TLR4-mediated inflammatory activation of human coronary artery endothelial cells by LPS

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Abstract

Objective: Blood levels of cytokines are commonly elevated in severe congestive heart failure (CHF) and in coronary artery disease (CAD). While the adverse effects of cytokines on contractile function and myocardial cell integrity are well studied, little is known on whether cardiac cells are only targets or active players in these inflammatory reactions. Methods and results: We tested if human coronary artery endothelial cells (HCAEC) may become a source of cytokine and adhesion molecule expression when stimulated with bacterial lipopolysaccharide (LPS). Analysis of HCAEC supernatants by ELISA identified enhanced secretion of IL-6, IL-8, and MCP-1 while endothelin-1 was not increased. IL-1\textbeta, IL-10, or TNF-\alpha were not detectable by ELISA while RT-PCR revealed enhanced mRNA expression of IL-1\textbeta and TNF-\alpha but not IL-10. FACS analysis showed an LPS-induced upregulation of ICAM-1, VCAM, and ELAM-1. LFA-1 could not be detected. We further characterized receptors involved in LPS-induced signaling. Our results indicate that activation of HCAEC by LPS requires Toll-like receptor (TLR) 4. Pretreating the cells with the 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase inhibitor Cerivastatin reduced IL-6 release. Conclusions: Taken together, our results indicate that activated HCAEC may act as inflammatory cells and thus directly contribute to the progression of CHF and CAD.

Keywords: Heart failure; Endotoxins; Infection/inflammation; Endothelial factors; Cytokines

1. Introduction

Growing evidence supports the role of local and systemic inflammation as a common pathophysiological mechanism in different cardiovascular diseases like congestive heart failure (CHF) or coronary artery disease (CAD). Elevated plasma levels of cytokines like interleukin (IL-) 6 and Tumor Necrosis Factor (TNF-) \textalpha were not only consistently detected in patients with CHF [1–3] but also in patients with stable or unstable angina and myocardial infarction [4–6]. Cytokine levels correlate with the extent of New York Heart Association (NYHA) functional classes, ventricular function and prognosis. In CAD, the IL-6 level is a marker of increased mortality in patients with unstable angina that can identify patients who will benefit most from invasive management [7]. Inflammatory mediators such as cytokines can modulate cardiac contractility and therefore might influence CHF progression [8–10]. Cytokines such as IL-1, IL-6, and TNF-\alpha exerted negative inotropic effects in isolated cells and hearts [11,12].

While the contribution of pro-inflammatory cytokines to progression of cardiovascular diseases is well established, little is known about the sources of enhanced cytokine secretion and the signal transduction pathways within the target cells. It was proposed that enhanced blood levels of cytokines in CHF might originate from inadequate tissue perfusion of non-vital organs [13]. Circulating blood cells such as monocytes were also identified as a source for secretion of cytokines like IL-1, IL-6, and TNF-\alpha [1,14].

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However, there is growing evidence that cardiac cells are not only targets but, following proper stimulation, contribute to cytokine secretion themselves [15,16].

Bacterial cell wall components like lipopolysaccharide (LPS) can activate inflammatory processes [11]. LPS interacts with CD14 [17], a GPI-linked molecule unable to mediate transmembrane signaling. However, due to its high affinity for LPS and its interaction with additional transmembrane receptors it still is an important regulator of LPS signal transduction. Complexes of soluble CD14 (sCD14) and LPS are able to stimulate a variety of cells lacking membrane-bound CD14, including endothelial cells [18]. The transmembrane signaling receptor for LPS has been recently identified as Toll-like receptor (TLR) 4 [19–21]. CD14 and TLR4 were found to be expressed by cardiomyocytes [15,22,23] and elevated levels of LPS and soluble CD14 could be measured in patients with CHF [24–26].

