Basic Science

Carvedilol Modulates In Vitro Lipopolysacchalride-Induced Interleukin-10 Production in Monocytic Cells

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Background: Interleukin-10 (IL-10) is an important mediator regulating the inflammatory responses. We wanted to understand how carvedilol modulates in-vitro lipopolysaccharide (LPS)-induced IL-10 expression in monocytic cells.

Methods: Both human peripheral blood monocytic cells and purified $CD14^+$ monocytes belonging to primary cells were isolated from whole blood of healthy donors. U937 cells, one monocytic cell line, were cultured. Initially, these three kinds of monocytic cells were pre-treated with carvedilol (concentrations varied from 0.1-1.0 μM) for 2 hours, and then LPS was added to induce IL-10 production for 3-24 hours before collection of supernatant. On the other hand, some U937 cells were long-term pretreated with both carvedilol and labetalol of varied concentrations for 1 to 2 months before LPS stimulation.

Results: We found that one-time carvedilol pretreatment at concentrations between 0.1 and 1.0 μM could not upregulate LPS-induced IL-10 production in human peripheral blood monocytic cells, purified $CD14^+$ monocytes or U937 cells. Long-term labetalol pretreatment of U937 cells for 1-2 months at concentrations of 0.3-1.0 μM had no effect on LPS-stimulated IL-10. However, long-term carvedilol pretreatment for U937 cells up to 1 to 2 months at concentrations of 0.1-1.0μM could dose-dependently upregulate the IL-10 expression. Both carvedilol and labetalol pretreatment in concentrations of 0.1-1.0 μM showed no cytotoxic effects to U937 cells.

Conclusion: In conclusion, one-time carvedilol treatment, if acting as an immune modulatory role on U937 cells and human monocytic cells, produced neither inhibition nor upregulation on IL-10 expression. Our observations of carvedilol modulating LPS-induced IL-10 production, due to its special antioxidant effect, might have some implications in understanding the broad-spectrum effects of carvedilol in regulating inflammatory reactions on atherosclerosis.

Key Words: Carvedilol • U937 cells • Interleukin-10

INTRODUCTION

Carvedilol is a nonselective β-adrenergic receptor antagonist with both vasodilating properties that are mediated by α-1 receptor inhibition and antioxidant properities that have been demonstrated previously.[1,2] Carvedilol has recently been approved for the treatment of hypertension and heart failure; it has beneficial effects on the symptoms and prognosis of patients with coronary artery disease, including myocardial infarction and angina pectoris.[3] In patients with ischemic heart disease in status of chronic stable heart failure, the effects of carvedilol on left ventricular function were maintained for at least a year from the start of treatment, with no apparent loss of the initial short-term improvement.[4] Carvedilol also can

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improve the endothelial function.[5] Modulation of activities in human monocytes can be considered as a therapeutic tool in coronary restenosis.[6]

Inflammation has a major role at every stage of atherosclerosis, which has been known as an inflammatory disease in vascular pathology.[7,8] The inflammatory reaction is initiated by endothelial activation induced by cytokines, oxidized low-density lipoprotein and/or changes in endothelial shear stress. Interleukin-10 (IL-10) is considered to have potent anti-inflammatory properties, exerting many biological activities depending on the cellular target.[9] Monocytes, the precursors of macrophages in all tissues, are present in every phase of atherogenesis. Exposure of laboratory animals or cells in culture to lipopolysaccharide (LPS) triggers gene induction and the generation of reactive oxygen intermediates (ROIs) by monocytes and macrophages. The relationship between monocytes and immunity in atherosclerosis has been emphasized, and the expression of IL-10 can delay the reaction of lipopolysaccharide-stimulated monocytes.[10] Current theories about IL-10 expression in monocytes suggest a triggering effect from proinflammatory cytokines.[11] In our study, we tried to understand whether carvedilol was able to achieve clinically beneficial effects through the modulation of activated monocytic cells by LPS-induced IL-10 expression.

