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**Caspase-3 is activated and rapidly released from human umbilical vein endothelial cells in response to lipopolysaccharide.**

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**Key words:** apoptosis, Toll-like receptor, sepsis, endotoxin, infection

**Abbreviations used:** LPS: lipopolysaccharide; HUVEC: human umbilical vein endothelial cells; CHX: cycloheximide; LBP: LPS-binding protein; LDH: lactate dehydrogenase; TLR: Toll-like receptor; HE-SFM: human endothelial-serum free medium; FBS: fetal bovine serum; sCD14: soluble CD14; sMD-2: soluble MD-2; PBS: phosphate-buffered saline; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

## Summary

Endothelial cell injury/dysfunction is considered to play a critical role in the pathogenesis of severe sepsis and septic shock. Although it is considered that endothelial cell apoptosis is involved in endothelial injury/dysfunction, physiological involvement remains ambiguous since the induction of apoptosis requires the inhibition of endogenous apoptosis inhibitors. Here we show that caspase-3 activation, a biological indicator of apoptosis, is observed in response to lipopolysaccharide (LPS) stimulation even under the influence of endogenous apoptosis inhibitors, and that activated caspase-3 is rapidly released from human umbilical vein endothelial cells (HUVEC). In the presence of cycloheximide (CHX), an increase in intracellular caspase-3/7 activity in response to LPS was not detected in HUVEC up to 24 hr following stimulation even in the presence of LPS-binding protein (LBP), soluble CD14 and soluble MD-2, whereas the decrease in cell viability and increase in release of the cellular enzyme lactate dehydrogenase (LDH) were observed in a soluble CD14/LBP-dependent manner. On the other hand, even in the absence of CHX, a significant increase in caspase-3/7 activity and a cleaved caspase-3 fragment with a slight increase in LDH release was observed in culture supernatants in response to LPS. This increase in caspase-3/7 activity was observed even when LDH release was undetected. These results indicate that caspase-3 is activated by LPS under physiological conditions and suggest that HUVEC escape from cell death by rapidly releasing activated caspase-3 into extracellular space. Failure of this escape mechanism may result in endothelial injury/dysfunction.

## Introduction

Sepsis, the systemic inflammatory response to infection, is manifested by two or more of the criteria that define systemic inflammatory response syndrome [1]. Severe sepsis is a condition complicated by organ dysfunction and septic shock (hypotension despite adequate fluid resuscitation). The end of the spectrum is multiple organ dysfunction syndrome, which is defined as the presence of altered organ function in an acutely ill patient and homeostasis that cannot be maintained without intervention [2]. Although infectious diseases can be successfully treated with medications and other therapeutic measures such as intensive care, sepsis is a leading cause of mortality among severely ill patients. Angus et al estimate that 750,000 cases of severe sepsis occur each year in the United States, with a mortality rate of 28.6% [3]. Other studies suggest that 28-day mortality rates of severe sepsis may be 50% or greater [4].

Lipopolysaccharide (LPS), a constituent of the outer membrane of the cell wall of Gram-negative bacteria, is the primary factor in the development of sepsis [5-6]. LPS-induced cell activation depends on the presence of at least four proteins; Toll-like receptor (TLR) 4, MD-2, CD14 and LPS-binding protein (LBP) [7-8]. LPS initially binds to LBP in the blood and is transferred to CD14, a 55-kDa glycoprotein [9]. The CD14/LPS complex then interacts with the TLR4/MD-2 complex, leading to cell activation. MD-2 is produced by several cells as a 20- to 25-kDa glycoprotein and binds to LPS to confer LPS responsiveness to TLR4-expressing cells [10].

Endothelial injury/dysfunction is also critical in sepsis pathogenesis [11]. The vascular endothelium is a pervasive organ; the human body contains approximately  $10^{13}$  endothelial cells, covering a surface area of 4,000 m<sup>2</sup> to 7,000m<sup>2</sup> and weighing 1 kg [12]. It serves a multitude of functions that help to maintain organ homeostasis,

including vasoregulation and selective vascular permeability, and provision of an anticoagulant surface [13]. In sepsis, the endothelium undergoes functional and mechanical changes that contribute to pathogenesis. Apoptosis is a form of cell death that is morphologically characterized by chromatin condensation, nuclear fragmentation, cell shrinkage, and blebbing of the plasma membrane. The end result of apoptosis is fragmentation of the cell into small membrane-bound bodies. These changes are accomplished by a specialized family of cysteine-dependent aspartate-directed proteases, termed caspases; hence apoptosis is biochemically characterized by the activation of caspases [14]. The caspase gene family consists of 15 mammalian members classified based on the structure and function of their prodomains [15]. The caspase family can be divided into two functional subgroups based on their roles. Inflammatory caspases (caspase-1, -4, -5, 11, -12, -13 and -14) play a role in cytokine maturation and inflammatory responses. The caspases involved in apoptosis are further divided into two functional subgroups, initiator of apoptosis caspases (caspase-2, -8, -9, -10 and -15) and effector caspases (caspase-3, 6, 7) [16-17].

