, and normal functional eNOS activity significantly decreased, and uncoupling eNOS activity significantly increased in the BCNU group. In the LPS + DTT group, MDA level and uncoupling eNOS activity significantly decreased, and NO concentration and normal functional eNOS activity significantly increased. During sepsis, the main mechanism for VEC OS was eNOS uncoupling mediated by eNOS glutathionylation.

Key words: Endothelial nitric oxide synthase, glutathionylation, synthesis, uncoupling, vascular endothelial cells.

Introduction

Sepsis refers to the systemic inflammatory response syndrome caused by infections; further development can cause very severe conditions including septic shock, severe sepsis, multiple organ dysfunction syndromes, and even death (1, 2). In recent years, the mortality rate of sepsis in the young population has decreased (3) because of the implementation of Surviving Sepsis Campaign: International Guidelines for Management of Severe Sepsis and Septic Shock (4-6). However, the statistical analysis from intensive care units have indicated that the mortality rates of sepsis and septic shock are still as high as 30% and 60%, respectively (7, 8).

The pathogenesis mechanism of sepsis is very complicated. The major pathophysiological processes include cytokine storm, inflammatory waterfall-like reaction, enteral bacterial translocation, intestinal-derived endotoxin, interplay between coagulation and inflammation systems, oxidative stress (OS), immune cell apoptosis, microcirculation, and mitochondrial dysfunction(9-11). Of these, vascular endothelial cell (VEC) dysfunction plays a major role in the pathogenesis for sepsis. During chronic inflammation, the VEC dysfunction mainly shows endothelial nitric oxide (NO) synthase (eNOS) dysfunction. As we know that NO, as the most important diastolic regulation factor, plays an important role in the regulation of VECs. At present a lot of researches have confirmed that the decreased NO concentration is the most important reason to aggravate vascular endothelial dysfunction in the pathological process of chronic inflammatory vascular disease (12). Malondialdehyde (MDA), as a product of lipid oxidative damage, is a classic biomarker to evaluate the degree of oxidative stress damage (13). Typically, eNOS exists in the form of dimer, and electron transfer and NO synthesis are closely related, so it can catalyze the synthesis of NO. However, in pathological conditions, the dimer dissociates into monomers which can not complete the chain reaction in the process of electron transfer, and emergence of uncoupling electron is likely to be abnormally passed to O_2, generating superoxide anion (O_2^-). In the end, eNOS results in the production of superoxide instead of NO (14). It results in decreased bioavailability of NO and increased OS, further causing or aggravating endothelium relaxation dysfunction, which is called eNOS uncoupling (15).

The mechanism of eNOS uncoupling includes (16, 17): (1) lack of NO-generating substrate, L-arginine (L-Arg); (2) insufficient supply of important cofactor, tetrahydrobiopterin (BH4), generated *from* NO; and (3) changed structure of eNOS induced by OS, such as decreased activity induced by eNOS glutathionylation. Recently, Chen et al. (18) found that the site of eNOS glutathionylation was Cys 689 and Cys 908. Meanwhile, it was also proved that glutathione reductase inhibitor, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) or oxidized glutathione could result in eNOS glutathionylation. 2-mercaptoethanol or dithiothreitol (DTT) could reversibly reduce the glutathionylated eNOS.

In the study on sepsis, Huet et al. reported using *in vitro* culture (19, 20) of human umbilical vein endothelial cell (HUVEC) and *incubation of serum from sepsis patients*, the reduced glutathione concentration in VECs played important roles in cell damage caused by ROS during sepsis. It was found that not only ROS generation mediated by sepsis serum, but also the death rate were related to the decrease in intracellular glutathione

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Table 1. Group of EA.hy926 cell line.

Group	Abbreviation	Treatment factor
Control group	DMEM	DMEM high-glucose (25 mmol/L) culture medium (Gibco, USA, 592)
Sepsis group	LPS	1 mg/L LPS (Sigma, USA) (22) + DMEM high-glucose (25 mmol/L) culture medium (Gibco, USA)
Glutathione depletion group	BCNU	5 mg/L BCNU (Sigma, USA) + DMEM high-glucose (25 mmol/L) culture medium (Gibco, USA)
Deglutathionylation sepsis group	LPS + DTT	1 mg/L LPS + 2 mmol/L DTT (Sigma, USA) + DMEM high-glucose (25 mmol/L) culture medium (Gibco, USA)

level.

What is the relationship between VEC OS of sepsis and eNOS uncoupling? What is the mechanism for eNOS uncoupling during sepsis? Based on the cell model, the relationship between sepsis VEC OS and eNOS uncoupling and the possible mechanism for eNOS uncoupling were explored, which could provide evidence for treating OS of sepsis at an early stage.