MATERIALS AND METHODS

U937 cell culture

U937 cells (from ATCC CRL-1593.2), human monocyte-like cell line, were maintained in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum, 1000 U/mL penicillin-streptomycin and 2mM glutamine (Gibco) at 37 °C, 5% CO_2. The U937 cells were passed every 3 days with fresh culture medium.

Human monocytic cell isolation

Human peripheral blood monocytic cells and purified $CD14^+$ monocytes (CD: cluster of differentiation) were isolated from whole blood as described previously.[12] In brief, whole blood from healthy donors was mixed with Ficoll-Hypaque, and after centrifugation, the layer of mononuclear cells was collected. After lysis of red blood cells (RBC), purified human monocytes were positively selected by magnetic cells sorting (MACS) CD14+ microbeads following the manufacturer's instructions (Miltenyi Biotech, Auburn, California, USA). The cell stimulant, LPS, was purchased from Sigma Chemical Co.

Preparation of carvedilol

The purified drug "carvedilol" was kindly provided by Roche Pharmaceuticals. Initially, the drug was dissolved in dimethyl sulfoxide (DMSO) to make a 100 mM stock concentration. For experiments, the required concentrations of carvedilol were made by further dilution of the concentrated stock solution with culture medium. The final working concentrations of DMSO were consistently less than 0.05% in culture medium and there was not any influence on the detection of cytokines or cell viability.

Preparation of labetalol

The effective concentration of labetalol, a selective α-1 and nonselective β antagonist, was 0.13 μg/mL (0.356 μM) in plasma. It was purchased from Sigma Chemical Co. It was dissolved in DMSO to make a stock concentration. For experiments, the required concentrations of labetalol in cell cultures were adjusted to 0.3-1.0 μM, respectively. The final working concentrations of DMSO were consistently less than 0.05% in culture medium and there was not any influence on the detection of cytokines or cell viability.

Cell treatment

Human peripheral blood monocytic cells, $CD14^+$ monocytes and U937 cells (1×10^6cells/mL) were pretreated with carvedilol of various concentrations for 2 hours. Another group of U937 cells were long-term pretreated with 0.1-1.0 μM carvedilol or 0.3-1.0 μM labetalol for up to 1 to 2 months. Then 1 μg/mL of LPS was added in culture medium with cells (1×10^6 cells/mL) to stimulate the expression of IL-10. Cells were then incubated at 37 °C (5% CO_2) for indicated time of 3-24 hours to prepare for ELISA.

Measurement of nonspecific cytotoxicity of carvedilol and labetalol

We used trypan blue exclusion methods to examine drug toxicity. A defined viable cell Figure (1×10^6 cells/mL) was treated with carvedilol and labetalol of various concentrations for 24 hours and the cell viability was determined by the trypan blue exclusion method.

Determination of IL-10

The determination of IL-10 cytokine concentrations was performed according to the manufacturer's instructions (R&D), with some modifications. Briefly, a 96-well flat-bottom plate was coated with anti-IL-10 mAb (100 μl at 4 μg/mL) in phosphate-buffered saline (PBS; pH 7.3) at room temperature overnight. Then the plate was washed with PBS containing 0.05% Tween 20 (PBS-T) three times. After this, the plate was incubated with a blocking solution containing 1% bovine serum albumin, 5% sucrose, and 0.05% NaN_3 in PBS for more than one hour. After a wash with PBS-T, the collected 100-μl supernatant was then added into each well for 2 hours. After that, the plates were washed with PBS-T three times and then incubated with biotylated anti-IL-10 detection antibodies (100 μl at 500 ng/mL) for 2 hours at room temperature. Following the wash, 100 μl of streptavidin horseradish peroxidase (1/2000 dilution of a 1.25 mg/mL solution) was added and incubated for 20 minutes at room temperature. After a triple wash, 100 μl of substrate solution containing 1/1 mixture of H_2O_2 and tetramethylbenzidine was added and incubated for another 20 minutes at room temperature. The reactions were ceased by adding stop solution (0.5N H_2SO_4), and the cytokine concentrations were measured with a microplate reader (Dynatech).

