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Identification of plasma APE1/Ref-1 in lipopolysaccharide-induced endotoxemic rats: Implication of serological biomarker for an endotoxemia

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ABSTRACT

Apurinic/apyrimidinic endonuclease1/Redox factor-1 (APE1/Ref-1) is a multifunctional protein involved in base excision DNA repair and in transcriptional regulation of gene expression. We investigated whether APE1/Ref-1 increased in plasma of endotoxemic rats. Lipopolysaccharide (LPS) was used to induce endotoxemia in rats. Administration of LPS (10 mg/kg, i.p.) significantly induced plasma nitrite production and tumor necrosis factor-α (TNF-α). A 37 kDa immunoreactive band was detected in cell-free plasma of LPS-treated rats using anti-APE1/Ref-1, which reached a maximum at 12 h after the LPS injection. The 37 kDa immunoreactive band was identified as rat APE1/Ref-1 by liquid chromatography/tandem mass spectrometry. Interestingly, treatment with recombinant human APE1/Ref-1 protein (2–5 μg/ml for 18 h) inhibited TNF-α-induced vascular cell adhesion molecule-1 expression in human umbilical vein endothelial cells. Taken together, the level of plasma APE1/Ref-1 increased in LPS-induced endotoxemic rats, suggesting that plasma APE1/Ref-1 might serve as a serological biomarker for endotoxemia.

1. Introduction

Sepsis is a major clinical problem and is associated with high mortality [1]. Severe sepsis and septic shock are common indications for admission to an intensive care unit and the leading cause of morbidity and mortality in critically ill patients [2]. Endotoxin including lipopolysaccharide (LPS) is an important mediator of the inflammatory response during a Gram negative bacterial infection. Endotoxin activates inflammatory cells and causes an increase in proinflammatory cytokines including tumor necrosis factor-α (TNF-α) as well as increased nitric oxide (NO) production, which can lead to multiple organ failure [3]. Serological biomarkers can be useful for identifying or ruling out endotoxemia, identifying patients who may benefit from specific therapies, and assessing the response to therapy. Several molecules have been proposed as useful biological markers for endotoxemia, but it is unknown which of these provides truly useful information [4].

Apurinic apyrimidinic endonuclease 1/Redox factor-1 (APE1/Ref-1) is a multifunctional protein with a molecular weight of ~37 kDa. APE1/Ref-1 is involved in both base excision repair of abasic DNA lesions and in eukaryotic transcriptional regulation [5]. Most reports have shown that APE1/Ref-1 is localized to the nucleus but growing evidence indicates that APE1/Ref-1 can be also localized in cytoplasm, particularly during highly metabolic or proliferative states [5–7]. APE1/Ref-1 undergoes active shuttling between the cytoplasm and nucleus in response to oxidative stress [8–10]. ANO donor or S-nitrosating agent effectively stimulates nuclear export of APE1 in an exportin chromosome region maintenance 1-independent manner under nitrosative stress [11]. Furthermore, APE1/Ref-1 expression increases in several inflammatory disorders, which is closely linked to inflammatory signaling [12–14].

Secretome 2.0 [15,16], software to predict secreted proteins, has suggested that APE1/Ref-1 is a non-classically secreted protein. The APE1/Ref-1 autoantibody has been identified in serum from patients with systemic lupus erythematosus, suggesting extracellular release of APE1/Ref-1 [17]; however, little is known about APE1/Ref-1 under endotoxemic conditions. In the present study, we hypothesized that APE1/Ref-1 may enter into systemic circulation in a endotoxemic condition. Therefore, we investigated the change in plasma APE1/Ref-1 in LPS-induced endotoxemic rats.

