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# ENHANCEMENT EFFECT OF ETHANOL ON LIPOPOLYSACCHARIDE-INDUCED PROCOAGULANT STATUS IN HUMAN UMBILICAL ENDOTHELIAL CELLS

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## Summary

In spite of the inhibitory effects of ethanol (EtOH) on platelet function, soft blood clots are often observed in cadaveric blood in cases of sudden death after alcohol ingestion. In order to resolve this discrepancy, we have focused on the role of vascular endothelial cells. We tried to investigate the effects of EtOH and LPS on endothelial cells from various perspectives; thrombogenic factor (Von Willebrand factor, VWF), fibrinolytic factor (tissue plasminogen activator, tPA) and inflammatory factor (Interleukin-6, IL-6). Human umbilical vein endothelial cells (HUVECs) were incubated with various concentrations of EtOH (0~160 mM) with or without LPS. Treatment with EtOH and LPS increased VWF release from HUVECs without enhancement mRNA expression. Treatment with 40 mM of EtOH also increased IL-6 release from HUVECs without enhancement mRNA expression. Although EtOH inhibited LPS-induced IL-6 mRNA expression, 20 mM of EtOH still had an increasing effect on the release of IL-6. These doses of EtOH are consistent with a moderate drunkenness level in a normal person. On the other hand, mRNA expression and release reaction of tPA were not affected by EtOH and LPS addition. In conclusion, EtOH enhances procoagulant status via VWF release and IL-6 production cooperation with LPS and may contribute to soft blood clot formation in cadaveric blood.

**Key words:** cadaveric blood, ethanol, lipopolysaccharide, endothelial cells, inflammation

## Souhrn

### Potencující účinek ethanolu na lipopolysacharidy indukovaný prokoagulační stav lidských pupečníkových endoteliálních buněk

Navzdory inhibičnímu účinku ethanolu na funkci destiček, měkká krevní koagula jsou často pozorována v kadaverosní krvi v případech náhlého úmrtí po požití alkoholu. Ve snaze vysvětlit tuto diskrepanci, zaměřili jsme se na roli vaskulárních endotheliálních buněk. Snažili jsme se prozkoumat účinek ethanolu a lipopolysacharidů (LPS) na endotheliální buňky z různých hledisek: trombogenního faktoru (von Willebrandův faktor, vWF), fibrinolytického faktoru (tkáňový aktivátor plasminogenu, tPA) a zánětlivého faktoru (interleukin 6, IL-6). Endotheliální buňky lidské pupečníkové žíly byly inkubovány při různých koncentracích ethanolu (0 – 160 mmol) s nebo bez LPS. Působení ethanolu a LPS zvýšilo uvolňování vWF z endotheliálních buněk lidské pupečníkové žíly bez zvýšení exprese mRNA. Ačkoliv ethanol inhiboval expresi LPS indukovanou IL-6 mRNA, 20 mmol roztok ethanolu ještě měl potencující účinek na uvolňování IL-6. Tyto koncentrace ethanolu odpovídají mírné hladině opilosti u normální osoby. Z druhé strany, exprese mRNA a uvolňující reakce tPA nebyly ovlivněny přidáním ethanolu a LPS. V souhrnu, ethanol podporuje prokoagulační stav cestou uvolňování vWF a tvorbou IL-6 podpořenou LPS a může přispívat ke tvorbě měkkých krevních koagul v kadaverosní krvi.

**Klíčová slova:** kadaverosní krev, ethanol, lipopolysacharidy, endotheliální buňky, zánět

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## INTRODUCTION

Fluidity of the cadaveric blood is one of the most important characteristics of a sudden death. The prevailing view of this phenomenon attributes it to the release of tissue plasminogen activator (tPA) from endothelial cells (23, 24). Yet it is also empirically known that soft blood clots have been observed in cases of sudden death after alcohol ingestion. A number of studies have stated that ethanol attenuates platelet activation induced by several agonists (14, 19, 20). Moreover, it has been reported that expression of tPA and urokinase-type PA (uPA) are up-regulated in endothelial cells after ethanol (EtOH) treatment (4, 22). The underlying mechanism of the discrepancy between fluidity of cadaveric blood and soft-clot formation in sudden death after alcohol ingestion remains to be fully elucidated.