Materials and Methods

Endothelial cells

HUVECs (EA.hy269) were obtained from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured as previously described (21).

After EA.hy926 cell line was cultured to 60%–70%, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) without serum for 12 h, washed with phosphate-buffered saline (PBS) twice, treated according to Table 1, and cultured for 12 h.

Cell viability and apoptosis assays

HUVECs (2×10^4/well) in each group were seeded in 96-well plates (Costar, Corning, USA) and incubated for 12 h. The cell viability was determined from the reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) to formazan. The absorbance at 550 nm was recorded in each well using an enzyme-linked immunosorbent assay (ELISA) microplate reader. The results were expressed as % of viable cells ± standard error of means relative to cells in the culture medium alone (defined as 100% viability). Cell viability was monitored in all cases using the crystal violet assay (23).

OS levels

The activities of *malondialdehyde (MDA)* were measured in HUVECs after 12 h of exposure in each group. MDA levels were measured with a commercially available kit (Ransod, Randox Laboratories, Montpellier, France) based on the method of thiobarbituric acid colorimetry.

The activities of NO were measured in HUVECs after 12 h of exposure in each group. NO levels were measured with a commercially available kit (Ransod, Randox Laboratories) based on the method of nitrate reductase activity determination.

The activities of superoxide dismutase (SOD) were measured in HUVECs after 12 h of exposure in each group. SOD activities (T-SOD) were measured with a commercially available kit (Ransod, Randox Laboratories) based on the method developed by McCord and Fridovich (24).

Intracellular eNOS levels

The intracellular difference in eNOS levels were assessed after 12 h of exposure in each group. Total eNOS activities (teNOS) were measured with a commercially available kit (Ransod, Randox Laboratories) based on ELISA (25). Normal functional eNOS activities (feNOS) were measured by the conversion of L-arginine to NO using a nitric oxide synthase assay kit (Beyotime Institute of Biotechnology, Nanjing, China) as previously described (26). Uncoupling eNOS activity (ueNOS) was calculated as the difference between teNOS and feNOS activities.

Detection for BH4 and L-Arg level

After incubation for 12 h, the EA.hy269 cells were washed with 2 mL of cold PBS three times and cleaned. Then, cold extracting solution (50 mmol/L Tris-HCl, pH 7.4; 1 mmol/L EDTA) was added. The cells were subjected to repetitive freeze–thaw cycles three times (low temperature –80ºC, high temperature 40ºC), and centrifuged at 15,000 rpm for 10 min under 4ºC. The supernatant was extracted, and the protein concentration in the supernatant (10 μL) was detected using a bicinchoninic acid(BCA) kit (Beyotime Biotechnology, Nanjing, China). The supernatant (200 μL) was taken, added to reaction buffer (1.8 mL), and then poured onto a piece of absorbent filter paper (Matsumoto Institute of Life Science, Kanazawa, Japan). All samples were stored at –20°C, and within 1 week, they were sent to the Laboratory of Inborn Errors of Metabolism (Sixth Affiliated Hospital of Sun Yat-Sen University) for metabolite profile analysis.

A clean urine sample (2 mL) was collected from each participant within the first 24 h after birth, using a urine collection bag. The urine was poured either into a drying tube or onto a piece of absorbent filter paper (Matsumoto Institute of Life Science). All samples were stored at –20°C, and within 1 week, they were sent to the laboratory for metabolite profile analysis.

A JMS-Q1000GC UltraQuad (Akishima, Tokyo, Japan) gas chromatograph/mass spectrometer was used for GC/MS measurement. Aliquots (1 μL) of derived extracts were injected into the apparatus using an automatic injection mode. The separation was carried out using an Ultra ALLOY metal capillary column UA5-30M-0.25F (Koriyama, Fukushima, Japan). The oven temperature was programmed to increase 17°C/min from 60°C to 220°C, hold at 220°C for 2 min, and then

increase 15°C/min from 220°C to 325°C, with a final holding at 325°C for 10 min. The temperatures of the injection port, ion source, and transfer line were 260°C, 200°C, and 220°C, respectively. Electron ionization mass spectra were obtained by repetitive scanning at 2.5 cycles/s, from *m/z* 50 to *m/z* 650. Helium gas was used as the carrier at a flow rate of 1 mL/min.

The gas chromatography/mass spectrometry (GC/MS) data were analyzed and interpreted based on the JEOL GC-MS analysis system (MILS, Kanazawa, Japan) and National Institute of Standards and Technology database, and the positive cases were reviewed by experts from both sides. The laboratory routinely conducts internal quality control and participates in external quality assessments organized by the European Research Network for Evaluation and Improvement of Screening, Diagnosis and Treatment of Inherited Disorders of Metabolism.