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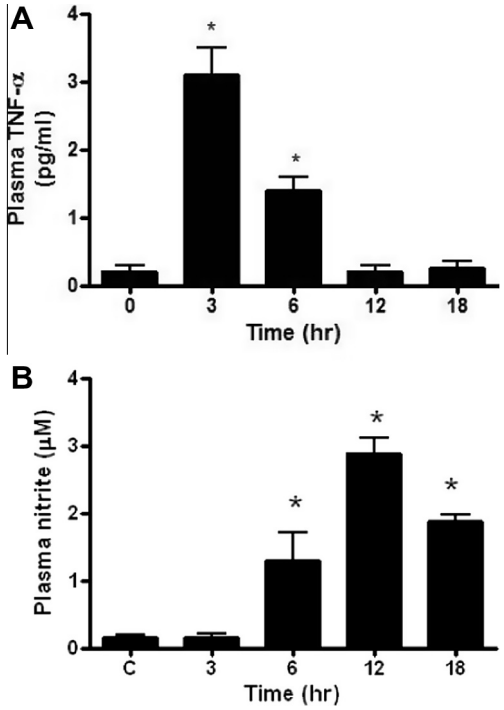

Fig. 1. Increased plasma nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) production in lipopolysaccharide (LPS) (10 mg/kg)-treated rats. (A) Time courses of plasma TNF-α production in LPS (10 mg/kg)-treated rats. Rats were exposed to LPS (10 mg/kg, i.p.) for the indicated time. Plasma TNF-α was determined with a rat TNF enzyme-linked immunosorbent assay kit. Each point shows the mean ± standard error (SE) ($n = 3$). $^*p < 0.05$ *vs.* control. (B) Time course of plasma NO production in LPS (10 mg/kg)-treated rats. Rats were exposed to LPS (10 mg/kg, i.p.) for the indicated time. NO production was spectrophotometrically determined by assaying plasma nitrite using the modified Griess reaction. Each point shows the mean ± SE ($n = 3$). $^*p < 0.05$ *vs.* control.

2. Materials and methods

2.1. Cell culture and reagents

Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial growth medium (EGM-2, Gibco, Grand Island, NY, USA). An APE1/Ref-1 antibody was obtained from Abcam (Cambridge, MA, USA). Anti-vascular cell adhesion molecule-1 (VCAM-1) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). LPS (*E. coli* serotype O26:B6) and anti-β-actin antibodies were purchased from Sigma (St. Louis, MO, USA).

2.2. Endotoxemic animal model

Animal experiments were performed with male Sprague–Dawley rats weighing 150–200 g. All animals were fed normal rat chow, had free access to water, and were kept under a 12 h light–dark cycle. Water and rodent chow were provided *ad libitum*. The animal care committee of Chungnam National University approved the animal care and experimental procedures conducted in this study (IACUC #2010-2-16). All experiments involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th edition, 2011). All instrumentation was conducted using aseptic procedures. Rats were injected intraperitoneally (i.p.) with LPS (10 mg/kg) or the same volume of phosphate-buffered saline as a control. At the designated time points (0, 3, 6, 12, and 18 h after LPS injection), rats were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (12 mg/kg, i.p.). Heparinized blood samples were obtained from the abdominal aorta to determine plasma APE1/Ref-1. Plasma samples were separated by centrifugation at 3000 rpm for 10 min at 4 °C, re-centrifuged at 5000 rpm for 5 min to obtain cell-free plasma, and stored at −70 °C until use.

2.3. Measurement of plasma nitrite and TNF-α levels

For measurement of plasma NO metabolites and TNF-α concentrations, 0.8–1 ml of whole blood was obtained by direct puncture of the abdominal aorta from anesthetized animals. NO production was spectrophotometrically determined by assaying plasma nitrite using the modified Griess reaction as reported previously [18]. The level of TNF-α in plasma was determined with a rat TNF enzyme-linked immunosorbent assay kit (BD Pharmigen, San Diego, CA, USA) with standard concentrations ranging from 4 to 1000 pg/ml according to the manufacturer's instruction. Absorbance was measured at a 450 nm wavelength.