Endothelial apoptosis is considered to be involved in septic endothelial injury/dysfunction [18]. However, whether apoptosis is induced in a physiological condition remains ambiguous since in most studies [19-20] LPS does not induce apoptosis unless the synthesis of endogenous apoptosis inhibitors [21-23], such as FLICE-like inhibitory protein [24], is blocked by protein synthesis inhibitors. Here, we investigated LPS-induced endothelial cell apoptosis by systematically examining the activity of effector caspases (caspase-3/7), cellular viability as well as LDH release, which reflects cellular cytotoxicity. We found that caspase-3 is activated by LPS in HUVEC even in a physiological condition and that activated caspase-3 is rapidly

released from cells.

## Materials and methods

### *Cell culture and reagents*

RAW 264, a mouse macrophage-like cell line (obtained from the Riken Cell Bank, Tsukuba, Japan), was maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Invitrogen), penicillin (100 U/ml), and streptomycin (100 µg/ml) as described earlier [25]. Human umbilical vein endothelial cells (HUVEC) were purchased from Kurabo (Tokyo, Japan) and maintained in human endothelial-serum free medium (HE-SFM; Gibco-BRL, Rockville, MD) supplemented with 0.5% (v/v) heat-inactivated FBS, recombinant human epidermal growth factor (10 µg/ml; Invitrogen), recombinant human basic fibroblast growth factor (20 µg/ml; Wako Pure Chemical Industries, Osaka, Japan), amphotericin B (250 µg/ml; Wako Pure Chemical Industries) and gentamicin (50 µg/ml; Gibco-BRL) at 37 °C in 5% CO<sub>2</sub>. HUVEC were used from passage 4 to 8. LPS from *Escherichia coli* O111:B4 and cycloheximide (CHX) were purchased from Sigma-Aldrich (St. Louis, MO). Soluble CD14 (sCD14) was obtained from R&D Systems (Minneapolis, MN). LBP and soluble MD-2 (sMD-2) were prepared as described [26]. Anti-caspase-3 (No. 9662) and anti-caspase-7 (No. 9492) polyclonal antibodies were purchased from Cell Signaling Technologies (Danvers, MA), and anti-caspase-7 polyclonal antibody N-17 and anti-LDH polyclonal antibody H-160 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

### *Cell stimulation*

RAW 264 cells were seeded in 6-well cell culture plates. The following day, the cells were washed twice with 200  $\mu$ l of cold phosphate-buffered saline (PBS) and stimulated in the culture medium with the indicated concentrations of LPS with or without 0.2-20  $\mu$ g/ml of CHX. HUVEC were seeded into 96-well cell culture plates. After a 16-hr incubation, the cells were washed twice with 200  $\mu$ l of cold PBS and stimulated with the indicated concentrations of LPS in HE-SFM containing LBP (100 ng/ml) in the presence or absence of 20  $\mu$ g/ml of CHX, sCD14 (1  $\mu$ g/ml) and/or sMD-2 (1  $\mu$ g/ml).

### *Cell viability assay*

Cell viability was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega). HUVEC culture medium was collected following stimulation and subjected to cytotoxicity and caspase-3/7 assays as described below. Cells were washed twice with 200  $\mu$ l of cold PBS and incubated in a 20 % solution of [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] in FBS-free HE-SFM for 1hr at 37 °C in 5% CO<sub>2</sub>. The amount of formazan converted by viable cells was determined by measuring absorbance at 490 nm after subtracting the absorbance at 650 nm on a 96-well microplate reader.

### *LDH release*

LDH release was assessed by determining LDH activity in culture supernatants using the CytoTox 96 Cytotoxicity Assay System (Promega). Culture supernatants were collected after stimulation and centrifuged at 4000 rpm for 5 min. The supernatants were incubated with the assay buffer and substrate mix at room temperature for 30 min



and the absorbance at 490 nm was measured using a 96-well microplate reader. The background (spontaneous LDH release) value was measured in non-stimulated cells and subtracted from each measurement. Maximum LDH release was measured by incubating non-stimulated cells in a lysis solution (1% Triton X-100, PBS) at 37 °C for 1 hr.

#### *Caspase-3/7 activity assay*

The caspase-3/7 activity in cell extracts and culture supernatants was determined using Caspase-Glo 3/7 Assay (Promega). After stimulation, RAW 264 cells were washed twice with cold PBS and lysed with 100  $\mu$ l of lysis buffer [10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 5 mM EDTA, 40 mM  $\beta$ -glycerophosphate, 0.5 % Nondiet P-40 (NP-40), 30 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$ ] containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The lysates were centrifuged at 4000 rpm for 5 min and caspase-3/7 activity was measured in the resulting supernatants. HUVEC were washed twice following stimulation with cold PBS and directly lysed with 50  $\mu$ l of caspase-3 /7 detection reagent. Culture supernatants collected after stimulation were centrifuged at 4000 rpm for 5 min. An equal volume of caspase-3/7 detection reagent was added to RAW 264 cell extracts and culture supernatants, and the mixture as well as the HUVEC lysates prepared above were incubated at 25 °C for 30 min. The luminescence emitted by each sample was measured with a luminometer.