Statistical analysis

Statistical analyses were conducted using the SPSS software (version 13.0; SPSS, IL, USA). Normally distributed variables were presented as mean ± standard deviation, and the differences between the two groups were analyzed using the *t* test. A P value < 0.05 was considered statistically significant. The comparisons of average values among groups were analyzed using analysis of variance, and the comparisons between two average values with statistical significance were tested. Those with homogeneity of variance were tested by least significant difference, and those without homogeneity of variance were tested by Games-Howell test. P less than 0.05 was considered statistically significant.

Results

eNOS uncoupling cell model

BCNU (concentrations 0.1, 0.5, 2, 5, 20, and 40 mg/L) was used to culture the EA.hy269 cell line for 12 h, and the MTT method was used to test cell viability. Figure 1A shows that as the BCNU concentration increased, the cell viability gradually decreased. The group treated with 20 and 40 mg/L showed a significant difference compared with other groups ($P < 0.05$). Figure 1B shows that the degree of eNOS uncoupling was increased with the concentration of BCNU. The group treated with 5, 20 and 40 mg/L showed no difference ($P > 0.05$).

By optimizing, 5 mg/L BCNU was chosen to build the cell model. Uncoupling eNOS levels at 6, 12, and 24 h were detected. As showed in Figure 2, compared with the DMEM group, the uncoupling eNOS level in the BCNU group significantly increased ($P < 0.05$), and showed a significant difference 12 h after incubation ($P < 0.001$). The desirable eNOS uncoupling VECs model was built using 5 mg/L BCNU after 12-h incubation.

Cell model of sepsis OS

The EA.hy269 cell line incubated with LPS is the common sepsis VECs model. In the present study, 1 mg/L LPS was used to incubate the EA.hy269 cell line and OS levels at 6, 12, and 24 h were detected. As illustrated in Figure 3A–B, compared with the DMEM culture medium group, the ROS level in the LPS group

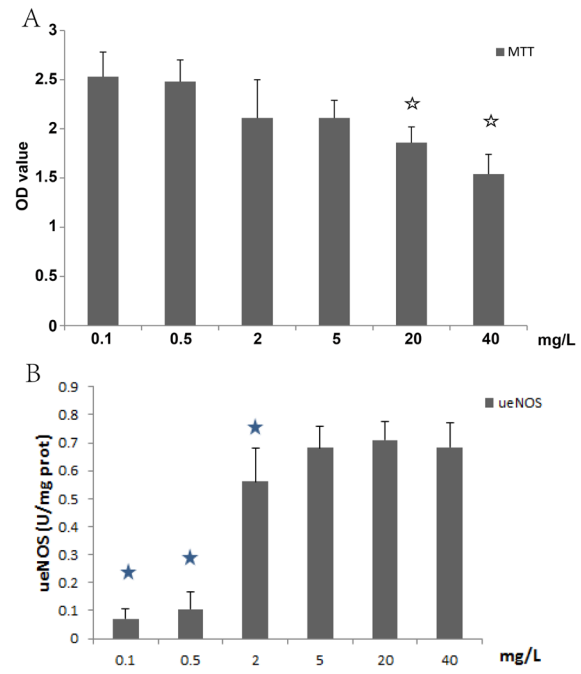

Figure 1. Influence of different BCNU concentrations on EA.hy926 cell viability and ueNOS level. The EA.hy926 cell line was cultured with different concentrations (0.1, 0.5, 2, 5, 20, and 40 mg/L) of BCNU for 12 h. MTT was used to detect cell viability, the total eNOS and eNOS activity was detected, the difference was the uncoupled eNOS activity expressed as ueNOS(25,26), and the results were expressed as mean ± standard deviation (SD). The experiment was repeated three times. Figure A shows that as the BCNU concentration increased, the cell viability gradually decreased. When the BCNU concentration was higher than 20 mg/L, the cell viability significantly decreased ($P < 0.05$). ☆ Represents $P < 0.05$ compared with other groups. Figure B shows that the degree of eNOS uncoupling was increased with the concentration of BCNU. The group treated with 5, 20 and 40 mg/L showed no difference ($P > 0.05$). ★ Represents $P < 0.05$ compared with the 20mg/L group.