2.4. Immunoblotting

Five microliters of cell-free plasma were diluted 1:4 in phosphate buffer saline. Samples were treated in Laemmli loading buffer at 95 °C for 10 min. Samples were electrophoretically separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (18 × 16 cm) in duplicate. One gel was stained with 0.1% Commassie Blue R250 (Sigma Chemical Co., St. Louis, MO, USA) to observe protein bands for liquid chromatography mass spectroscopy analysis. APE1/Ref-1 protein levels were assessed by densitometric analysis of the immunoreactive bands, compared with immunoreactivity of the recombinant human APE1/Ref-1 protein.

HUVECs were harvested in 80 μl of lysis buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1% NP-40, 1% Na deoxycholate, 2.5 mM Na pyrophosphate, 1 mM Na_3VO_3, 1 mM β-glycerophosphate, and a protease inhibitor cocktail. The lysate was centrifuged at 12,000 rpm for 20 min, and the supernatant was collected. Prepared proteins were separated on 10% SDS–PAGE and transferred onto a PVDF membrane. After blocking with 5% nonfat dry milk in TBS containing 0.05% Tween 20, the membrane was incubated with the APE1/Ref-1 (1:2000) and VCAM-1 (1:1000) antibodies for 18 h at 4 °C. The membrane was then treated with an appropriate peroxidase-conjugated secondary antibody, and the chemiluminescent signal was developed using the Super Signal West Pico or the Femto substrate (Pierce Biotechnology, Rockford, IL, USA). Each membrane was stained with anti-β-actin antibody to normalize loading.

2.5. Isolation and purification of recombinant APE1/Ref-1

We purified the recombinant human APE1/Ref-1 protein using a pET28 expression system to determine the functional role of plasma APE1/Ref-1 [18]. Recombinant human APE1/Ref-1 and enhanced green fluorescent protein (EGFP), as a control protein, were prepared under native conditions. In brief, following a 4 h induction with IPTG, cells were sonicated in buffer solution (500 mM NaCl, 50 mM Tris pH 8.0), and the recombinant protein was purified on a Ni-NTA agarose column (Qiagen, Valencia, CA, USA). After washing with a 50 mM imidazole-containing buffer solution, the APE1/Ref-1 and EGFP proteins were eluted from a 250–500 mM imidazole-containing buffer solution followed by desalting with a PD-10 column (Amersham Pharmacia Biotech, Parsippany, NJ, USA) into PBS. Purified proteins were aliquoted and frozen in 10% glycerol with liquid nitrogen.

