Therapeutic hypothermia mitigates the sepsis-increased permeability in EA. hy926 cells by preserving Rap1 expression

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Research

Keywords: Therapeutic hypothermia, Rap1, Rho, VE-cadherin, Cell permeability

Posted Date: April 15th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-22450/v1

Abstract

Background: To determine the effect and potential mechanisms of therapeutic hypothermia (TH) on the permeability of septic cells.

Main methods: Human EA. hy926 cells were transfected with, or without, control or Rap1-specific siRNA and treated with 2 μg/ml of lipopolysaccharide (LPS), followed by cultured in normal temperature (NT) or a temporary therapeutic hypothermia (TH) for 10 h. The cellular permeability of each group of cells was determined by transwell permeability assay and the relative levels of ras-proximate-1 (Rap1), RhoA (a small GTP enzyme of the Rho family), Ve-cadherin expression and myosin light chain (MLC) phosphorylation were quantified by western blot and immunofluorescent assays.

Results: Compared with the control group, LPS stimulation increased cellular permeability, which was enhanced by Rap1 silencing in EA. hy926 cells under a NT condition, but significantly mitigated by TH. Furthermore, LPS up-regulated RhoA expression and MLC phosphorylation, but reduced Rap1 and Ve-cadherin expression, which were also enhanced by Rap1 silencing, but significantly mitigated by TH. Immunofluorescent analyses indicated that LPS significantly increased phosphorylated MLC, but decreased Ve-cadherin expression, which were deteriorated by Rap1 silencing, but significantly mitigated by TH in EA. hy926 cells.

Conclusions: TH significantly mitigated the sepsis-increased permeability of EA. hy926 cells by enhancing the Rap1 expression to attenuate the RhoA/MLC signaling.

Background

Sepsis is a life-threatening condition and characterized by aberrant host responses to infection, leading to multiple organ dysfunction [1]. There are 1.5 million cases of sepsis in the United States yearly, accounting for about 30% of hospital deaths [2] and patients with septic shock have a mortality rate up to 50% [3]. The economic impact of sepsis on the United States is estimated for more than $20 billion yearly [4]. Although extensive studies have led to a great approach there is no effective therapies to control septic shock in the clinic [5]. During the pathogenesis of septic shock, infectious and inflammatory factors destroy endothelial barriers, damage vascular endothelial cells and activate the coagulation system, together with immunosuppression and immunoregulation, contributing to multiple organ dysfunction [6]. Our previous study has shown that temporary therapeutic hypothermia (TH) can regulate immune response, protect epithelial cells and reduce vascular endothelial injury, suggesting that TH may be a potential therapeutic strategy for septic shock [7]. However, whether and how TH regulate the sepsis-induced endothelial cell permeability have not been clarified.

Sepsis can result in severe endothelial dysfunction [8, 9], leading to increased permeability and tissue edema, which contribute to organ failure and mortality [10]. Cyclic adenosine monophosphate (cAMP) is an important intracellular signaling molecule that regulates the endothelial barrier function [11]. The cAMP can promote ras-proximate-1 (Rap1) and Ve-cadherin expression to enhance endothelial barrier

function [12]. The Rap1 can stabilize epithelial cell-cell connections [13]. Furthermore, Rap1 can inhibit the RhoA, a small GTP enzyme of the Rho family, and Rho-associated coiled coil-containing protein kinase (ROCK) signaling to preserve epithelial barrier function [14, 15]. Actually, ROCK can induce actin contraction, inhibit myosin light chain (MLC) phosphatase, prevent MLC dephosphorylation to increase MLC phosphorylation and vascular endothelial permeability [16, 17]. Accordingly, increased Rap1 activity can inhibit the RhoA/ROCK/MLC signaling to preserve endothelial barrier function and decrease endothelial permeability [15, 18–21]

We hypothesize that TH may mitigate the sepsis-increased cellular permeability by enhancing Rap1 activity to inhibit the RhoA /MLC signaling. To address the hypothesis, we employed human Ea. hy926 and Rap1 silenced Ea. hy926 cells to test the impact of TH on the LPS-induced cellular permeability and LPS-modulated Rap1, Ve-cadherin, RhoA expression and MLC phosphorylation. Our findings indicated that TH enhanced Rap1 and Ve-cadherin to mitigate the LPS-increased RhoA expression, MLC phosphorylation and cellular permeability in Ea. hy926 cells.