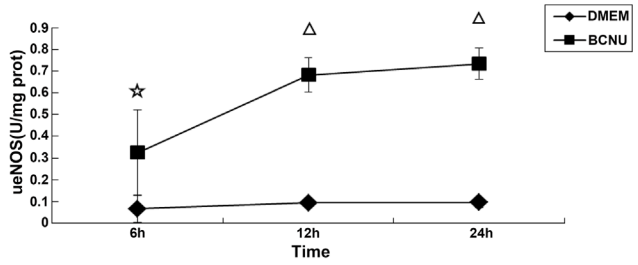

Figure 2. Changing trend for uncoupling eNOS. EA.hy269 was cultured with BCNU (5 mg/L), and the total eNOS and eNOS activity was detected at 6, 12, and 24 h. The difference was the uncoupled eNOS activity expressed as ueNOS(25,26). At the same time point, EA.hy269 cultured with DMEM was used as the control group, and the result was expressed as mean ± SD. The experiment was repeated three times. The results showed that compared with the DMEM group, the ueNOS activity at each time point significantly increased in the BCNU group ($P < 0.05$). ☆ Represents $P < 0.05$, and △ represents $P < 0.001$.

significantly increased ($P < 0.001$), and the NO concentration in the culture medium decreased with time ($P < 0.05$). It suggested that sepsis VEC OS model could be built by incubating the EA.hy269 cell line with LPS for 12 h.

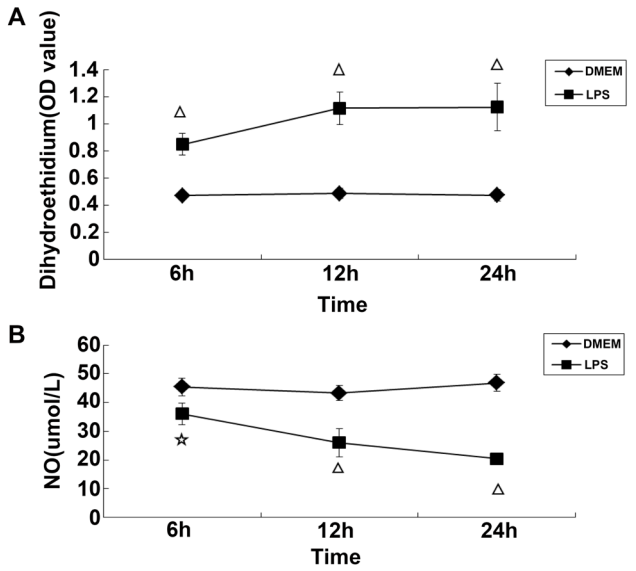

Figure 3. Changing trend for ROS and NO. LPS (1 mg/L) was used to culture EA.hy269, and intracellular ROS levels and NO levels in the culture medium were detected by the dihydroethidium fluorescence probe method at 6, 12, and 24 h. Figure A showed that compared with the DMEM group, ROS levels in the LPS group significantly increased from 6h to 12h ($P < 0.001$). Figure B showed that compared with the DMEM group, NO levels at each time point in the LPS group significantly increased ($P < 0.05$). The result was expressed as mean ± SD, and corrected by MTT. The experiment was repeated three times. ☆ Represents $P < 0.05$, and △ Represents $P < 0.001$.

Cell morphology and cell viability

EA.hy269 cells were given LPS (1 mg/L), BCNU (5 mg/L), LPS (1 mg/L) + DTT (2 mmol/L), and DMEM high glucose (25 mmol/L) for 12 h. The morphology of all the groups was similar. MTT was used to detect cell viability and repeated three times. Considering the detected optical density value as unit, the cell activity in each group was as follows: LPS group, 2.22; BCNU group, 2.11, DTT+LPS group, 2.36; and DMEM group, 2.53. No statistical difference was found among the cell activities in the groups ($P > 0.05$), as shown in Figure 4.

Relationship between eNOS uncoupling and sepsis OS

As shown in Figure 5 and Table 2, in the eNOS uncoupling cell model, compared with the DMEM group, MDA level and T-SOD activity in the BCNU group significantly increased ($P < 0.05$), the ROS level detected by dihydroethidium fluorescence probe also significantly increased ($P<0.05$), but the NO concentration significantly decreased ($P < 0.05$). As illustrated in Figure 6, in the sepsis VEC OS model, compared with the DMEM group, intracellular uncoupling eNOS activity significantly increased in the LPS group ($P < 0.05$).