In order to resolve this discrepancy, we focused on the role

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of vascular endothelial cells. It has long been understood that blood clot formation can be associated with the following factors known as Virchow's triad; reduction in blood flow, endothelial injury and alterations in the constitution of blood (9). Endothelial cells in normal condition provide various anticoagulants such as or nitric oxide, whereas they express thrombogenic activity when they are injured. The most common injury to endothelial cells is caused by inflammation due to infection.

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria and is the prototypical example of an endotoxin. LPS-induced inflammation causes various types of reaction, and the relationship between coagulation and inflammation have been well-discussed (7). Since absorption of LPS in the intestine is enhanced by alcohol ingestion (25), it is just conceivable that an interaction between EtOH and LPS affects the endothelial function.

In the present study, we tried to investigate the effects of EtOH and LPS on endothelial cells from various perspectives; thrombogenic factor, fibrinolytic factor and inflammatory factor. Von Willebrand factor (VWF) is one of the most important thrombogenic factor produced and released from endothelial cells. tPA is the most potent fibrinolytic factor which is also produced and released from endothelial cells. Interleukin-6 (IL-6) is the most important proinflammatory cytokine, which correlates with various aspects of hemostasis (15). In this paper, in order to explore potential mechanism of soft-clot formation after alcohol drinking, we determined mRNA expression and secretion of VWF, tPA and IL-6 by using human umbilical vein endothelial cells (HUVECs).

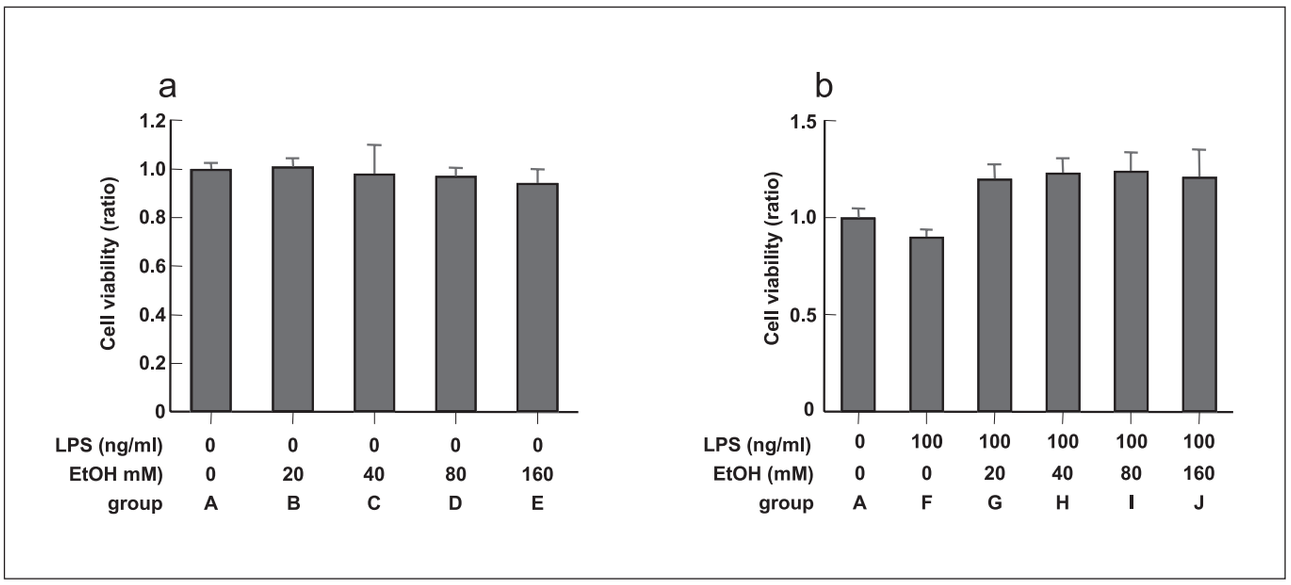



**Figure 1. Evaluation of cell viability by WST-8 assay.**
**a, Treatmet with EtOH alone.**
**b, Treatment with EtOH and LPS.**
**The data are presented as ratios to the response of Group A. Note that exposure to of EtOH and LPS did not reduce cell viability.**