The first cardiac cells that can come in contact with the circulating LPS may be the coronary endothelial cells. However, whereas human umbilical endothelial cells (HUVEC) were shown to respond to LPS, only little is known about the activation mechanisms of human coronary artery endothelial cells (HCAEC). As endothelial cells of different sources seem to be very heterogeneous [27,28], we aimed to identify inflammatory mediators produced by HCAEC and receptors involved in the signaling mechanism. We will show that HCAEC, presumably in a TLR4-dependent manner, respond to LPS leading to subsequent release of inflammatory cytokines like IL-6, IL-8, and MCP-1 and enhanced expression of adhesion molecules like ICAM-1, VCAM, and ELAM-1. Moreover, we will demonstrate that cytokine release can be partially inhibited by the HMG CoA reductase inhibitor Cerivastatin. In conclusion, our data show that HCAEC can act as ‘inflammatory cells’. We therefore propose that their activation could exert negative effects on contractile function of the failing heart.

2. Methods

2.1. Reagents and buffers

LPS was extracted from Salmonella friedenau with phenol–chloroform–petroleum-ether as described [29] (kindly provided by H. Brade, Research Center Borstel). The synthetic tetraacylated bisphosphate precursor of E. coli Lipid A (also known as Compound 406) was synthesized as described [30]. Cerivastatin was obtained from Bayer (Wuppertal, Germany).

2.2. Antibodies

The following antibodies and the given concentrations were used for flow cytometry analysis: mouse anti-human-intercellular adhesion molecule (ICAM)-1, IgG1 (2 μg/ml; Dianova, Hamburg, Germany); mouse anti-human-vascular cell adhesion molecule (VCAM), IgG1 (2 μg/ml; Dianova, Hamburg, Germany); mouse anti-human-E-selectin (ELAM)-1, IgG1 (2 μg/ml; Dianova, Hamburg, Germany); mouse anti-human-leucocyte functional antigen (LFA)-1 IgG2a (2 μg/ml; Dianova, Hamburg, Germany); FITC-conjugated mouse anti-human-CD14, IgG2a (2.5 μg/ml; Pharmingen, San Diego, CA, USA); mouse anti-human-CD55 IgG1 (50 μg/ml; Southern Biotechnology Associates, Birmingham, UK); mouse anti-human-TLR4 IgG2a, HTA125 (50 μg/ml; kindly provided by Dr K. Miyake, Saga Medical School, Nabeshima, Saga, Japan); mouse-IgG1,-Isotype Control (50 μg/ml; Dako, Hamburg, Germany); mouse-IgG2a,-Isotype Control (50 μg/ml; Dako, Hamburg, Germany); FITC-labeled goat-anti-mouse-IgG (15 μg/ml; Dianova, Hamburg, Germany).

2.3. Cell culture

HCAEC were obtained from Promo Cell (Heidelberg, Germany). Cells were grown in endothelial cell growth medium (EBM-2; Clonetics, St. Katharinen, Germany) supplemented with EGM-2 (2% FCS, hydrocortisone, human fibroblast growth factor, vascular endothelial growth factor, insulin-like growth factor, epidermal growth factor, gentamicin/amphotericin-1000, heparin; Clonetics, St. Katharinen, Germany). After reaching confluence, endothelial cells were detached from culture flasks using Trypsin–EDTA, washed and resuspended in complete medium. All experiments were done with cells kept in culture between three and six passages.

Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of healthy adult donors by Ficoll-Isopaque density gradient centrifugation. Isolated PBMCs were washed in PBS and cultured in RPMI 1640 containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin (Biochrom, Berlin, Germany).

2.4. Stimulation of cells with LPS

Endothelial cells were seeded in 500 μl complete medium with a density of 20 000 cells/well in 24-well plates or in 1000 μl complete medium with a density of 40 000 cells/well in 12-well plates. After growing to confluency, cells were washed once with complete medium. Different amounts of LPS were diluted in complete cell culture medium and added to the cells. Control cultures were incubated with complete medium only. After 24 h, cell culture supernatants were collected and frozen at −80 °C before analysis by ELISA.

All experiments were repeated at least three times with different preparations of endothelial cells. From each experiment, a representative result is shown. The values represent the mean of duplicate measurements of two distinct wells±S.D. Data were considered statistically
significant if P-values were less than 0.05 (Mann–Whitney t-test).