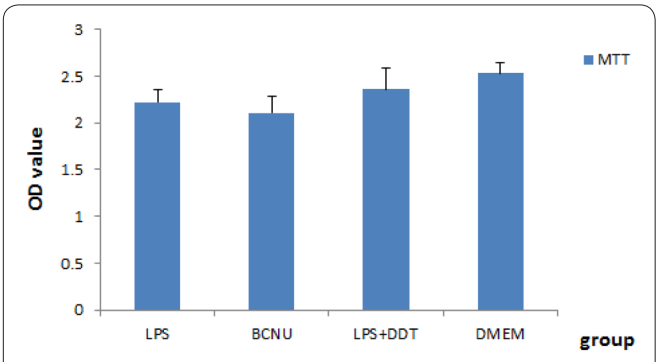

Figure 4. Comparisons of EA.hy926 cell viability in each group. EA.hy926 cell line was stimulated by LPS (1 mg/L), BCNU (5 mg/mL), LPS (1 mg/L) +DTT (2 mmol/L), and DMEM high glucose (25 mmol/L) for 12 h. MTT was used to detect cell viability, and no significant difference was found among the groups ($P > 0.05$). The experiment was repeated three times.

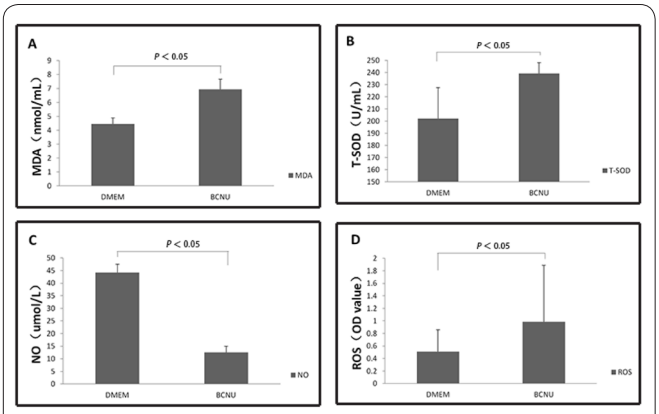

Figure 5. Oxidative stress condition at 12 h in the eNOS uncoupling cell model. EA.hy269 was cultured with BCNU (5 mg/L) and DMEM for 12 h, and MDA level, T-SOD activity, NO concentration and ROS level was detected. The results showed that in the eNOS uncoupling cell model, compared with the DMEM group, MDA level, T-SOD activity and the ROS level in the BCNU group significantly increased ($P < 0.05$), but the NO concentration significantly decreased ($P < 0.05$). The result was expressed as mean ± SD, and repeated three times.

Mechanism for sepsis uncoupling

As shown in Figure 7, 12 h after incubating the EA.hy269 cell line with LPS, intracellular BH4 and L-Arg levels did not change significantly compared with the DMEM group ($P > 0.05$).

As shown in Figure 8 and Table 3, compared with the DMEM group, at 12 h after incubation, the MDA levels in the LPS and BCNU groups both significantly increased ($P < 0.01$), and NO levels significantly decreased ($P < 0.01$). However, no significant difference was found in the T-SOD activity ($P>0.05$), suggesting that the OS in sepsis model and OS in the eNOS uncoupling model both could result in VECs dysfunction. Nevertheless, compared with the BCNU group, the MDA level in the LPS group more significantly increased (*P*

Table 2. Oxidative stress condition at 12 h in the eNOS uncoupling cell model (mean ± SD, $n = 3$).

Group	MDA (nmol/mL)	T-SOD (U/mL)	NO (μmol/L)	ROS (OD value)
DMEM	4.457 ± 0.431	202.16 ± 25.41	44.15 ± 3.39	0.510 ± 0.346
BCNU	6.941 ± 0.713★	239.28 ± 8.84★	12.56 ± 2.45★	0.987 ± 0.902★

Note: ★ Compared with the DMEM group, $P < 0.05$.

Table 3. Comparison between sepsis and eNOS glutathionylation (mean ± SD, $n = 3$).

	LPS	BCNU	DMEM	*F*	*P*
MDA	8.286 ± 2.058★▲	6.328 ± 1.299★	4.414 ± 0.682	10.561	0.001
T-SOD	220.15 ± 26.08	223.14 ± 28.58	203.68 ± 20.15	1.038	0.378
NO	31.29 ± 7.32★▲	15.81 ± 7.01★	43.73 ± 2.74	31.946	0.001
teNOS	1.048 ± 0.085	0.952 ± 0.155	0.890 ± 0.063	3.285	0.066
feNOS	0.502 ± 0.090★▲	0.301 ± 0.172★	0.774 ± 0.068	23.934	0.001
ueNOS	0.547 ± 0.107★▲	0.651 ± 0.078★	0.116 ± 0.035	77.518	0.001

Note: teNOS, total eNOS; feNOS, normal functional eNOS; ueNOS, uncoupled eNOS. Unit: MDA (nmol/mL); T-SOD (U/mL), NO (μmol/L), NOS (U/mg prot). ★ compared with DMEM group, $P < 0.05$; ▲ compared with BCNU group, $P < 0.05$.