## MATERIALS AND METHODS

*Cells and reagents*

HUVECs and culture medium were purchased from Cell Applications (San Diego, CA, USA). Absolute EtOH and LPS from *Escherichia coli* serotype O55 were purchased from Wako Pure Chemical Industries (Osaka, Japan).

*Cell culture*

HUVECs were grown for 2-3 days until reaching 80% confluence in a humidified chamber with a 5% $CO_2$-air mixture at 37 °C. For experiments, HUVECs were passaged using trypsin-EDTA. They were seeded into 24-well or 96-well tissue culture plates. The cells were confluent after 2 days. HUVECs were incubated with EtOH alone or EtOH with LPS. We employed 10 experimental groups; Group A (EtOH 0 mM), Group B (EtOH 20mM), Group C (EtOH 40 mM), Group D (EtOH 80 mM), Group E (EtOH160 mM), Group F (EtOH 0mM, LPS 100ng/ml), Group G (EtOH 20 mM, LPS 100 ng/ml), Group H (EtOH 40 mM, LPS 100ng/ml), Group I (EtOH 80 mM, LPS 100 ng/ml) and Group J (EtOH 160 mM, LPS 100 ng/ml). EtOH alone treatment groups (A~E) were incubated with various concentrations of EtOH for indicated hours, while EtOH and LPS treatment groups (F~J) were first incubated for 30 min at 37 °C in culture media containing various concentrations of EtOH and incubated for indicated hours with addition of LPS. All experiments were performed on second passage cells.

*WST-8 assay*

The cytotoxicity of EtOH and LPS were evaluated with a WST-8 colorimetric assay (Kishida Chemical, Osaka, Japan). HUVECs seeded into a 96-well plate were treated for 20 hours in 100 l of culture media containing various concentrations of EtOH with or without LPS. Next, 10 μl of WST-8 reagent were added and incubation conducted for 2 hours. Cell viability was determined according to the manufacturer's instructions. The data are presented as a ratio to the response in Group A and are expressed as mean ± SEM.

*Gene expression analysis*

After 4 hours of treatment, cell monolayers were washed twice in PBS, after which total RNA was extracted using RNeasy micro kit and treated with RNase free DNase (Qiagen, Valencia, CA, USA). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using 1 μg of total RNA, and Superscript RT kit (Invitrogen) with random hexamers. Quantitative real time RT-PCR analysis was performed with an Applied Biosystems StepOne Plus Real time PCR System

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using TaqMan® universal PCR master mix according to the manufacturer's specifications for human VWF, tPA and IL-6 genes (Applied Biosystems, Foster City, CA, USA). The TaqMan probes and primers for human VWF, tPA and IL-6 gene (assay identification number, Hs00169795_m1, Hs00938315_m1 and Hs99999032_m1, respectively) were assay-on-demand gene expression products (Applied Biosystems), while human GAPDH gene (assay identification number Hs99999905_m1) was used as an endogenous control. The data were analyzed by the ΔΔCt method (17) and were presented as normalized gene amount relative to Group A.

*Measurement of VWF, tPA and IL-6*

Culture supernatants (400 μl) from 4 hour-treated HUVECs were centrifuged at 15,000 g to remove cell debris and frozen at -80 °C until use. VWF was measured by ELISA as described elsewhere (3). The VWF values were presented as a percentage of pooled normal human plasma. tPA (Assaypro, St Charles, MO, USA) and IL-6 (Biosource International, Camarillo, CA, USA) were obtained with ELISA kits according to the manufacturer's instructions. The data are presented as a ratio to the response in Group A and are expressed as mean ± SEM.