#### *Electrophoresis and Western blotting*

HUVEC were seeded in 6-well collagen-coated cell culture plates and stimulated as indicated. Following stimulation, culture supernatants were collected and centrifuged at 4000 rpm for 5 min. SDS (final concentration: 0.25%), DTT (final concentration: 1 mM) and a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) were added to the

supernatants and the mixture was boiled for 5 min. The boiled mixture was cooled to room temperature and passed through AmiconUltra-4 Centrifugal Filter Devices (MW cutoff: 50,000). Proteins in the filtrate were concentrated using trichloroacetic acid precipitation. The precipitate was dissolved in 20  $\mu$ l of SDS-sample buffer and subjected to 15% SDS-PAGE followed by Western blot analysis as previously described [27].

#### *Statistical Analysis*

All data are presented as means  $\pm$  SEM. Statistical analyses were performed using the two-tailed Student's paired t-test. Differences were judged statistically significant when  $P < 0.05$ .

## **Results**

### *Intracellular caspase-3/7 activities of HUVEC and RAW 264 cells*

We first examined the intracellular caspase-3/7 activity in response to LPS stimulation. HUVEC were stimulated with the indicated concentrations of LPS in culture medium containing 100 ng/ml LBP with or without 1  $\mu$ g/ml sCD14 and/or sMD-2. After cell viability was measured, the cells were washed and cellular caspase-3/7 activity was determined. Although statistical significance was sporadically observed in some points, no dose-dependent effects in response to LPS were observed for either caspase-3/7 activity (Fig. 1A left) or the viability (Fig. 1B left) irrespective of the presence/absence of sCD14 and/or sMD-2. Since changes in viability affect total caspase-3/7 activity, measured caspase-3/7 activities were normalized by viability (Fig. 1C left). However, no significant effects were observed in the normalized caspase-3/7

activities.

We next examined the effect of LPS in the presence of CHX since endogenous apoptosis inhibitors may prevent apoptosis in HUVEC. Cells were stimulated as described above in the presence of 20  $\mu\text{g/ml}$  CHX, and viability and cellular caspase-3/7 activity were measured. No dose-dependent effects in response to LPS were observed in caspase-3/7 activity (Fig. 1A right) or viability (Fig. 1B right) regardless of the presence/absence of sMD-2. On the other hand, both were decreased in response to LPS when sCD14 was present. Normalization of caspase-3/7 activity by viability again produced no significant effect in the response to LPS (Fig. 1C right).

Cellular caspase-3/7 activity was also measured in RAW 264, a macrophage-like cell line used as a positive control (Fig. 1D). Activity was determined after cells were stimulated with the indicated concentrations of LPS. Very little activation of caspase-3/7 was observed with the addition of 0.2  $\mu\text{g/ml}$  CHX. However, stimulation with LPS significantly increased caspase-3/7 activity in the presence of 2 and 20  $\mu\text{g/ml}$  CHX.

We next asked whether the decrease in viability observed in response to LPS in the presence of CHX and sCD14 was caspase-dependent or not. After HUVEC were stimulated without or with 100 ng/ml LPS for 24 hr in culture medium containing 100 ng/ml LBP, 1  $\mu\text{g/ml}$  sCD14 and 20  $\mu\text{g/ml}$  CHX in the presence or absence of increasing concentrations of a broad spectrum caspase inhibitor z-VAD-FMK, cell viability was measured. LPS stimulation significantly decreased the viability and the decrease was inhibited by z-VAD-FMK in a concentration-dependent manner (Fig. 2). z-VAD-FMK at 50  $\mu\text{M}$  completely inhibited the decrease, indicating that the LPS-induced decrease in viability was caspase-dependent.

Since intracellular caspase-3/7 activity was not detected in HUVEC in response to LPS, caspase-3/7 activity was measured during a time course following LPS stimulation (Fig. 3). HUVEC were stimulated with 100 ng/ml LPS in culture medium containing 100 ng/ml LBP and 1  $\mu$ g/ml sCD14 in the presence or absence of CHX (20  $\mu$ g/ml). In the absence of CHX (left), no significant changes in intracellular caspase-3/7 activity were observed throughout the incubation period, and LPS stimulation did not increase activity. In the presence of CHX (right), a time-dependent increase in intracellular caspase-3/7 activity was observed. However, LPS stimulation did not further increase this activity.

*Caspase-3/7 and LDH activities were detected in HUVEC culture supernatants after LPS stimulation*

After HUVEC were stimulated without or with LPS for 24 hr in culture medium containing 100 ng/ml LBP and 1  $\mu$ g/ml sCD14 in the presence or absence of CHX (20  $\mu$ g/ml), culture supernatants were collected for the measurements of caspase-3/7 activity and LDH release (Fig. 4). Unexpectedly, we found that caspase-3/7 activity in the culture supernatants was significantly increased in response to LPS stimulation both in the absence (left-bottom) and presence (right-bottom) of CHX in a sCD14-dependent manner. Caspase-3/7 activity without LPS stimulation was lower than when CHX was present. However, a significant increase in caspase-3/7 activity was observed in response to LPS in the presence of sCD14. Consistent with the decrease in viability (Fig. 1), LDH release from HUVEC was increased in response to LPS in the presence of CHX in a sCD14-dependent manner (Fig. 4 right-upper). The LDH release parallel with caspase-3/7 activity in the culture supernatants (Fig. 4 right-lower). On the other

hand, only a small increase in LDH release was observed in the absence of CHX at higher concentrations of LPS in the presence of sCD14 (Fig. 4 left-upper).