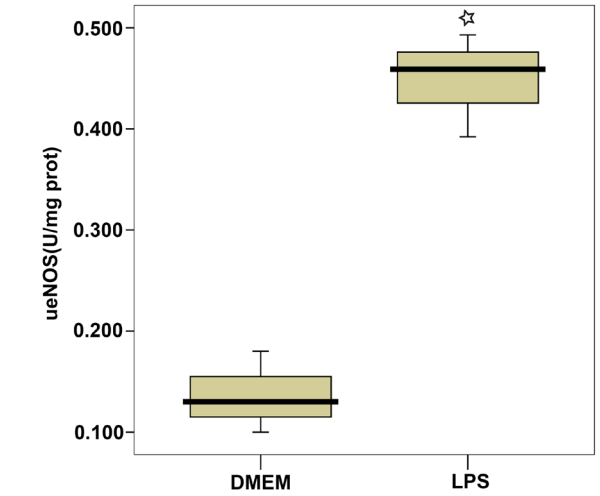

Figure 6. The uncoupled eNOS in the sepsis oxidative stress cell model. EA.hy269 was cultured with LPS (1 mg/L) for 12 h, and the total eNOS and the eNOS activity was detected. The difference was the uncoupled eNOS activity expressed as ueNOS. The results showed that compared with the DMEM group, the intracellular uncoupling eNOS activity in the LPS group significantly increased ($P < 0.05$). The result was expressed as mean ± SD, and corrected by MTT. The experiment was repeated three times. ☆ Represents $P < 0.05$.

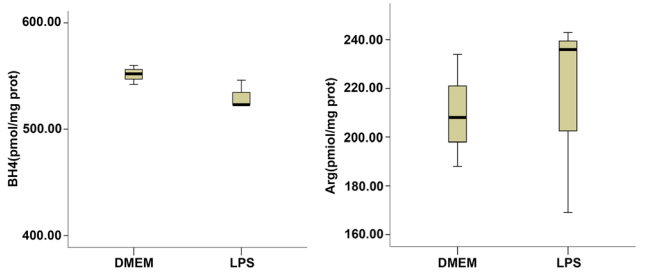

Figure 7. (A) BH_4 level. After culturing EA.hy269 with LPS (1 mg/L) for 12 h, the mass spectrum was used to detect the intracellular BH4 level. The result was corrected by MTT and expressed as mean. The experiment was repeated three times. The results showed that compared with the DMEM group, intracellular BH_4 in the LPS group did not change significantly ($P > 0.05$). **(B) L-Arg level.** After culturing EA.hy269 with LPS (1 mg/L) for 12 h, the mass spectrum was used to detect the intracellular L-Arg level. The result was corrected by MTT and expressed as mean. The experiment was repeated three times. No significant difference was found in the intracellular L-Arg level in DMEM and LPS groups ($P > 0.05$).

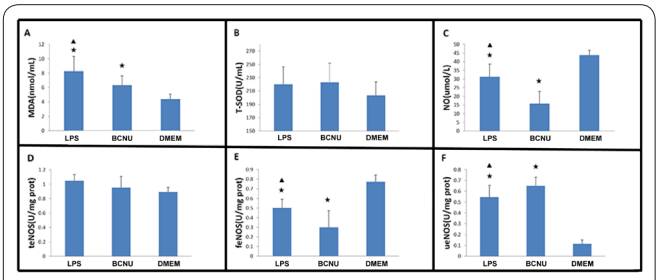

Figure 8. Comparison between sepsis and eNOS glutathionylation. EA.hy269 was cultured with LPS (1 mg/L), BCNU (5 mg/L) and DMEM for 12 h. Then MDA level, T-SOD activity, NO concentration, the total eNOS and eNOS activity was detected, and the uncoupled eNOS activity was calculated. The experiment was repeated three times. ★ compared with DMEM group, $P < 0.05$; ▲ compared with BCNU group, $P < 0.05$.

< 0.01), but T-SOD activity showed no significant difference ($P > 0.05$). It was suggested that the degree of VECs dysfunction induced by sepsis model was even more severe.

Meanwhile, compared with the DMEM group, intracellular total eNOS levels in the LPS and BCNU groups slightly increased, but with no statistical difference. Furthermore, normal functional eNOS (generating NO by catalytic reaction) levels significantly decreased and the uncoupling eNOS (generating ROS by catalytic reaction) level increased ($P < 0.01$), suggesting that glutathionylation of sepsis and eNOS both could result in eNOS uncoupling. Compared with the BCNU group, normal functional eNOS activity in the LPS group was higher ($P < 0.01$), the uncoupling eNOS activity was not as high as that in the BCNU group ($P < 0.01$), but the total eNOS activities in both the groups were equal, indicating that the degree of eNOS uncoupling caused by sepsis was not as good as single eNOS glutathionylation, as shown in Figure 8 Table 3.