*Statistical analyses*

According to the results of WST assay and ELISA, statistical analysis was performed using Statview software (SAS Institute, Cary, NC, USA). Differences between the groups were assessed by a Kruscal-Wallis test with post-hoc testing (Tukey-Kramer test). A value of $p < 0.05$ was considered statistically significant.

**Tab. 1**

**a. Normalized gene amount relative to group A in treatment with EtOH**

| group | VWF | tPA | IL-6 |
|---|---|---|---|
| A | 1.00 (0.45-2.21) | 1.00 (0.73-1.36) | 1.00 (0.80-1.24) |
| B | 0.67(0.51-0.90) | 1.00 (0.80-1.26) | 0.56 (0.21-1.48) |
| C | 1.24 (0.48-3.20) | 0.89 (0.49-1.63) | 0.85 (0.41-1.75) |
| D | 0.67 (0.56-0.79) | 1.01 (0.83-1.23) | 1.14 (0.83-1.57) |
| E | 1.16 (0.42-3.23) | 1.07 (0.90-1.27) | 0.93(0.70-1.23) |

Numbers in parentheses indicate the range of relative

**b. Normalized gene amount relative to group A in treatment with EtOH and LPS**

| group | VWF | tPA | IL-6 |
|---|---|---|---|
| A | 1.00 (0.80-1.24) | 1.00 (0.81-1.24) | 1.00 (0.80-1.24) |
| F | 0.97 (0.68-1.37) | 0.90 (0.81-1.00) | 1.56 (1.32-1.83) |
| G | 1.04 (0.86-1.26) | 0.90 (0.75-1.07) | 1.06 (0.91-1.23) |
| H | 1.17 (0.91-1.50) | 0.90 (0.85-0.96) | 1.06 (0.82-1.37) |
| I | 0.97 (0.77-1.24) | 0.99 (0.80-1.22) | 0.81 (0.65-1.00) |
| J | 0.87 (0.55-1.38) | 0.94 (0.82-1.06) | 0.98 (0.69-1.39) |

Numbers in parentheses indicate the range of relative

## RESULTS

At first, to rule out the possibility that a high concentration of EtOH and LPS had a lethal effect on endothelial cells, we assessed cell viability following EtOH and LPS treatment. We found that incubation of HUVECs for 20h with EtOH with or without LPS did not significantly affect cell viability, as determined by WST-8 assay (Figure 1). These results suggested that EtOH and LPS did not induce protein release due to necrotic cell collapse.

The results of real time quantitative PCR are summarized in Table 1. EtOH alone did not show remarkable change in mRNA expression of VWF, tPA and IL-6 (Table1. a). Co-stimulation with LPS and EtOH (Table 1. b) also did not show significant change in mRNA expression of VWF and tPA. mRNA expression of IL-6 was up-regulated by LPS stimulation (Group F). This is because LPS stimulates the synthesis of inflammatory cytokines by activating nuclear factor kappaB(NF-μB)-dependent signaling mechanisms. However, addition of EtOH inhibited LPS-induced mRNA expression (Group G-J).

In view of protein secretion, the mean value of concentrations of VWF, tPA and IL-6 in group A were 5.73%, 0.34 ng/ml and 41.3 pg/ml, respectively. EtOH alone did not significantly affect the release of VWF and tPA in conditioned media (Figure. 2 a, c).

However, by addition of LPS, VWF release was significantly increased (Figure.2 b). On the other hand, tPA release was not affected at all by the addition of LPS (Figure. 2 d). IL-6 secretion in conditioned media was significantly increased by addition of 40mM EtOH (Figure.2 e, Group C). Naturally, LPS treatment increased IL-6 secretion (Figure.2 f, Group F). Although real time PCR analysis demonstrated that addition of EtOH inhibited LPS-induced mRNA expression of IL-6, co-stimulation with 20 mM of EtOH and LPS (Group G) still increased IL-6 secretion compared to Group A. There were no significant difference between group F and Group G in IL-6 secretion.