To exclude the possibility that proteases other than caspase-3/7 were responsible for the cleavage of the caspase-3/7-specific substrate used to assess activity, we measured caspase-3/7 activity after adding a specific caspase-3/7 inhibitor (Z-DEVD-FMK) to culture supernatants after collection. Caspase-3/7 activity was completely inhibited by 5  $\mu$ M of inhibitor (data not shown), indicating that measured activity was derived from caspase-3/7.

We also used Western blot analysis to measure caspase-3/7 proteins in culture supernatants (Fig. 5 upper panel). Caspase-3 was hardly detectable in the culture supernatant of non-stimulated cells but cleaved caspase-3 (p17 fragment) was clearly observed in culture supernatant from LPS-stimulated cells. The addition of CHX also increased the level of cleaved caspase-3, which was further enhanced by LPS stimulation in the presence of CHX. A protein band was detected at the molecular weight size close to full-length caspase-3, however the appearance was not reproducible (data not shown).

Cytotoxicity was also evaluated during incubation periods via examination of LDH protein released into culture supernatants (Fig. 5 lower panel). The trace amount of LDH detected in the culture supernatant of non-stimulated cells was slightly increased by LPS stimulation. The addition of CHX increased the LDH level, which was further augmented by LPS stimulation in the presence of CHX. Two different anti-caspase-7 antibodies that detected procaspase-7 in HUVEC cellular extracts did not detect caspase-7 in the culture supernatants (data not shown).

*Caspase-3/7 activity detected in culture supernatants without accompanying LDH release*

Since the increase in cleaved caspase-3 fragment in culture supernatants was much greater than the increase in LDH release in the absence of CHX (Fig. 5), we examined caspase-3/7 and LDH activities in culture supernatants at various time points following LPS stimulation (Fig. 6). HUVEC were stimulated without or with 100 ng/ml LPS for the indicated time in culture medium containing 100 ng/ml LBP and 1  $\mu$ g/ml sCD14 in the presence (right) or absence (left) of CHX (20  $\mu$ g/ml), and the supernatants were collected for the measurements of caspase-3/7 activity and LDH release. Caspase-3/7 activity gradually increased with time and LPS stimulation significantly enhanced the activities both in the absence (left upper) and presence (right upper) of CHX. A statistically significant enhancement was observed after 12 hr and 8 hr following LPS stimulation in the absence and presence of CHX, respectively. No LDH release was observed in the absence of CHX (left lower) regardless of the presence or absence of LPS stimulation. In the presence of CHX (right lower), LDH release gradually increased over time and tended to be enhanced by LPS.

## **Discussion**

Although LPS-induced endothelial cell apoptosis has been reported in some studies, discrepancies exist regarding the inducibility of apoptosis and the requirement of protein synthesis inhibitors for the induction of apoptosis. The endothelial cell lines and/or methods used to evaluate apoptosis may influence these differences. Although it has been shown that LPS directly induces cell death in a bovine endothelial cell line

[28-29], most studies using human endothelial cells showed that these cells require protein synthesis inhibitors such as CHX for the induction of apoptosis in response to LPS [24, 30]. Various methods were employed to measure endothelial injury in these studies. For example, Hu et al. examined cell viability by MTT assay [30] and Bannerman et al. examined caspase activities [24]. In the present study, we systematically evaluated endothelial injury with the MTS viability assay, LDH release (cytotoxicity) and caspase-3/7 activity both intracellularly and in culture supernatants in the presence and absence of a protein synthesis inhibitor, CHX. As reported previously, we also observed in this study that LPS induces decrease in viability (Fig. 1) and an increase in cytotoxicity (Fig. 4) in the presence of CHX and that viability and intracellular caspase-3/7 were not affected by LPS stimulation in the absence of CHX (Fig. 1) although a small increase in cytotoxicity was observed (Fig. 4). However, surprisingly, we found that LPS induced a significant increase in caspase-3/7 activity (Fig. 4 and 6) in culture supernatants even in the absence of CHX. This activity was completely inhibited by a specific caspase-3/7 inhibitor (data not shown) and the cleaved p17 caspase-3 fragment was detected in the supernatant (Fig. 5), indicating that at least active caspase-3 was released into the supernatant. Because only intracellular caspase-3/7 activities were examined in most studies, activation of caspase-3 in the absence of CHX might not have been detected.