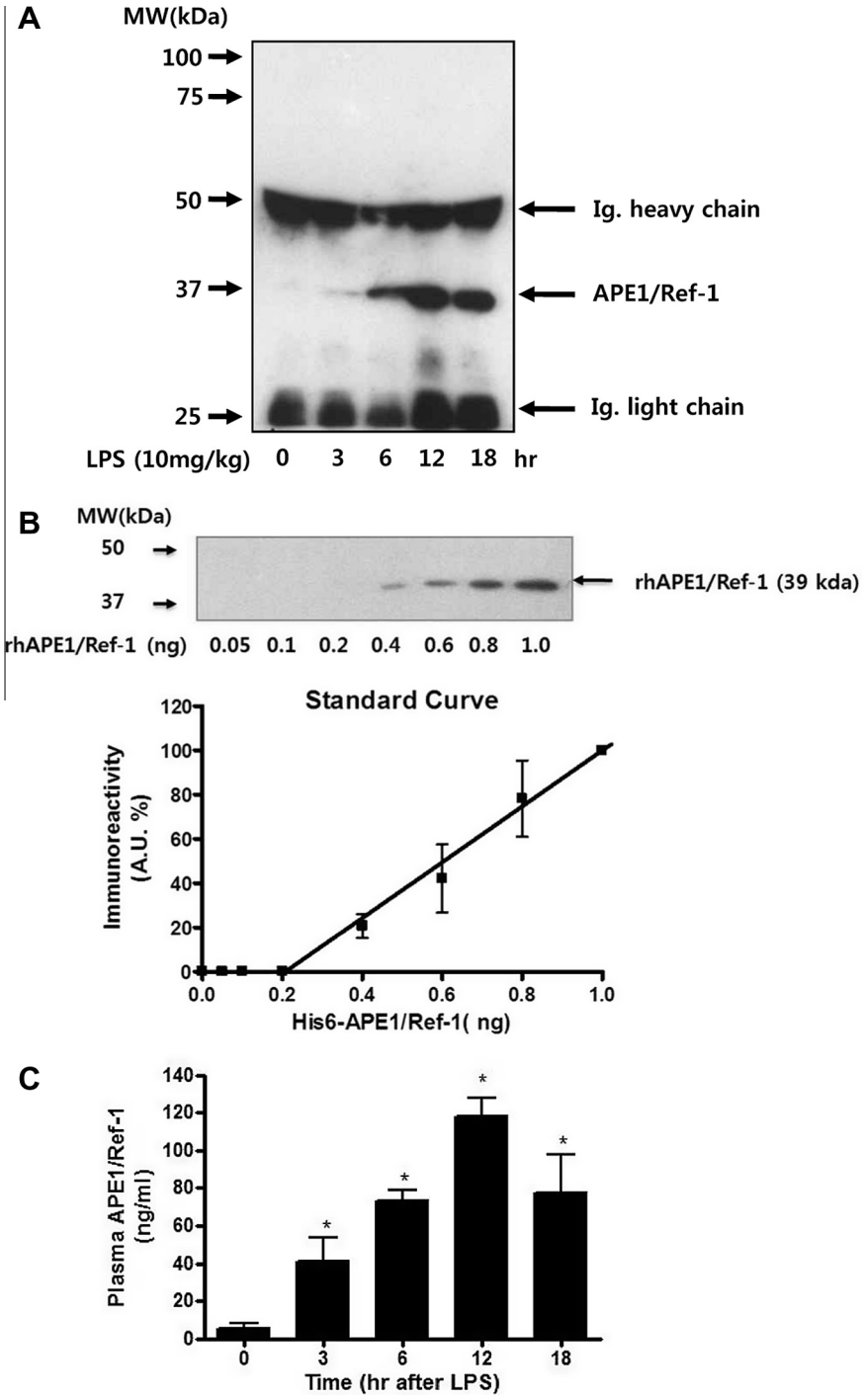

Fig. 2. Apurinic/apyrimidinic endonuclease1/Redox factor-1 (APE1/Ref-1) is detected in the plasma of lipopolysaccharide (LPS)-induced endotoxemic rats. (A) Immunoreactivity to APE1/Ref-1 in the plasma of LPS-treated rats. Western Blot analysis for APE1/Ref-1 was performed in plasma of rats treated with LPS (10 mg/kg, i.p. for 3–18 h). APE1/Ref-1 bands of 37 kDa were detected in plasma of LPS-treated rats. Non-specific immunoglobulin bands (heavy chain of 50 kDa, and light chain for 25 kDa) were observed in the immunoblot analysis. (B) Immunoreactivity of recombinant human APE1/Ref-1 (rhAPE1/Ref-1, 39 kDa). Standard curve of immunoreactivity for the APE1/Ref-1 antibody showed linear correlation in the range of 0.05–1 ng rhAPE1/Ref-1. (C) Estimate of plasma APE1/Ref-1 concentrations in LPS-treated rats. The concentration of plasma APE1/Ref-1 was estimated by immunoreactivity using a recombinant human APE1/Ref-1 protein. Each point shows the mean ± standard error ($n = 5$). $^{*}p < 0.05$ *vs.* control.

2.6. LC-MS/MS

A band separated from plasma by SDS–PAGE, which was identified as immunoreactive with antiAPE1/Ref-1, was excised for LC-MS/MS analysis. The gel piece was subjected to in-gel digestion with sequencing grade trypsin in 25 mM ammonium bicarbonate buffer overnight at 37 °C. The prepared tryptic peptides from gel slices were analyzed by LC-MS/MS (ProteomeTech, Seoul, Korea). Individual spectra from the LC-MS/MS were analyzed with the NCBI non-redundant protein data base against APE1/Ref-1 using MASCOT software (Matrix Science Ltd., London, UK at http://www.matrixscience.com).