Statistics

The concentrations of IL-10 cytokine under various experimental conditions were compared with the LPS-treated alone condition. Data were expressed as means ± SD and subjected to ANOVA. $p < 0.05$ denoted statistical significance between the different conditions.

RESULTS

Short-term effects of carvedilol on LPS-induced IL-10 production

Human peripheral blood monocytic cells, $CD14^+$ monocytes and U937 cells (1×10^6 cells/mL) were cultured with various concentrations of carvedilol for 2 hours. Then LPS (1 μg/mL) was added to stimulate the production of IL-10 for 3-24 hours. Finally, the supernatant were collected for detection of IL-10 level by ELISA as described above. As shown in Figure 1, 2 and 3, un-stimulated cells secreted very low levels of IL-10. In the presence of LPS, the production of IL-10 was induced. We found that short-term pretreatment of cells with carvedilol of various concentrations could not up-regulate IL-10 production no matter what the time courses of stimulation were.

The cytotoxic effect of carvedilol and labetalol in U937 cells

We treated U937 cells with various doses of carvedilol

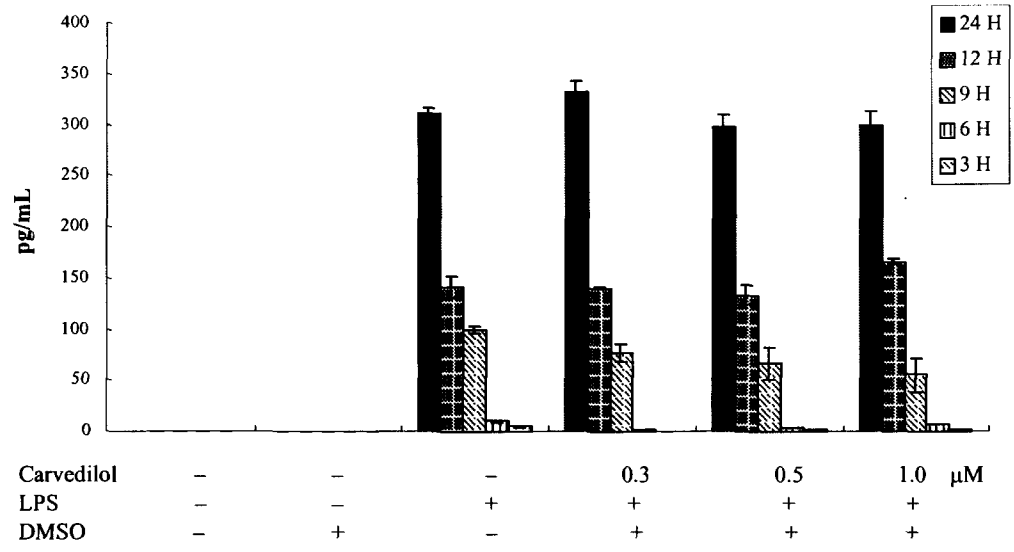

Figure 1. IL-10 production in PBMC after LPS stimulation. The PBMC (1×10^6 cells/mL) were pretreated with various concentrations of carvedilol for 2 hours, and then LPS 1μg/mL for PBMC (1×10^6 cells/mL) was added for incubation of 3-24 hours. The production of IL-10 was measured by ELISA. The data are representative of at least three consistent individual experiments and are shown as means ± SD.