It is likely that cleaved active caspase-3 is rapidly released from cells in the absence of CHX because the activation of intracellular caspase-3/7 in response to LPS was not observed at any of the time points examined (Fig. 3). In the presence of CHX, it is plausible that most of the cleaved caspase-3 was derived from cells that lost membrane integrity because a considerable decrease in viability (Fig. 1) and increase in LDH

release (Fig. 4, 5 and 6) were observed. However, cleaved caspase-3 was also detectable (Fig. 5) in response to LPS in the absence of CHX although no significant changes in viability were observed (Fig. 1). Apparently viability may remain unchanged if LPS increases cell growth or cellular NADH activity, which is used to evaluate viability in this study. Thus, we measured the release of LDH into culture supernatants and found that it was only slightly increased by higher concentrations of LPS in the absence of CHX at 24 hr following stimulation (Fig. 4). LDH protein in culture supernatants, examined in parallel with the detection of cleaved caspase-3, was also slightly increased in response to LPS (Fig. 5). Therefore, at least some parts of cleaved caspase-3 may be derived from cells that have lost membrane integrity. It is currently unknown if this loss of membrane integrity is caused by the activation of caspase-3. LPS may induce the production of substances that cause necrotic cell death in HUVEC. However, the LPS-induced increase in cleaved caspase-3 protein is obviously greater than the increase in LDH protein in culture supernatants (Fig. 5). Furthermore, the LPS-induced increase in caspase-3/7 activity in culture supernatants occurred without increase in LDH release at earlier time points (Fig. 6). Therefore, it is probable that a certain amount of cleaved caspase-3 was derived from cells that had not lost membrane integrity. It is widely known that interleukin-1 $\beta$  and caspase-1, which lack a signal peptide sequence for secretion, are secreted from cells via an unconventional endoplasmic reticulum/Golgi-independent pathway [31-32]. Other proteins that have no signal peptides but are secreted have also been identified [32]. Further study may reveal an as yet unknown secretion mechanism for cleaved caspase-3.

In HUVEC, the decrease in viability, increase in LDH release and activation of



caspase-3/7 in response to LPS were dependent on the presence of sCD14 and LBP but were independent of the presence of sMD-2 (Fig. 1 and 4). It is reported that endothelial cells lack membrane CD14 and only TLR4 and MD-2 are expressed [33-34]. We also confirmed by real time RT-PCR that HUVEC expressed TLR4 and MD-2 mRNAs but have limited expression of CD14 mRNA (data not shown). Since it has been reported that serum sCD14 levels are increased in septic patients [35], the reduction of sCD14 level may suppress the induction of endothelial cell apoptosis and contribute to improvement of endothelial cell injury/dysfunction.

Since most studies have reported that LPS-induced apoptosis develops only in the presence of protein synthesis inhibitors, such as CHX, it has been pointed out that the results do not always reflect physiological reactions and it has been unclear whether apoptosis is involved in endothelial injury/dysfunction that occurs under sepsis. Although there is evidence to suggest that the survival pathway may fail due to inhibition by other inflammatory mediators or cytokines during sepsis [36-37], our results clearly demonstrate that LPS activates caspase-3 even in the absence of CHX and that activated caspase-3 is rapidly released from cells without causing cell death. It is likely that HUVEC escape from cell death by releasing activated caspase-3 induced by LPS stimulation. In the presence of CHX, this protection mechanism appears to fail because active caspase-3 accumulated in HUVEC (Fig. 3) and caspase-dependent cell death occurred (Fig. 2 and 5). Studying this mechanism will clarify the role of LPS-induced apoptosis in the pathogenesis of endothelial injury/dysfunction under sepsis.

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### Figure legends

**Fig. 1. Effect of LPS on cell viability and intracellular caspase-3/7 activity in HUVEC and RAW 264 cells.** HUVEC were stimulated with the indicated concentrations of LPS in HE-SFM containing 100 ng/ml LBP with (left) or without

(right) 20  $\mu\text{g/ml}$  CHX in the presence or absence of 1  $\mu\text{g/ml}$  sCD14 and/or sMD-2. After 24 hr, intracellular caspase-3/7 activity (**A**) and viability (**B**) were measured. The measured caspase-3/7 activities were corrected with the corresponding viabilities (**C**). Measured caspase-3/7 activity, viability and corrected caspase-3/7 activity values obtained without LPS in the absence of CHX were defined as 100%. **D**: RAW 264 cells were treated with the indicated concentrations of LPS with or without CHX (0.2-20  $\mu\text{g/ml}$ ). Intracellular caspase-3/7 activity was measured after a 7-hr incubation. The activity obtained without LPS in the absence of CHX was defined as 100%. Values are the means  $\pm$  S.E.M. from at least four independent experiments. \* $P < 0.05$ : compared with the respective value without LPS by a two-tailed Student's paired t-test. Note that a statistically significant increase was observed at following points: A (-CHX): LPS 1, 10, 100 ng/ml in the presence of sCD14; LPS 1, 10 ng/ml in the presence of sCD14 plus sMD-2; A (+CHX): LPS 1000 ng/ml in the absence of sCD14 and sMD-2; B (+CHX): LPS 0.1, 1000 ng/ml in the absence of sCD14 and sMD-2; LPS 1, 10, 100 ng/ml in the presence of sMD-2; C (-CHX): LPS 1, 10 ng/ml in the presence of sCD14; LPS 1, 10, 1000 ng/ml in the presence of sCD14 plus sMD-2 and that a statistically significant decrease was observed at following points: A (+CHX): LPS 100, 1000 ng/ml in the presence of sCD14 and sCD14 and sMD-2; B (+CHX): LPS 100, 1000 ng/ml in the presence of sCD14 and sCD14 and sMD-2.