Results

TH mitigates the LPS-increased cell permeability

First, we tested whether TH could modulate the LPS-increased cell permeability in EA. Hy926 cells using transwell chamber assay. As shown in Fig. 1, in comparison with that in the control cells, LPS stimulation significantly increased cell permeability (** $P < 0.01$), which was significantly mitigated by TH (# $P < 0.05$) although the permeability in the TH group remained significantly higher than that in the control (* $P < 0.05$). Furthermore, transfection with control siRNA did not alter the permeability in EA. Hy926 cells while Rap1 silencing significantly elevated the LPS-increased cellular permeability (# $P < 0.05$) and it significantly reduced the effect of TH (& $P < 0.05$). Hence, TH mitigated the LPS-increased cellular permeability in EA. Hy926 cells in a Rap1-dependent manner.

TH significantly enhances the Rap1 expression to attenuate the LPS-enhanced RhoA/MLC signaling in EA. Hy926 cells.

The Rap1 can modulate the RhoA/MLC signaling and Ve-cadherin expression [14, 15]. Accordingly, we characterized the relative levels of Ve-cadherin and RhoA expression and MLC phosphorylation in the different groups of EA. Hy926 cells by western blot. As shown in Fig. 2, in comparison with that in the control group, LPS stimulation significantly decreased Rap1 and Ve-cadherin expression, but increased RhoA expression and MLC phosphorylation in the NT group of EA. Hy926 cells (** $P < 0.01$ for all), which were further enhanced in the Rap1 silenced EA. Hy926 cells (# $P < 0.05$ or ## $P < 0.01$). In contrast, TH significantly enhanced the Rap1 and Ve-cadherin expression, relative to that in the NT condition (# $P < 0.05$ or ## $P < 0.01$), which were decreased in the Rap1 silenced EA. Hy926 cells (& $P < 0.05$ or && $P < 0.01$). Furthermore, TH also decreased the RhoA expression and MLC phosphorylation in EA. Hy926 cells,

relative to that in the NT condition (# $P < 0.05$), while the therapeutic effect were weakened by Rap1-specific siRNA1 and Rap1-specific siRNA2 silence respectively .

Immunofluorescent analyses indicated that compared with that in the control, LPS stimulation significantly decreased Ve-cadherin expression (** $P < 0.01$), but increased phosphorylated MLC signals even in the Rap1-silenced EA. Hy926 cells under a NT condition (** $P < 0.01$) (Fig. 3). In contrast, TH significantly mitigated the effects of LPS on Ve-cadherin expression and phosphorylated MLC signaling in EA. Hy926 cells (# $P < 0.05$), and the effect of TH on Ve-cadherin expression was significantly reduced in the Rap1 siRNA2-silenced EA. Hy926 cells (& $P < 0.05$). Thus, TH significantly enhanced the Rap1 expression to attenuate the LPS-enhanced RhoA/MLC signaling in EA. Hy926 cells.

Discussion

Sepsis can increase cell permeability, and lead to capillary leakage syndrome (CLS), which causes severe hypoproteinemia, hypovolemia, tissue hypoperfusion, edema, shock and multiple organ dysfunction syndrome [22–24]. Our previous studies have shown that TH can improve the permeability of septic cells [7]. In this study, we found that TH significantly mitigated the LPS-increased permeability in EA. Hy926 cells. Given that cellular permeability is crucial for the pathogenesis of CLS the decreased permeability by TH suggests that TH may be valuable for control of septic shock, like VEGF (vascular endothelial growth factor) antagonist [25], nitric oxide inducer [26], inhibition of MLC phosphorylation [27], protection of cell connection [28].

The Rap1/Rho/MLC signaling is a critical regulator of cell permeability. We found that compared with the control group, LPS stimulation significantly decreased Rap1 and Ve-cadherin expression, but increased RhoA expression and MLC phosphorylation in EA. hy926 cells, which were enhanced by Rap1 silencing. In contrast, TH significantly mitigated the effects of LPS by preserving Rap1 and Ve-cadherin expression and reducing the LPS-stimulated RhoA expression and MLC phosphorylation in EA. hy926 cells. Such inhibitory effects of TH were attenuated by Rap1 silencing. Such novel data demonstrated that TH mitigated the LPS-increased permeability by preserving Rap1 expression in EA. hy926 cells.