Table 1
Peptide analysis of plasma apurinic/apyrimidinic endonuclease1/Redox factor-1 (APE1/Ref-1). The peptide sequence of the band corresponding to the molecular weight of APE1/Ref-1 on sodium dodecyl sulfate polyacrylamide gel electrophoresis was analyzed by liquid chromatography tandem mass spectroscopy. Six peptides matching the APE1/Ref-1 protein sequence were identified. The matched peptide showing the highest query coverage was confirmed as *Rattus norvegicus* APE1/Ref-1 (Accession no. AAG49922.1).

Identified peptide sequence	Size	Position
R↓AAA EDGEEPKSEPETKK↓S	17	8–24
K↓EEAPDILCLQETKCSENK↓L	18	85–102
K↓EGYSGVGLLSRQCPLK↓L	16	125–140
R↓KPLVLCGDLNVAHEEIDLRNPKGNK↓K	25	202–226
K↓KNAGFTPQERQGFGEMLQAVPLADSFR↓H	27	227–253
K↓ALGSDHCP ITLYLAL*	15	303–317

Note: Total 117 matched peptides (refined *Rattus norvegicus*) was detected.

2.7. Statistical analysis

All data are expressed as mean ± standard error. Statistical evaluation was conducted using a one-way analysis of variance, followed by a Tukey's post hoc test. A p-value <0.05 was considered significant.

3. Results

3.1. LPS increased plasma NO and TNF-α production in rats

Endotoxemia-induced inflammation was associated with an increase of plasma NO and inflammatory cytokine production. We examined the time courses of plasma NO and TNF-α production in response to LPS (10 mg/kg, i.p.) in rats. As shown in Fig. 1, administration of LPS (10 mg/kg, i.p.) increased the levels of plasma NO and TNF-α, indicating LPS-induced systemic inflammation in the rats. LPS-induced plasma TNF-α levels peaked at 3 h and returned to basal level at 12 h; however, LPS-induced plasma NO production reached a peak at 12 h with about a 3 h lag time.

3.2. Plasma APE1/Ref-1 increased in plasma of LPS-induced rats

We were interested in whether LPS-induced endotoxemia was associated with increased plasma APE1/Ref-1. As shown in Fig. 2A, the APE1/Ref-1 protein was not detected in the plasma of normal rats; however, it was detected in the plasma of LPS (10 mg/kg)-treated rats, suggesting that LPS-induced endotoxemia was associated with the presence of APE1/Ref-1 in the circulating blood plasma.