2.5. Inhibition of LPS by Compound 406 or Cerivastatin

Confluent monolayers of HCAEC cultured in 24-well plates with 500 μl complete medium were washed once and then pre-incubated for 30 min with complete medium containing Compound 406 or Cerivastatin. Control cultures were incubated with complete medium alone. After 30 min of pre-incubation, 10 or 100 ng/ml LPS was added and cells were cultivated for 24 h. Cytokine content in cell culture supernatants was measured by ELISA.

2.6. Cytokine ELISA

Cytokine concentrations in cell culture supernatants were quantified by sandwich-ELISA using specific pairs of mAb against IL-1α, IL-1β, IL-6, IL-8, IL-10 and TNF-α (Biosource, Nivelles, Belgium) and monocyte chemotactic protein-1 (MCP)-1 (R&D, Wiesbaden, Germany). The assays were carried out according to the protocols given by the manufacturers. Endothelin (ET)-1 was measured by an antisense: 5'-AGCGGAAAAATCGTGTGG-3' and TLR4 (sense: 5'-CAGGGTACATGGTGCCAGCG-3'). TNF-α (sense: 5'-GAGTGAACAGCTGAG-3' and antisense: 5'-CCCTTCTCAGCTGAAAGG-3'). IL-1β (sense: 5'-CAGGTCCATGAAAGTACCTCC-3' and antisense: 5'-GACATACCCAGCAGCTGG-3'), IL-10 (sense: 5'-CTGAGAATCAGACACATCAAGG-3' and antisense: 5'-GTCAACCTAGTCGCATGTCCAT-3'), CD14 (sense: 5'-GGTCCGCTGTTAGAAAGAG-3' and antisense: 5'-TTTCCATCACAAGGTTTTT-3'). As positive control, we used RNA generated from LPS-stimulated PBMCs or unstimulated PBMCs. PCR products were confirmed by ethidium bromide staining after agarose gel electrophoresis.

3. Results

3.1. LPS induces the release of IL-6, IL-8 and MCP-1

In all cell culture supernatants of unstimulated HCAEC, a basal secretion of IL-6, IL-8, MCP-1, and ET-1 could be detected. Stimulation of HCAEC with different amounts of LPS for 24 h induced a dose-dependent increase in IL-6, IL-8, and MCP-1 (Fig. 1A, B, C) but not of ET-1 (Fig. 1D). As little as 1 ng/ml LPS led to a significant rise in cytokine release. Additionally, earlier (3, 6, 9 h) and later (48 h) time points have been analyzed. A detectable increase in IL-6, IL-8, and MCP-1 concentrations was detected as early as 3 h after stimulation (data not shown). An induction of IL-1α, IL-1β, IL-10 and TNF-α (detection limit of all ELISA, 7.5 pg/ml) release after stimulation with LPS was not observed at all analyzed time points (data not given).

3.2. LPS induces mRNA expression of IL-1β and TNF-α but not of IL-10

Since the stimulation of HCAEC with LPS did not lead to a detectable release of IL-1β, IL-10, and TNF-α by ELISA analysis, their mRNA expression was analysed by RT-PCR. In contrast to IL-10 mRNA, IL-1β and TNF-α mRNA were time-dependently upregulated (Fig. 2). Com-
pared to TNF-α, mRNA expression of IL-1β was more pronounced but delayed until 4 hours after stimulation.

3.3. LPS induces expression of ICAM-1, VCAM and ELAM-1

We also investigated the response of HCAEC with respect to the expression of adhesion molecules. Cells were stimulated with increasing concentrations of LPS for 6 h and expression of ICAM-1, VCAM, ELAM-1 and LFA-1 were measured by flow cytometry. HCAEC showed a basal expression of ICAM-1 and VCAM which was increased following stimulation with LPS (Table 1). ELAM-1 was only detected in stimulated cells (Table 1).
Table 1  
Expression of adhesion molecules by LPS-stimulated HCAEC