The available data indicated that Rap1 were crucial for cell-matrix adhesion and cell-cell adhesion. Rap1 inhibits the RhoA activity, which can activate ROCK to promote non-muscle myosin II activation and actin contraction, and stress fiber formation to enhance local adhesion [16]. In addition, activated ROCK can promote MLC phosphorylation to increase permeability [18]. Therefore, Rap1 can reduce vascular permeability under both resting and stress conditions and dynamically regulate the barrier function of endothelial cells [29]. During the process of sepsis, Rap1 inhibits RhoA and Rac activities to preserve endothelial cell permeability [30]. Actually, inhibition of Rap1 can increase vascular permeability to deteriorate ARDS (acute respiratory distress syndrome) [31] while enhancement of Rap1 activity can accelerate the recovery of LPS-induced lung injury and vascular endothelial cell function [32].

Previous studies have shown that many factors, such as histamine, bradykinin, platelet activating factor and thrombin, increase vascular permeability by regulating Ve-cadherin expression in endothelial cells

[16, 17, 33, 34]. We found that LPS stimulation increased the MLC phosphorylation in EA. hy926 cells, which explained why LPS increased cell permeability [35], consistent with previous observations [36, 37]. However, TH mitigated the LPS-decreased Ve-cadherin expression such data indicated that TH preserved Rap1 expression to inhibit the LPS-increased RhoA expression and MLC phosphorylation and LPS-decreased Ve-cadherin expression in EA. hy926 cells [14, 20, 21, 38]. Thus, the Rap1/RhoA/MLC signaling may be valuable targets for intervention of LPS-induced high endothelial cell permeability. We are interested in further investigating the therapeutic effects of TH in vivo and the potential mechanisms underlying the action of TH during the process of septic shock.

Conclusion

Our data indicated that TH significantly mitigated the LPS-increased cell permeability in EA. hy926 cells by preserving Rap1 and Ve-cadherin expression to reduce the LPS-increased RhoA expression and MLC phosphorylation. Therefore, TH may be valuable for control of septic shock by mitigating cell permeability.

Methods

Human EA. Hy926 cells were from Zhong Qiao Xin Zhou Biotechnology(ZQ0079) and cultured in 10% FBS DMEM at 37℃ in 5% CO2. The cells (5 × 104 cells/well) were cultured in 6-well plates for 24 h and treated with, or without, lipopolysaccharide (2 μg/ml) at 37℃ for 4 h. The cells were further cultured at 32℃ (therapeutic hypothermia, TH) for 4 h, followed by cultured at 37℃ for 2 h. The control cells were cultured at 37℃ (normal temperature, NT) for 10 h.

Transwell analysis of cell permeability

The impact of TH on LPS-increased cell permeability in EA. Hy926 cells was determined as described previously [39]. Briefly, EA. Hy926 cells (5 × 104 cells/well) were cultured in 24-well transwell plates up to formation of a monolayer and stimulated in triplicate with LPS (2 μg/ml) for NT or TH culture. Subsequently, each of the upper and bottom chambers was loaded with 300 μl PBS and the upper chamber was added with 50 mg/L of horseradish peroxidas (HRP, Beyotime Biotechnology, A0208, Shanghai, China). Two min later, 10 μl of sample was collected from the inferior chamber and reacted with 200 μl TMB in a 96-well plate for 10 min. The absorbance at 450 nm in individual wells was measured in a microplate reader. The optical density (OD) values of individual wells are properly correlated to the degrees of permeability of cells.

Transfection

EA. Hy926 cells (5 × 104 cells/well) were cultured in antibiotic-free medium in 6-well plates overnight and transfected with control or Rap1-specific siRNA (Table 1) using Lipofectamine™ 2000 (Invitrogen, 11668-019, USA). Two days later, the efficacy of specific gene silencing was determined by Western blot.