## DISCUSSION

We have investigated the role of endothelial cells in EtOH administration. We demonstrated that moderate dose of EtOH enhanced IL-6 release and co-stimulation with EtOH and LPS increased VWF release without an enhancement of tPA production.

Since LPS is a potent substance that causes inflammation, while a high dose of EtOH also may denature the cell membrane, there was a possibility that the release reactions were due to cell collapse. We therefore confirmed that EtOH and LPS did not induce cell death even after 20h incubation and consider that the increase of release reactions were not due to cell collapse.

VWF release from HUVECs would promote soft clot formation, since VWF plays a crucial role in hemostasis as a mediator for platelet aggregation and carrier protein for coagulation factor VIII. Although expression of VWF mRNA was not affected by EtOH with or without LPS, co-stimulation with EtOH and LPS increased release of VWF from HUVECs. These results suggest that co-stimulation with EtOH and LPS induces VWF release from internal store without de novo synthesis.

Inflammatory cytokines contribute to coagulation activation by increasing fibrinogen concentration and inducing tissue factor expression on the cell surface (7). Conversely, coagulation activation also stimulates production of inflammatory cytokines (10-12). There exist many reports using various kinds of cells, such as osteoblast-like cells, hepatocytes, monocytes, and dermal microvascular endothelial cells. However, it is still controversial whether EtOH activates NF-kB (16, 18, 21, 27). It may be that the various cell lines give rise to the differences seen in the effect of EtOH on NF-μB activation. However, Jonsson et al. reported that EtOH inhibited NF-μB activation in HUVECs (13). In our experiments, HUVECs significantly increased IL-6 release at

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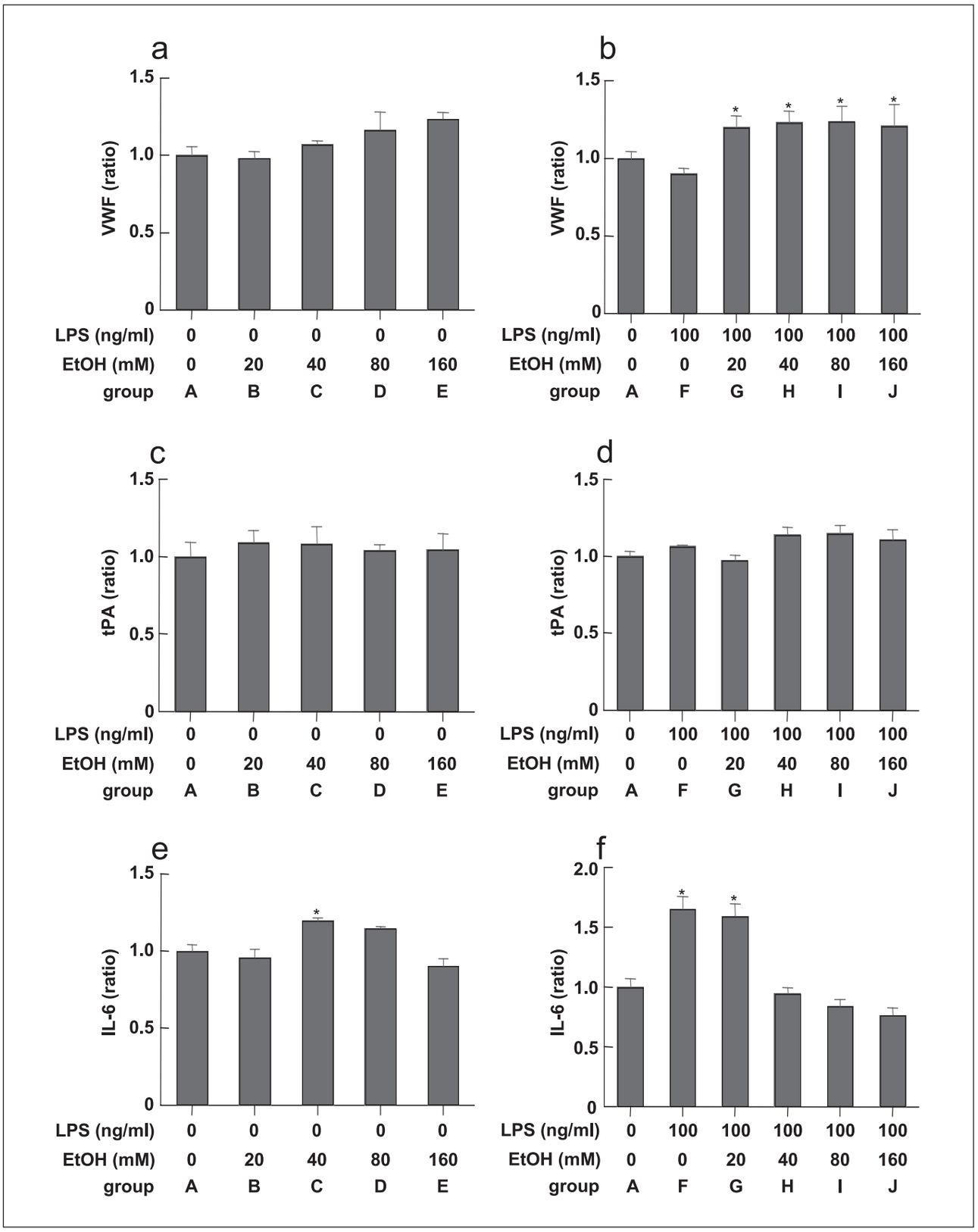