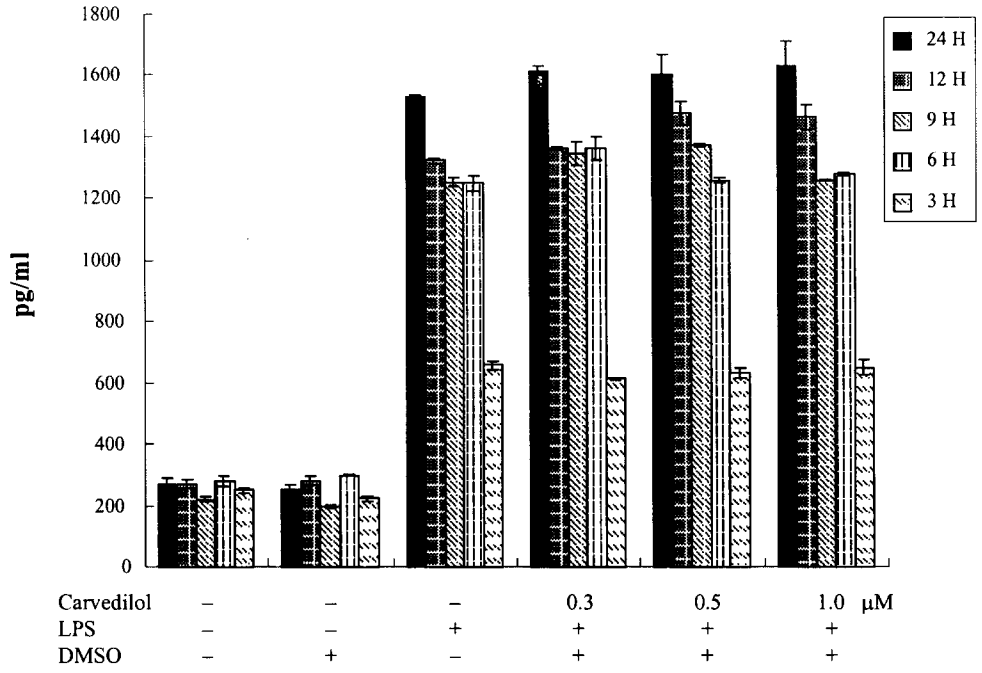

Figure 2. *IL-10 production in monocytes after LPS stimulation. The monocytes (1 × 10^6 cells/mL) were pretreated with various concentrations of carvedilol for 2 hours, and then LPS 1 μg/mL for monocytes (1 × 10^6 cells/mL) was added for incubation of 3-24 hours. The production of IL-10 was measured by ELISA. The data are representative of at least three consistent individual experiments and are shown as means ± SD.*