Western blot

The relative levels of Rap1, RhoA, Ve-cadherin, MLC expression and MLC phosphorylation in individual groups of cells were quantified by Western blot. Briefly, the different groups of cells were harvested and lyzed in lysis buffer, followed by centrifuged. After quantification of protein concentrations, the cell lysates (50 μg/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and transferred onto polyvinylidene difluoride (PVDF) membranes. After being blocked, the membranes were probed with primary antibodies including anti-Rap1 (Abcam, ab181858, UK), anti-RhoA, (Proteintech, 10749-1-AP, USA), anti-MLC (Proteintech, 10906-1-AP), anti-p-MLC (Cell Singnaling Technology, 3671, USA), anti-Ve-cadherin (Cell Singnaling Technology, 2500) and anti-GAPDH (Yeasen Biotech, 33106ES60, China). After being washed, the bound antibodies were detected with HRP-conjugated second antibodies and visualized with enhanced chemiluminescent reagents. The relative levels of each target to the control protein were quantified by densitometric analysis using Image-Pro Plus software (Media Cybernetics).

Immunofluorescence

EA. Hy926 cells (1 × 104 cells /ml) were cultured on glass coverslips for 24 h, and fixed with 4% paraformaldehyde, and permeabilized, followed by blocked with 1% BSA. The cells were probed with anti-Ve-cadherin 1:400, or anti-p-MLC 1:50 overnight at 4℃. After being washed, the cells were incubated with fluorescent second antibodies and stained intracellularly with DAPI. The fluorescent signals were observed under a fluorescent microscope (OLYMPUS, Japan).

Statistical analysis

Data are expressed as the mean ± SD. The difference among groups was analyzed ANOVA and post hoc least significant test and the difference between groups was determined by Student's T test using SPSS software window 17. Statistical significance was defined when a P-value of < 0.05.

Abbreviations

TH
therapeutic hypothermia
NT
normothermia
LPS
lipopolysaccharide
Rap1
ras-proximate-1
MLC
myosin light chain
cAMP
cyclic adenosine monophosphate

ROCK
Rho-associated coiled coil-containing protein kinase
OD
optical density
SDS-PAGE
sodium dodecyl sulfate polyacrylamide gel electrophoresis
PVDF
polyvinylidene difluoride

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests. No potential conflicts of interest exist with any companies/organizations whose products or services may be discussed in this article.

Funding

No.

Authors' contributions

SY and DW made conception and design of the study and completed the experiment. SY and DW analyzed and interpreted the data. SY drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This work was performed at the Department of Emergency Medicine, Second Affiliated Hospital, Zhejiang University, School of Medicine and Research Institute of Emergency Medicine, Zhejiang University, Hangzhou, China. We thank Dr. Jiang Libing, MD, for providing guidance on the study. We also thank all the relevant staff of the emergency departments for assisting in the implementation of this study.

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Tables

Table 1. The sequences of Rap1-specific siRNAs

Title	Sequence（5'-3'）
SiRNA1	GAGGGAUUUAUACAUGAAATT
	UUUCAUGUAUAAAUCCCUCTT
SiRNA2	CCACAUUUAACGAUUUACATT
	UGUAAAUCGUUAAAUGUGGTT
SiRNA3	GUGCGGCAAAUUAACAGAATT
	UUCUGUUAAUUUGCCGCACTT