**Fig. 2. Decrease in viability in response to LPS is caspase-dependent.** HUVEC were stimulated without or with 100 ng/ml of LPS in HE-SFM containing 100 ng/ml LBP, 1  $\mu\text{g/ml}$  sCD14 and 20  $\mu\text{g/ml}$  CHX in the presence or absence of increasing concentrations of z-VAD-FMK. After 24 hr, cellular viability was measured. Values are

the means  $\pm$  S.E.M. from four independent experiments Viability obtained without LPS in the absence of z-VAD-FMK was defined as 100%.

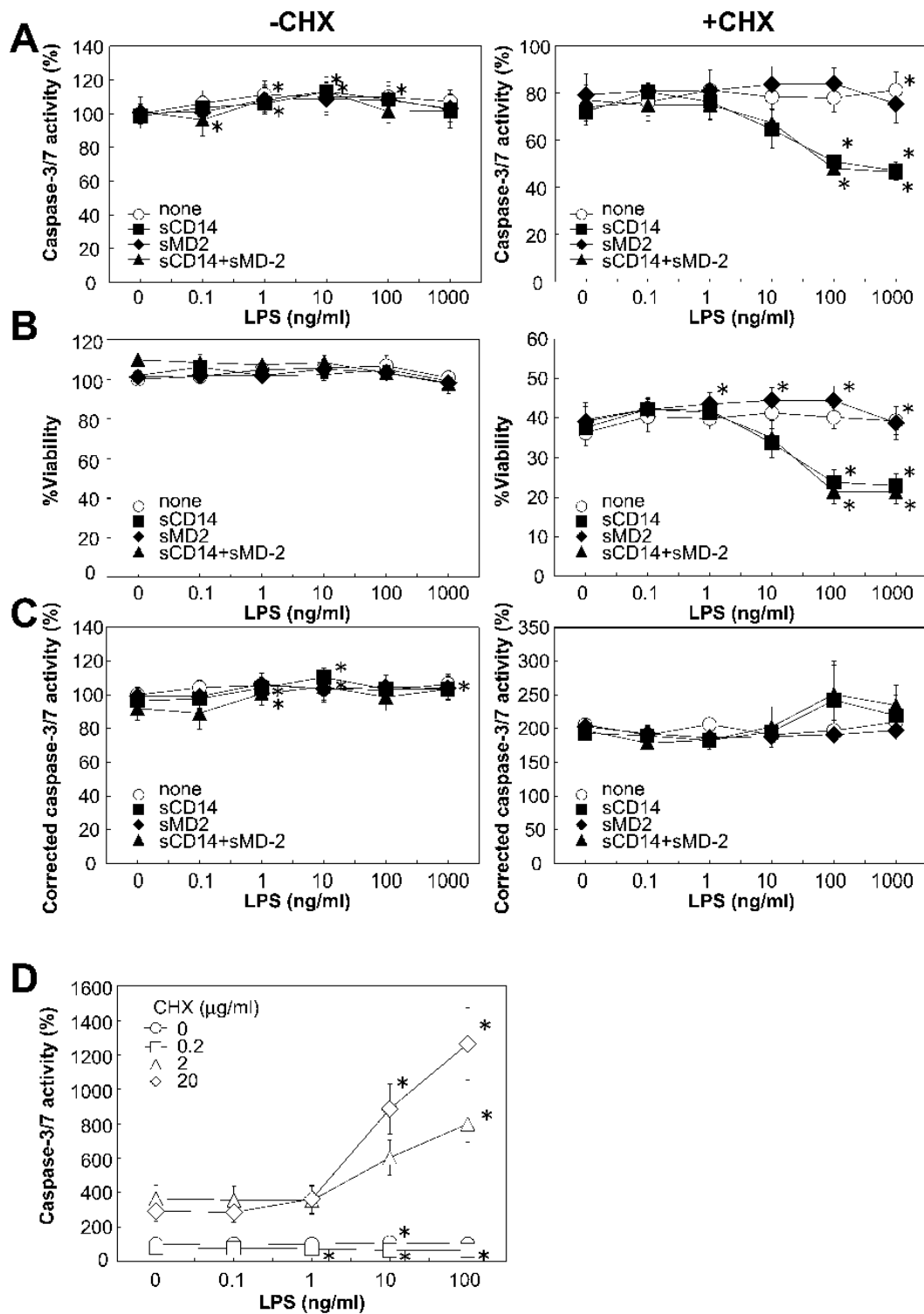
**Fig. 3. Time course profile of intracellular caspase-3/7 activity in response to LPS in HUVEC.** HUVEC were stimulated without or with 100 ng/ml of LPS in HE-SFM containing 100 ng/ml LBP and 1  $\mu$ g/ml sCD14, in the absence (left) and presence (right) of 20  $\mu$ g/ml CHX. Caspase-3/7 activity and viability were measured at the indicated time points. Measured caspase-3/7 activities were corrected using the corresponding viabilities and %caspase-3/7 activity was expressed by defining the corrected caspase-3/7 activity obtained without LPS in the absence of CHX at time 0 hr as 100%. Values are the means  $\pm$  S.E.M. from three independent experiments \*P < 0.05: compared with activity in the absence of LPS by a two-tailed Student's paired t-test.