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LPS 1 ng/ml</th>
<th>LPS 10 ng/ml</th>
<th>LPS 100 ng/ml</th>
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<tbody>
<tr>
<td>IgG₁</td>
<td>19.5</td>
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<tr>
<td>IgG₂</td>
<td>18.6</td>
<td></td>
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<tr>
<td>ICAM-1</td>
<td>238.8</td>
<td>285.7</td>
<td>330.5</td>
<td>358.8</td>
</tr>
<tr>
<td>VCAM</td>
<td>75.4</td>
<td>83.6</td>
<td>128.1</td>
<td>160.5</td>
</tr>
<tr>
<td>ELAM-1</td>
<td>26.7</td>
<td>28.1</td>
<td>35.9</td>
<td>48.0</td>
</tr>
<tr>
<td>LFA-1</td>
<td>23.1</td>
<td>24.5</td>
<td>24.5</td>
<td>22.2</td>
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</tbody>
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The table shows the geometric mean fluorescences (%) measured by flow cytometry; S.D. of all samples was less than 10%.

while LFA-1 was not expressed at a detectable level in stimulated or unstimulated cells (Table 1).

3.4. LPS-induced cytokine expression is mediated by TLR4

On monocytes, LPS interacts with a receptor complex consisting of CD14, CD11/CD18, TLR4, and CD55 [17,19,31,32]. However, on the cell surface of HCAEC, we could detect CD55 but neither CD14 nor TLR4 by flow cytometry (Fig. 3A). In contrast, these three receptors were identified on monocytes. Consequently, expression of CD14 and TLR4 was tested at the mRNA level. Using RT-PCR, TLR4 mRNA was detected in all HCAEC preparations while CD14 was not (Fig. 3B).

To test the hypothesis that LPS-induced activation in HCAEC is mediated by TLR4, we antagonized LPS-induced activation with Compound 406, the synthetic lipid A precursor lipid IVα. Recently, it has been demonstrated that LPS antagonists act at the level of TLR4 [33]. Preincubation of HCAEC with Compound 406 completely inhibited LPS-induced IL-6 release (Fig. 4), thus demonstrating on a functional level the involvement of TLR4 in the LPS-induced activation of HCAEC. Inhibition was also observed for IL-8 and MCP-1 secretion (data not shown).

3.5. Cerivastatin inhibits LPS-induced IL-6 release

HMG CoA reductase inhibitors modulate inflammatory processes [34,35]. We therefore tested whether the HMG CoA reductase inhibitor Cerivastatin affects LPS-induced cytokine expression in HCAEC. Pre-incubation of the cells with different concentrations of Cerivastatin reduced both basal and LPS-induced expression of IL-6 (Fig. 5).

4. Discussion

Despite the different causal mechanisms, CHF and CAD are both associated with clinical and biochemical indices of inflammation. This relates to elevated levels of acute phase proteins and various cytokines and chemokines [1–3]. The levels of these mediators change with severity of disease possibly indicating a causal hint for the degree of inflammatory activity and the progression of cardiovascular disease [6–9].

It is believed that most of the elevated blood levels of cytokines possibly result from activated monocytes in circulation and myocardial tissue [36,37] but significant contribution to blood levels may also originate from reduced tissue perfusion due to restricted blood flow [13]. There is also increasing evidence that the loss of endoluminal integrity of the intestinal system may lead to transmigration of Gram-negative bacteria into circulation when right heart failure contributes significantly to the disease progress. This has been proposed as the endotoxin hypothesis of CHF [24–26].

Several cytokines exert negative effects on cardiac contractile performance. Among the cytokines which deteriorate contractility are IL-6 and TNF-α [8,12]. These cytokines may result from activated monocytes in the myocardium and insufficiently perfused non-vital tissues. However, it is highly likely that cardiac cells may also become activated when potent stimulants circulate. In fact, these cells may no longer act as mere targets of the circulating cytokines but may become active players in the pro-inflammatory vicious circle.

In this investigation, we are able to confirm that indeed HCAEC become activated when exposed to LPS as was shown earlier by other groups [38–40]. By actively secreting multiple chemokines and cytokines, the activated HCAEC may contribute to the cardiovascular disease progression. The endothelial cells closely neighbor myocardial cells. Thus, it is reasonable to speculate that the local concentrations of cytokines secreted by HCAEC may contribute to the symptomatic response in addition to the circulating cytokines. Activation by LPS also results in a distinct expression of adhesion molecules, a function of the chemokine and cytokine elevations observed in CHF and CAD [41,42]. It may be that with different stimuli, different results may be found.