Figures

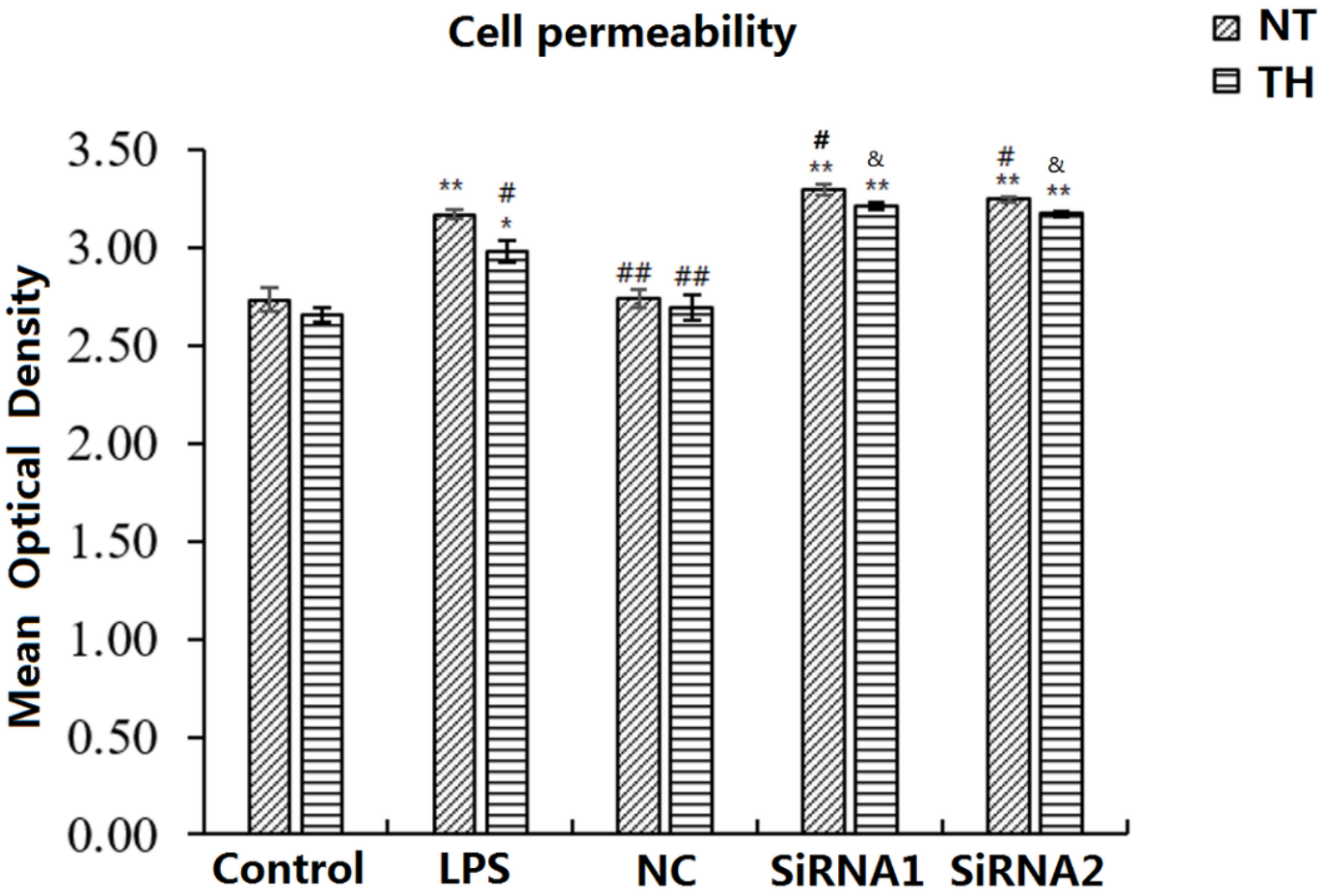

Figure 1

TH mitigates the LPS-increased cellular permeability in EA. Hy926 cells, dependent on the Rap1 signaling. EA. Hy926 cells were transfected with, or without, control or Rap1-specific siRNA for 48 h and the cells were stimulated in triplicate with, or without, LPS, followed by cultured in NT or TH for 10 h. The cellular permeability of individual groups of cells was measured by transwell permeability assay. Data are expressed as the mean ± SD of each group of cells from three separate experiments. NT: normothermia group; TH: therapeutic hypothermia group; LPS: LPS stimulation group; NC: control group with control siRNA transfection; siRNA1: the Rap1-specific siRNA1-transfected cells; siRNA2: the Rap1-specific siRNA2-transfected cells. *P < 0.05, **P < 0.01, vs. the NT control group; #P < 0.05, ##P < 0.01 vs. the NT LPS group; &P < 0.05, &&P < 0.01, vs. the TH LPS group.