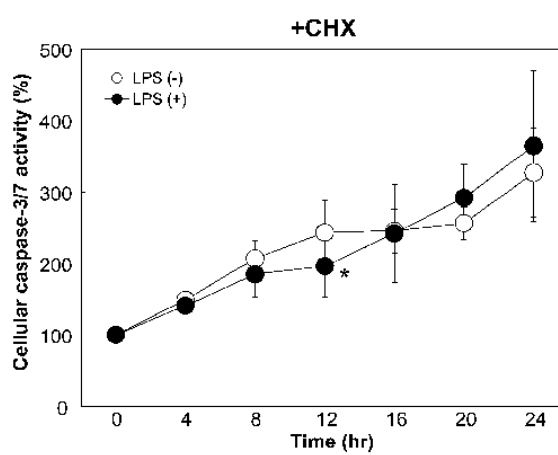
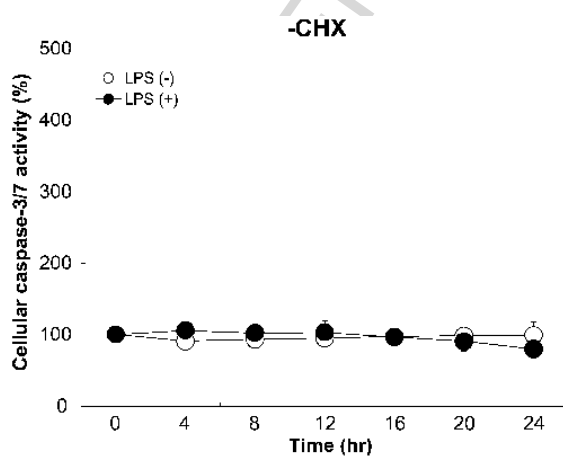
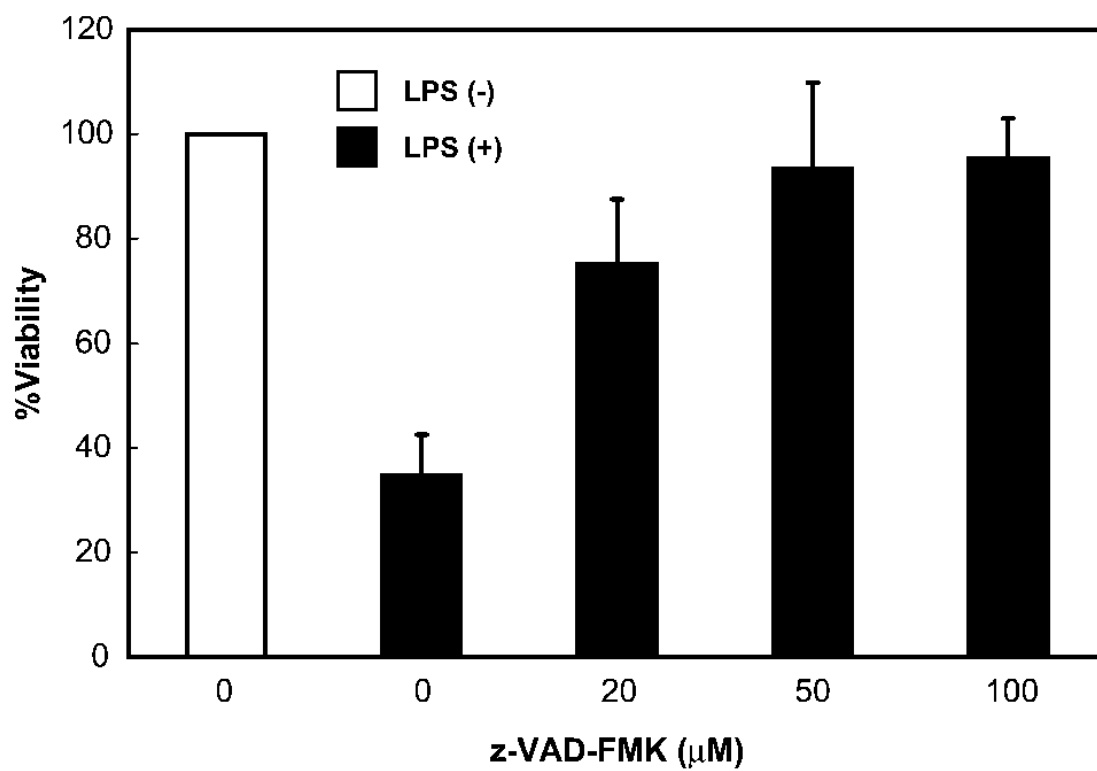
**Fig. 4. LDH and caspase-3/7 activities in culture supernatants of HUVEC in response to LPS.** HUVEC were stimulated with the indicated concentrations of LPS in HE-SFM containing 100 ng/ml LBP without (left) or with (right) 20  $\mu$ g/ml CHX in the presence or absence of 1  $\mu$ g/ml sCD14 and/or sMD-2 . After 24 hr, LDH level (upper) and caspase-3/7 activity in the culture supernatant (lower) were measured. Values are the means  $\pm$  S.E.M. from at least three independent experiments Each value for LDH activity obtained without LPS in the presence and absence of CHX were defined as 0%. Caspase-3/7 activity obtained without LPS in the absence of CHX was defined as 100%. \*P < 0.05: compared with the respective response in the absence of LPS by a two-tailed Student's paired t-test.