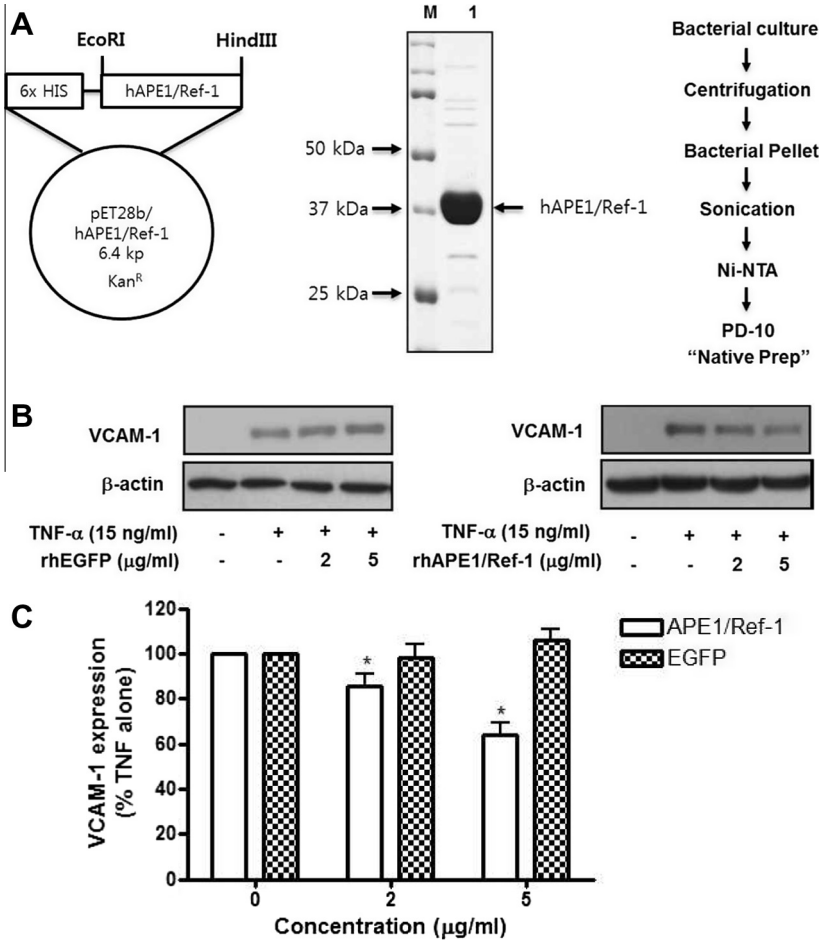

Fig. 3. Recombinant human apurinic/apyrimidinic endonuclease1/Redox factor-1 (APE1/Ref-1) inhibits TNF-α-induced vascular cell adhesion molecule-1 (VCAM-1) expression in cultured endothelial cells. (A) Purification of recombinant human APE1/Ref-1. Human full length APE1/Ref-1 cDNA was inserted in the EcoRI and HindIII restriction enzyme site in the pET28b system. Recombinant human APE1/Ref-1 was purified under native conditions and was identified by Commassie Blue staining. M, Size marker, lane 1 shows human APE1/Ref-1. (B) Effect of recombinant human APE1/Ref-1 on tumor necrosis factor-alpha (TNF-α)-induced VCAM-1 in human umbilical vein endothelial cells. Cells were treated with rhAPE1/Ref-1 (2–5 μg/ml) or enhanced green fluorescent protein (EGFP) (2–5 μg/ml) as a control. TNF-α-induced VCAM-1 expression was significantly suppressed by recombinant APE1/Ref-1 (2–5 μg/ml). (C) Summarized data for A. Each bar shows the mean ± standard error (n = 5). $^*p < 0.05$ *vs.* each basal condition.

To estimate the level of plasma APE1/Ref-1, the immunoreactivity of APE1/Ref-1 on Western Blot was compared with recombinant APE1/Ref-1. The standard curve showed a linear correlation at 0.1–1 ng of recombinant APE1/Ref-1, as shown in Fig. 2B. Basal plasma APE1/Ref-1 was estimated to 5 ± 2 ng/ml by the quantitative immunoblotting analysis, and the peak plasma APE1/Ref-1 level in response to LPS was 118 ± 10 ng/ml, suggesting a serological maker for LPS-induced endotoxemia.

3.3. Peptide analysis of secreted APE1/Ref-1

Next, we investigated whether a 37 kDa immunoreactive band was rat APE1/Ref-1 in the plasma of LPS-treated rats. The peptide sequence of the band, separated on SDS–PAGE, was analyzed by LC-MS/MS. Six oligopeptides, which were matched with amino acid sequences of rat APE1/Ref-1, were detected in LPS-treated rats. The matched peptide was confirmed to be *Rattus norvegicus* APE1/Ref-1 (Accession no. AAG49922.1) (Table 1). This finding indicated that the pathophysiological stress of endotoxemia resulted in systemically circulating APE1/Ref-1, and suggests that plasma APE1/Ref-1 may serve as a serological biomarker for systemic inflammation such as endotoxemia.