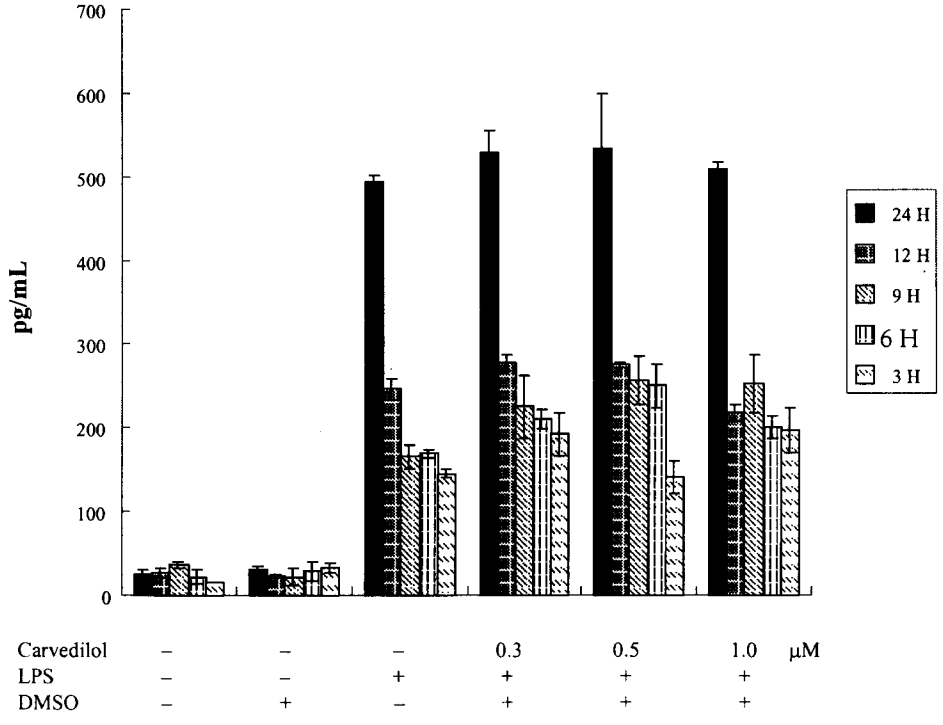

Figure 3. *IL-10 production in U937 cells after LPS stimulation. The U937 cells (1 × 10^6 cells/mL) were pretreated with various concentrations of carvedilol for 2 hours, and then LPS 1 μg/mL for U937 cells (1 × 10^6 cells/mL) was added for incubation of 3-24 hours. The production of IL-10 was measured by ELISA. The data are representative of at least three consistent individual experiments and are shown as means ± SD.*

and labetalol. In Figure 4, we found that carvedilol and labetalol at concentrations varying from 0.1 or 0.3 to 1.0 μM had no cytotoxicity to U937 cells no matter how long they had been treated with drugs.

The effects of long-term carvedilol and labetalol on LPS-induced IL-10 production in U937 cells