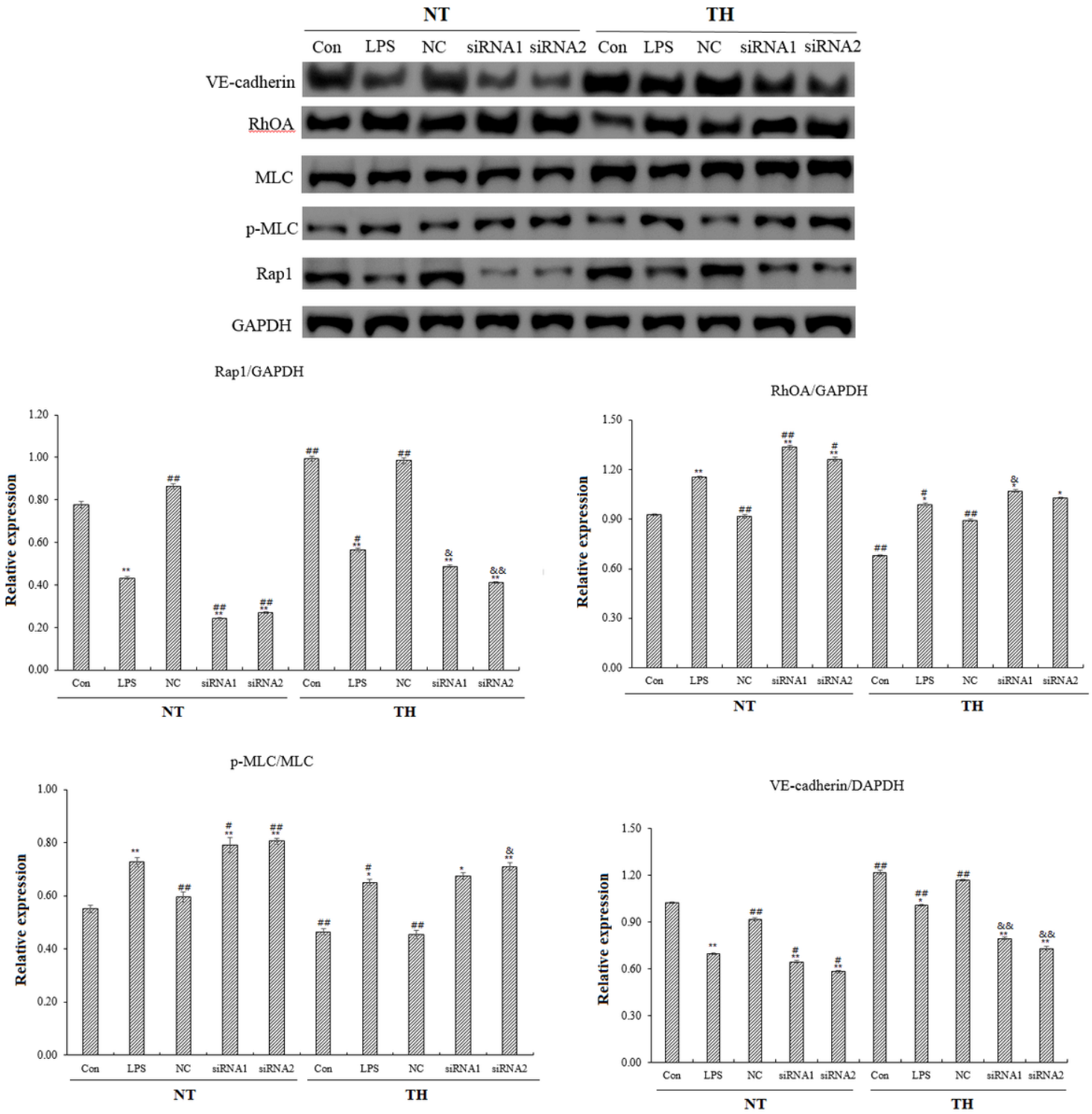

Figure 2

TH significantly enhances the Rap1 expression and attenuates the LPS-enhanced RhoA/MLC signaling in EA. Hy926 cells. Following transfected with control or Rap1-specific siRNA and treated with, or without, LPS, the different groups of EA. Hy926 cells were cultured in NT or TH for 10 h and the relative levels of Rap1, RhoA, Ve-cadherin expression and MLC phosphorylation were quantified by Western blot. Data are representative images or expressed as the mean ± SD of each group of cells from three separate

experiments. *P < 0.05, **P < 0.01 vs. the NT control group; #P < 0.05, ##P < 0.01 vs. the NT LPS group; &P < 0.05, &&P < 0.01 vs. the TH LPS group.