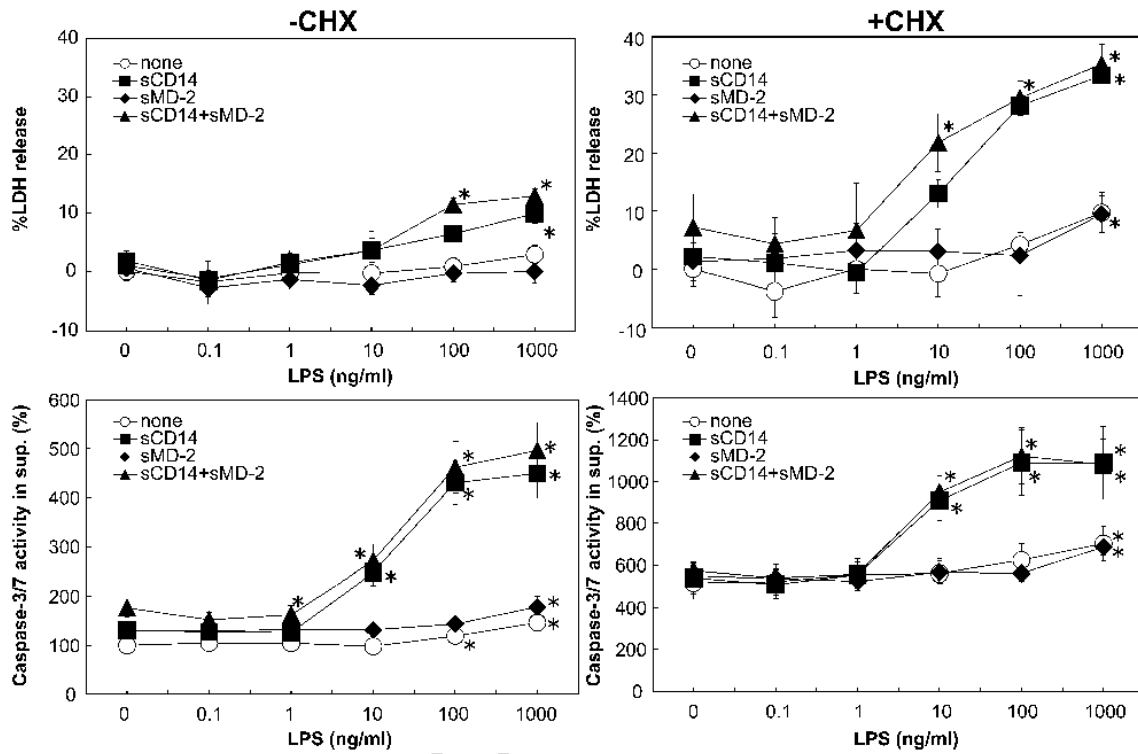


**Fig. 5. LDH proteins and cleaved caspase-3 were detected in HUVEC culture supernatants.** HUVEC were stimulated without or with 100 ng/ml of LPS in HE-SFM containing 100 ng/ml LBP and 1  $\mu$ g/ml sCD14 in the presence and absence of 20  $\mu$ g/ml CHX. After 24 hr, an equal volume of culture supernatants was collected and concentrated as described in the Materials and Methods and caspase-3 (upper) and LDH (lower) were detected by Western blotting. A representative of three independent experiments with similar results is shown.

**Fig. 6. Time course profile of caspase-3/7 and LDH activities in HUVEC culture supernatants in response to LPS.** HUVEC were stimulated with or without 100 ng/ml of LPS in HE-SFM containing 100 ng/ml LBP and 1  $\mu$ g/ml sCD14, in the absence (left) and presence (right) of 20  $\mu$ g/ml CHX. Caspase-3/7 (upper) and LDH (lower) activities in culture supernatants were measured at the indicated time points. Net caspase-3/7 activities are shown. In LDH releases, each LDH activity value obtained at time 0 in the presence or absence of CHX was defined as 0%. Values are the means  $\pm$  S.E.M. from three independent experiments. \* $P < 0.05$ : compared with activity in the absence of LPS at the respective time points by a two-tailed Student's paired t-test.







culture sup.

CHX