Initially, U937 cells (1×10^6 cells/mL) were cultured with

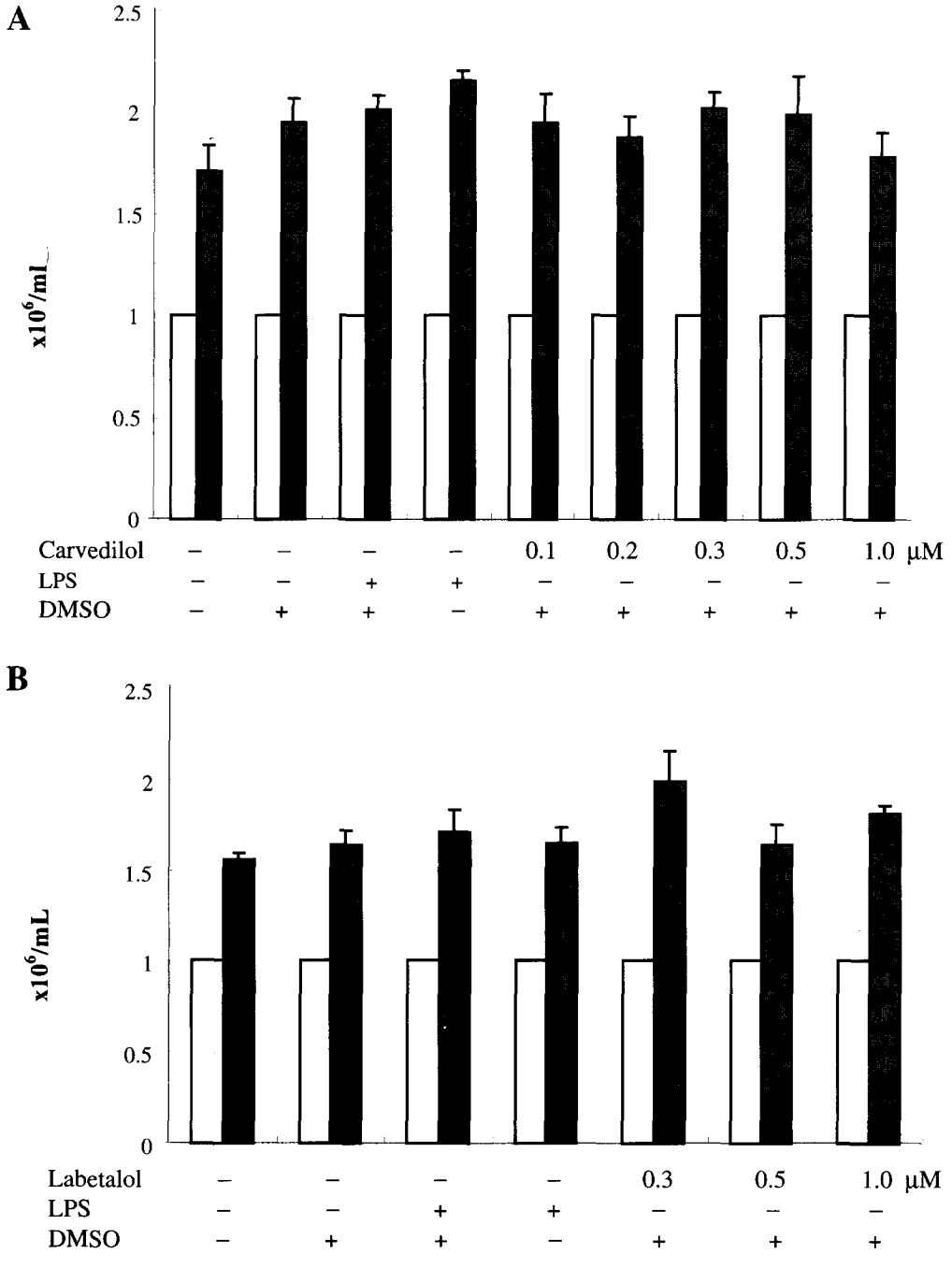

Figure 4. (A) The viable count of U937 cells after carvedilol treatment. U937 cells at 1 × 10^6 cells/mL (white bars) were treated with various concentrations of carvedilol for 24 hours. Using trypan blue exclusion method, the cell number of U937 cells was determined at 24th hour (dark bars). Data shown are the means ± SD from at least three consistent individual experiments. (B) The viable count of U937 cells after labetalol treatment. U937 cells at 1 × 10^6 cells/mL (white bars) were treated with various concentrations of labetalol for 24 hours. Using trypan blue exclusion method, the cell number of U937 cells was determined at 24th hour (dark bars). Data shown are the means±SD from at least three consistent individual experiments.