As shown in Figure 9 and Table 4, compared with the DMEM group, the MDA level significantly increased in the LPS group ($P < 0.01$), the NO level significantly decreased ($P < 0.01$), and no significant difference was observed in the T-SOD activity (P>0.05). Also, the MDA level and T-SOD activity in the LPS + DTT group did not change significantly ($P > 0.05$) and the NO level significantly increased ($P < 0.01$). Compared with the LPS group, the MDA level in the LPS + DTT group significantly decreased, the NO concentration significant-

Table 4. Comparison between sepsis and eNOS deglutathionylation (mean ± SD, $n = 3$).

	LPS	LPS + DTT	DMEM	*F*	*P*
MDA	8.286 ± 2.058★	5.046 ± 1.114▲	4.414 ± 0.682	13.072	0.001
T-SOD	220.15 ± 26.08	198.28 ± 14.62	203.68 ± 20.15	1.797	0.200
NO	31.29 ± 7.32★	57.24 ± 15.33★▲	43.73 ± 2.74	10.244	0.002
teNOS	1.048 ± 0.085	1.054 ± 0.184	0.890 ± 0.063	3.500	0.057
feNOS	0.502 ± 0.090★	0.815 ± 0.108▲	0.774 ± 0.068	21.291	0.001
ueNOS	0.547 ± 0.107★	0.240 ± 0.178▲	0.116 ± 0.035	19.921	0.001

Note: teNOS, total eNOS; feNOS, normal functional eNOS; ueNOS, uncoupling eNOS. Unit: MDA (nmol/mL), T-SOD (U/mL), NO (μmol/L), NOS (U/mg prot). ★ compared with DMEM group, $P < 0.05$; ▲ compared with the LPS group, $P < 0.05$.

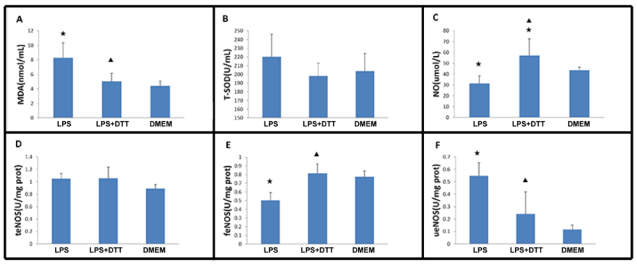

Figure 9. Comparison between sepsis and eNOS deglutathionylation. EA.hy269 was cultured with LPS (1 mg/L), BCNU (5 mg/L) and DMEM for 12 h. Then MDA level, T-SOD activity, NO concentration, the total eNOS and eNOS activity was detected, and the uncoupled eNOS activity was calculated. The experiment was repeated three times. ★ compared with DMEM group, $P < 0.05$; ▲ compared with BCNU group, $P < 0.05$.

ly increased ($P < 0.01$), and the T-SOD activity showed no significant difference ($P > 0.05$). It was suggested that after glutathionylation modification of eNOS was removed by sepsis, the uncoupling of eNOS improved. Furthermore, the OS of cells was also significantly alleviated, VECs dysfunction was recovered, and even the NO level increased.

Compared with the DMEM group, the intracellular total eNOS level in the LPS group did not change significantly ($P > 0.05$), the normal functional eNOS level significantly decreased, and the uncoupling eNOS level increased ($P < 0.01$). However, total eNOS, normal functional eNOS, and uncoupling eNOS levels in the LPS + DTT group did not change *significantly*. Compared with the LPS group, the normal functional eNOS activity in the LPS + DTT group was higher ($P < 0.01$) and the uncoupling eNOS activity significantly decreased ($P < 0.01$). It was suggested that the removal of glutathionylation modification of eNOS by sepsis could lead to the recovery of uncoupled eNOS activity, and decrease of uncoupled eNOS activity, as shown in Figure 9 and Table 4.

Discussion

This study explored the relationship between VECs dysfunction and OS in sepsis and the pivotal role of glutathione depletion in septic endothelial eNOS uncoupling. Using stable LPS-induced cell model and BCNU-induced cell model, the study demonstrated a mutually promoting, reciprocal relationship between OS and eNOS uncoupling, which was in line with the findings of previous studies (15, 27). Moreover, it was found that during sepsis, the glutathione level not only reflected the OS degree, but was significantly related with the prognosis of patients (19, 20). However, no significant differences were found in the concentrations of BH_4 and L-Arg in the case of septic endothelial eNOS uncoupling; rather the rate of glutathione depletion was enhanced in acute sepsis.