**Figure 2. Levels of VWF, tPA and IL-6 in conditioned media treated with EtOH alone (a, c, and e) and EtOH with LPS (b, d, and f). The data are presented as ratios to the response of Group A.**
**a and b, WF concentration.**
**c and d, tPA concentration. Note that tPA release did not increase, even under EtOH and LPS stimulation.**
**e and f, IL-6 concentration. Note that IL-6 release is still enhanced in Group G. There were no significant difference between group F and Group G.**
**The data are expressed as mean ± SEM. * $p < 0.05$.**

Patologie 4-09 12.10.2009 14:31 Str. 48
proLékaře.cz | 16.11.2026

40 mM EtOH treatment (Group C). Although EtOH inhibited LPS-induced IL-6 mRNA expression, we have demonstrated that 20 mM of EtOH has an increasing effect on the release of IL-6. These concentrations of EtOH are often seen in autopsy cases. A recent report has revealed that soft clot formation in cadaveric blood is associated with a blood ethanol level having a mean value of 0.237 mg/ml (around 40 mM) (8). This is consistent with our results; i.e., that moderate EtOH concentration supports soft blood clot formation. Taken together, LPS acts synergistically with EtOH to release VWF and IL-6 from HUVECs. Although further investigation should be required to clarify the underlying mechanism of this phenomenon, it is possible that EtOH and LPS would change the membrane permeability.

On the other hand, mRNA expression and secretion of tPA remained unchanged, even under co-stimulation with EtOH and LPS. Since tPA is the most important fibrinolytic factor, unchanged tPA release would contribute to soft blood clot formation. According to a previous report, tPA mRNA was up-regulated by EtOH stimulation (4). However, in that study, relatively low levels of EtOH were used (< 20 mM). In our present study, we used relatively high levels of EtOH (> 20 mM, moderate ebrious ~ non physiological levels). As EtOH affects many kinds of proteinkinase as well as NF-kB (1, 2, 5, 26), tPA production mechanism are likely to be modified intricately by the difference in the concentration of EtOH. Unlike VWF, EtOH and LPS did not induce tPA secretion. We considered that the stimuli for their secretion may be different because tPA and VWF are stored in different vesicles each other (6).

In conclusion, a moderate dose of EtOH enhances procaogulant status via VWF and IL-6 release cooperation with LPS. These actions do not involve an enhancement of tPA production. Enhancement of LPS absorption due to alcohol ingestion would contribute to soft blood clot formation in cadaveric blood.

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