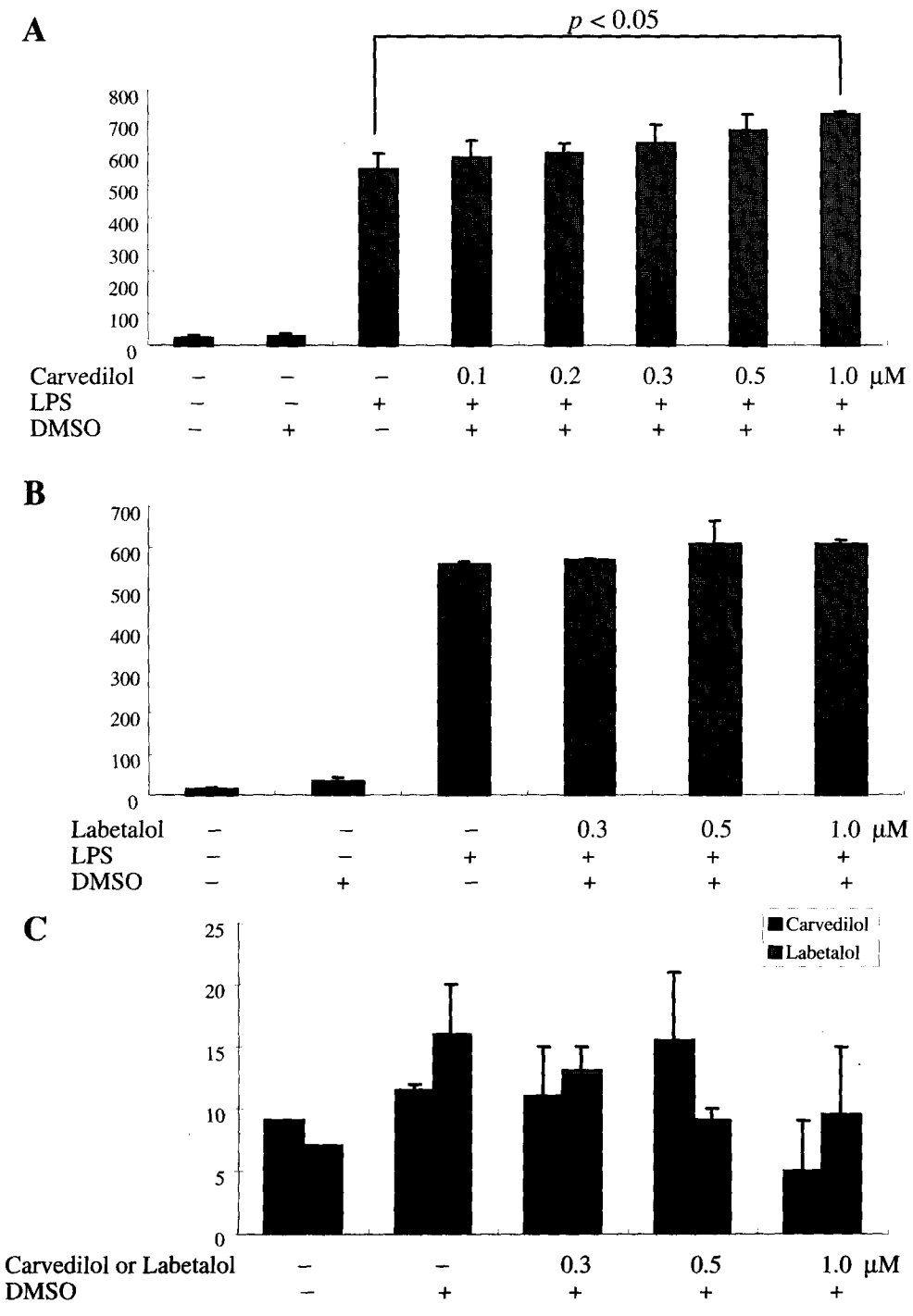

Figure 5. *(A) IL-10 production in long-term carvedilol-treated U937 cells after LPS stimulation. The U937 cells (1 × 10^6 cells/mL) were pretreated with various concentrations of carvedilol for 1-2 months, and then LPS 1 μg/mL for U937 cells (1 × 10^6 cells/mL) was added for incubation of 24 hours. The production of IL-10 was measured by ELISA. The data are representative of at least three consistent individual experiments and are shown as means ± SD. (B) IL-10 production in long-term labetalol-treated U937 cells after LPS stimulation. The U937 cells (1 × 10^6 cells/mL) were pretreated with various concentrations of labetalol for 1-2 months, and then LPS 1 μg/mL for U937 cells of 1 × 10^6 cells/mL was added for incubation of 24 hours. The production of IL-10 was measured by ELISA. The data are representative of at least three consistent individual experiments and are shown as means ± SD. (C) IL-10 production in long-term carvedilol- or labetalol-treated U937 cells without LPS stimulation. The U937 cells (1 × 10^6 cells/mL) were treated with various concentrations of carvedilol or labetalol for 1-2 months without LPS stimulation and regularly passed with new medium every 3 days. The production of IL-10 was measured by ELISA. The data are representative of at least three consistent individual experiments and are shown as means ± SD.*

various concentrations of carvedilol or labetalol for 1 to 2 months. Then, LPS (1 μg/mL) was added to stimulate the production of IL-10 for 24 hours. Finally, the supernatant were collected for detection of IL-10 level by ELISA. We found that pre-treatment of cells with carvedilol could up-regulate IL-10 production in a dose-dependent fashion, but this phenomenon was not found in the labetalol group (Figure 5). Figure 5c discloses that there was no increase of IL-10 amount only in long-term drug-treated groups without LPS stimulation.