A previous study confirmed the mechanisms for eNOS uncoupling in chronic inflammations (such as coronary artery atherosclerotic plaque and diabetes) (16, 17) as follows. (1) The insufficient BH_4 level for eNOS uncoupling was the main reason. The decrease in the BH_4 level was mainly caused by the decreased activity of dihydrofolate reductase and guanosine triphosphate cyclohydrolase I (GTPCH-I) during its biological synthesis (28). In the mice fed a high-salt diet, the decreased BH_4 level caused eNOS uncoupling, and the increase in O2- from eNOS caused a decrease in the vasodilation ability (29). Contrarily, the supply of BH_4 could reverse eNOS dysfunction. For example, BH_4 given to aorta separated from the hypertension model could decrease ROS derived from NOS. BH_4 from ergogenic supplement could decrease eNOS uncoupling after myocardial infarction, decrease generation of peroxides, and slow down cardiac remodeling process (30). (2) The L-Arg level was insufficient to cause eNOS uncoupling. L-Arg not only was the substrate of eNOS, but also played a competitive effect with asymmetric dimethylarginine (ADMA), a kind of endogenous inhibitor. Under OS, the activity of protein-arginine methyl transferases (PRMT) increased and the activity of ADMA degrading enzyme, dimethylarginine dimethylaminohydrolase, decreased, leading to an increase in the ADMA level (31, 32). Furthermore, 24 h after stimulating HUVECs using thrombin or inflammatory factor, the expression level of arginase II in endothelial cells did not change, but its activity significantly increased, resulting in a decreased L-Arg level (33). (3) eNOS glutathionylation could result in eNOS uncoupling. Two conserved Cys (Cys689 and Cys908) in NOS family played a very important role in maintaining the normal activity of eNOS. Once they were glutathionylated, NOS generated O_2^- by catalysis, and it could not be inhibited by L-NAME and removing Ca^{2+}. Furthermore, BCNU could also cause

eNOS glutathionylation and result in endothelial dysfunction (16). Moreover, eNOS glutathionylation in rats with spontaneous hypertension increased, and the endothelium dysfunction was also reversed by thiol-specific antagonists (such as dithiothreitol) (18).

The present study found that in the sepsis cell model based on EA.hy926 stimulated by LPS, the mechanism for eNOS uncoupling was different from that in chronic inflammations. Under sepsis, BH_4 and L-Arg levels did not significantly decrease, suggesting that in the acute pathological processes such as sepsis, BH_4 and L-Arg were not the main reason for eNOS uncoupling.

This study used methods with different testing principles for eNOS. ELISA could detect all the intracellular eNOS *level.* The fluorescence probe could only detect normal functional eNOS activity. The difference between them was mainly uncoupling eNOS, *so ueNOS could be used for analysis*. It has been reported that (18) BCNU and DTT were used to culture EA.hy926 for simulating eNOS glutathionylated *and* eNOS deglutathionylated cells, and the relationship between eNOS glutathionylation and eNOS uncoupling under sepsis was confirmed. In order to construct the stable eNOS uncoupling cell model in our study, we cultured the EA.hy269 cell line for 12 h with 0.1, 0.5, 2, 5, 20, and 40 mg/L BCNU respectively, and then the MTT method was used to test cell viability. As a result, the cell viabilities of EA.hy269 cell line incubated in 20 or 40mg/L BCNU decreased significantly compared to in 0.1, 0.5, 2 or 5mg/L. However, the degree of eNOS uncoupling was increased with the concentration of BCNU. Accordingly, we chose 5 mg/L as the threshold of BCNU concentration, which is the concentration could be ensured both the viability of cells and the decoupling effect as far as possible. Furthermore, in our study compared with the DMEM group, the uncoupling eNOS level in the 5mg/L BCNU group significantly increased ($P<0.05$), and showed a significant difference 12 h after incubation ($P<0.001$)

In the present study, intracellular eNOS was divided into two types (NO generation and ROS generation), but besides the two types, a few nonfunctional eNOS were found as well. However, they were ignored due to their small levels. Hence, the detailed data might have been influenced, but the main conclusion remained the same. It is presumed that with the development of related technologies, the nonfunctional eNOS can be removed, resulting in enhanced reliability.

Although there are still some problems to be solved in our research, our research has shown that during sepsis simulated by LPS-treatment, the main mechanism for VEC OS is eNOS uncoupling mediated by eNOS glutathionylation.

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