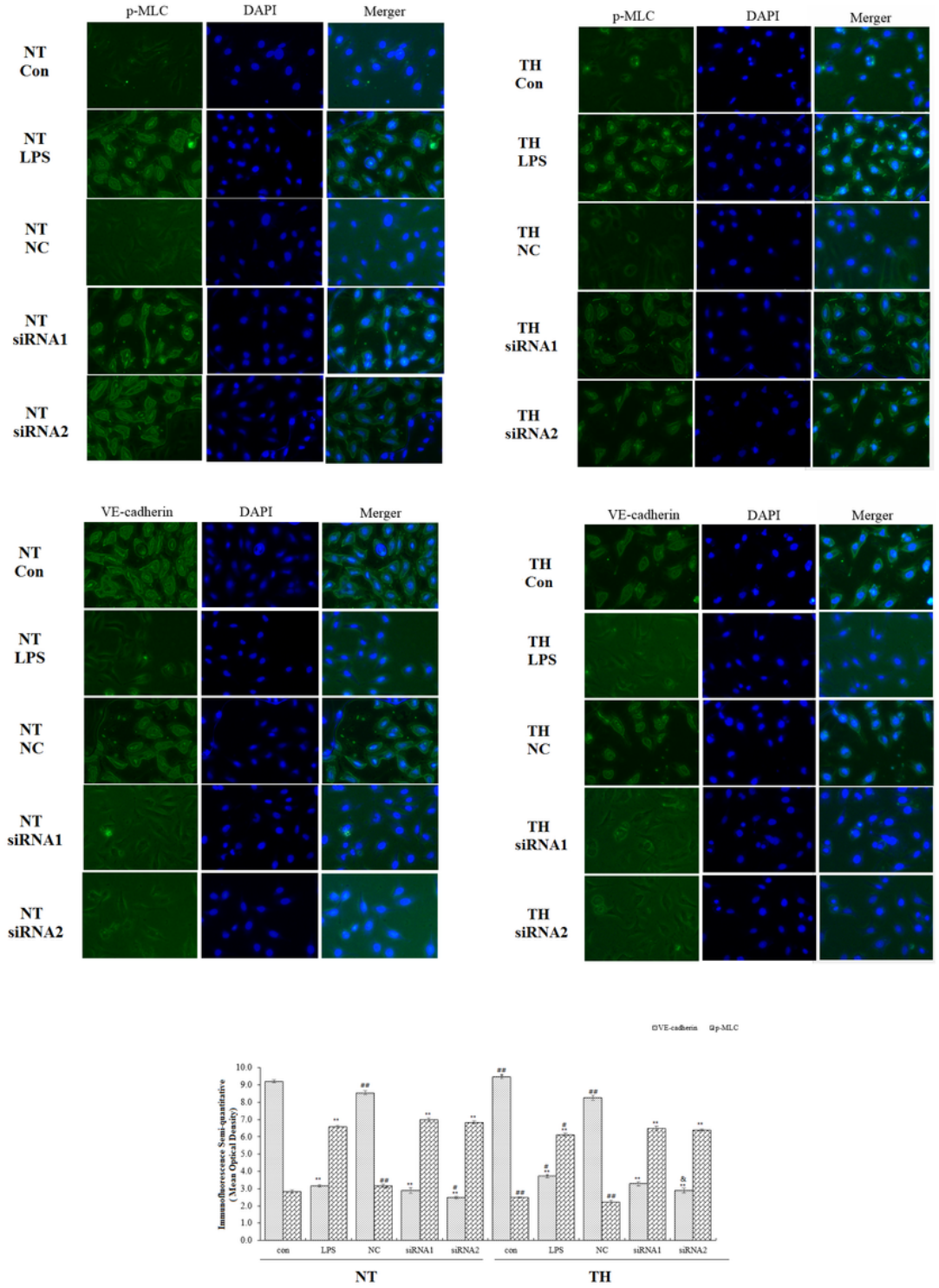

Figure 3

TH mitigates the LPS-modulated Ve-cadherin expression and MLC phosphorylation in EA. Hy926 cells. Following transfected with control or Rap1-specific siRNA and treated with, or without, LPS, the different groups of EA. Hy926 cells were cultured in NT or TH for 10 h and stained with fluorescent anti-Ve-

cadherin or anti-phosphorylated MLC, followed by photoimaged under a fluorescent microscope. Data are representative images (magnification x 200) or expressed as the mean ± SD of each group of cells from three separate experiments. *P < 0.05, **P < 0.01 vs. the NT control group; #P < 0.05, ##P < 0.01 vs. the NT LPS group; &P < 0.05, &&P < 0.01 vs. the TH LPS group.