Although the activation of HCAEC by bacterial cell wall components of Gram-negative bacteria is well established, the signal transduction pathways involved remain unclear. Recently, a low CD14 expression on umbilical vein endothelial cells was reported [43] but our findings confirm earlier results that CD14 is not expressed on endothelial cells [18]. Furthermore, we could demonstrate the expression of TLR4 mRNA in HCAEC. However, we failed to detect TLR4 protein expression on the cell surface as determined by flow cytometry which might be due to a very low numbers of receptors expressed. Nevertheless, the contribution of TLR4 in the activation of HCAEC by LPS could be demonstrated by pretreatment of the cells with the synthetic lipid A antagonist Compound 406, which antagonizes LPS at the level of TLR4 [33].

In previous investigations, our group could demonstrate a profound anti-inflammatory effect of HMG CoA reductase inhibitors in HUVECs infected with Chlamydia.
Fig. 3. (A) Comparison of LPS-receptor expression on HCAEC and monocytes. HCAEC were tested for the expression of CD14, CD55, and TLR4 by flow cytometry. One representative result is shown. The closed line represents the isotype control. Results were confirmed in two subsequent experiments on different days (n=3, data not shown) with similar results. (B) Expression of TLR4 and CD14 mRNA in HCAEC. RT-PCR for TLR4 with mRNAs of five separate preparations of HCAEC (A–E) is shown. β-Actin mRNA generated from PBMCs served as a positive control (+), H2O as a negative control (−).

pneumonia [34]. These experimental results were confirmed and reported by many others and also correspond to clinical observations that the beneficial effects of statins may not be due only to the lipid lowering properties [44]. Albert et al. and Jialal et al. reported a decrease in CRP protein in patients treated with statins [45,46]. In patients with CAD, statins not only lower CRP levels but also levels of IL-6 and soluble P-selectin [47]. In murine [48] and rat [49] models of myocardial infarction, statins have been shown to improve left ventricular remodeling and heart failure. We therefore wanted to know if statins may also reduce the inflammatory effect induced by LPS. In our present investigation, we could demonstrate that also during pro-inflammatory activation of HCAEC by LPS, statins are highly effective in reducing the inflammatory activation. The levels of Cerivastatin plasma concentration reached during conventional therapy are around 3.3–3.8 μg/l [50], which equals a concentration of 6.9–7.9 nM Cerivastatin. Although this is just below the effective concentration range in our experiments, the effects of Cerivastatin in vivo compared to the effects obtained in cell culture may not necessarily be achieved within the
same concentration range, and might depend on a variety of factors, e.g. plasma proteins that influence the bioavailability of Cerivastatin. Thus, our observations support the idea that, although lipoproteins may have some protective effects in binding and neutralizing endotoxin released from the intestine, patients with CHF might also benefit from the treatment with statins [51].

However, the mechanisms how statins reduce IL-6 release from LPS-activated HCAEC are unclear. The pleiotropic effects of Cerivastatin include the inhibition of the small GTP-binding proteins Rho, Ras, and Rac [44]. However, the main intracellular TLR4 signaling cascade includes the binding of the adaptor protein MyD88, the activation of IRAK and TRAF 6 that finally leads to the translocation of the transcription factor NF-κB [51]. Signaling through TLR4 also initiates the activation of mitogen-activated protein kinases (MAPK), although the exact mechanism is not known [44]. Since MAPK can also be activated by small GTP-binding proteins, it may be hypothesized that the connection of both signal transduction pathways is possible at this point. This hypothesis is supported by the findings of Takata et al. who demonstrated the inhibition of the p42/p44 MAPK by pravastatin in human aortic endothelial cells stimulated with thrombin [52]. The question if the action of Cerivastatin is related to or independent from TLR4 signaling pathways has to be addressed in further experiments.

In summary, we have found evidence for a direct involvement of coronary endothelial cells in LPS-mediated inflammatory activation that could facilitate progression of CHF.

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