3.4. Recombinant APE1/Ref-1 inhibits TNF-α-induced endothelial inflammation

We hypothesized that plasma APE1/Ref-1 in endotoxemia plays a part in regulating vascular inflammation; thus, we investigated the effect of recombinant APE1/Ref-1 on vascular endothelial inflammation. Recombinant human APE1/Ref-1 was purified under native conditions. The schematic diagram of human APE1/Ref-1 cDNA in the pET28b system and the typical Commassie Blue staining of purified APE1/Ref-1 is shown in Fig. 3A. Cells were treated with TNF-α (15 ng/ml) in the presence of recombinant human APE1/Ref-1 (2–5 μg/ml) and EGFP as a control protein. As shown in Fig. 3B, TNF-α treatment (15 ng/ml) induced increased VCAM-1 expression in HUVECs; however, the level of TNF-α-induced VCAM-1 expression was significantly suppressed by recombinant human APE1/Ref-1 (2–5 μg/ml). These findings suggest an important role for extracellular APE1/Ref-1 during vascular endothelial inflammation.

4. Discussion

We demonstrated that APE1/Ref-1 increased in the plasma of LPS-induced endotoxemic rats, and that recombinant APE1/Ref-1 had anti-inflammatory activity by suppressing proinflammatory molecules.

APE1/Ref-1 is a 37 kDa nuclear protein containing nuclear localization signals. As 20 N-terminal residues of APE1/Ref-1 implicate the nuclear localization signal peptide for nuclear translocation, APE1/Ref-1 mutants lacking the nuclear localization signals are localized outside of the nucleus [19]. Many secreted proteins have a signal peptide sequence for secretion [20]. The presence of these signal peptides can be used to predict possible protein secretion. In a preliminary study, an APE1/Ref-1 secretome analysis using Secretome 2.0 showed APE1/Ref-1 as a possible non-classically secreted protein. We detected the APE1/Ref-1 protein in the plasma of endotoxemic rats, and a 37 kDa immunoreactive band was identified as *Rattus norvegicus* APE1/Ref-1 (Accession no. AAG49922.1) by LC-MS/MS (Table 1). Therefore we suggest that plasma APE1/Ref-1 can be used as serological biomarker for endotoxemia.

In the experimentally induced endotoxemic rats, the level of plasma TNF-α reached a peak at 3 h and returned to basal levels at 12 h. Plasma APE1/Ref-1 reached a peak at 12 h and showed a pattern similar to that of plasma NO levels. This result suggests that plasma APE1/Ref-1 secretion might be strongly correlated with plasma NO levels which is induced by TNF-α or LPS. Post-translational protein modification is a crucial mechanism to regulate protein function in eukaryotic cells. LPS causes acetylation of histones and represses a number of histone deacetylases [21,22]. Histone deacetylase may act as an intra-nuclear gatekeeper based on their ability to antagonize NO-induced APE1/Ref-1 trafficking [11]. APE1/Ref-1 is a target of SIRT1 protein deacetylases [23].

It is well established that endothelial cells lining blood vessels are highly metabolically active, and provide an important frontline of host defense. TNF-α is an important mediator of systemic inflammation and is produced in response to LPS [24,25]. TNF-α is a potent activator of endothelial cells and activates the expression of adhesion molecules that promote binding of circulating monocyte [26,27]. APE1/Ref-1 has been identified as a protein capable of nuclear redox activity, including DNA binding activity of several transcriptional factors [28]. Extracellular thioredoxin, a type of redox regulating protein, has anti-inflammatory effects through its abilityto suppress neutrophil infiltration at the inflammatory site [29,30]. In the present study, the recombinant human APE1/Ref-1 treatment suppressed TNF-α-induced VCAM-1 expression in endothelial cells, suggesting a functional role for extracellular APE1/Ref-1. Although the precise mechanism underlying extracellular APE1/Ref-1 in cells has yet to be elucidated, one possibility is that biologically active extracellular APE1/Ref-1 may influence the redox status of cytokine receptors in endothelial cells.

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