DISCUSSION

Nowadays, atherosclerosis is considered to be an inflammatory disease.[7,8,13,14] IL-10 has been reported with potent anti-inflammatory properties and ability to inhibit a broad array of immune parameters.[10,15] It has the ability to inhibit activation and effector function of T cells, monocytes and macrophages.[16-19] IL-10 is expressed in both early and advanced human atherosclerotic plaques and limits the local inflammatory process.[20,21] In humans, systemic inflammation has been recognized as a major risk factor for the occurrence of acute coronary syndrome that is due to plaque rupture-induced thrombosis in the coronary arteries.[22] In light of the significance of IL-10 in inflammatory atherosclerosis, and modulatory activities of carvedilol on monocytic cells, we tested whether carvedilol could mediate any effect on those aspects.

In the present study, we first demonstrated that carvedilol, as one-time treatment in U937 cells and human monocytic cells, seemed to have no inhibitory or up-regulatory effects on LPS-induced IL-10 production, no matter what the time courses were. In these test concentrations of carvedilol, there was no cytotoxicity to U937 cells and human monocytic cells. These results let us recognize that one-time carvedilol effect in patients with coronary artery disease, hypertension or heart failure might not go through the up-regulation of IL-10 on monocytic cells. As studied, the peak plasma concentration of carvedilol in human volunteers has been reported to be about 0.3 μM after a single oral dose of 50 mg.[23] At least, in our study, it was good news that one-time low-dose carvedilol treatment (0.1-1.0 μM) in U937 cells and human monocytic cells exhibited no inhibitory effect on LPS-induced IL-10 production. Because carvedilol has many clinically beneficial effects proved by several large-scale clinical trials, it is worthwhile to observe and study long-term carvedilol effects on IL-10 production in cells. Since primary cells such as human monocytes cannot be cultured for a long time, U937 cells were used as a tool to study long-term carvedilol effect on IL-10 expression. In the following, we proved long-term carvedilol pretreatment up to 1 to 2 months on U937 cells could upregulate IL-10 production. At the same time, we found that there was no upregulatory effect on IL-10 production if long-term labetalol-treated U937 cells were stimulated by LPS. These results mean that carvedilol may raise IL-10 production on LPS-activated U937 cells through its antioxidant properties. In another study not published, we found that the amount of IL-10 increased due to modulation of carvedilol on IL-10 mRNA level. At the same time, we also found that high-level carvedilol up to 5-10 μM might inhibit IL-10 expression in both U937 cells and monocytes and show some cytotoxicity to monocytes. From the above views, we should apply with care in patients to identify clinically whether regular and long-term treatment with carvedilol might alter the response of activated cells on IL-10 expression both in vivo and in vitro.

We are interested in IL-10 for its role in limiting the local inflammatory process as an antiatherogenic cytokine. In this study, we identified that carvedilol could modulate the IL-10 expression on LPS-stimulated U937 cells depending on the time and concentrations of carvedilol pretreatment. From another view, however, we also need to understand that IL-10 does have potential for double-edged regulatory influences on inflammation.[24] The article presented by Pinderski et al. is a large step beyond the earliest evidence for a role of IL-10 in modulating atherosclerosis.[25] IL-10 potentially possesses both immunosuppressive and anti-inflammatory roles. Increased blood concentrations of IL-10 may augment susceptibility to repeated or continuous microbial invasion and have been associated with an adverse clinical outcome in patients with infectious disease.[26-28] We should keep in mind these potential adverse properties of increased IL-10 level. Nevertheless, in regards to anti-atherosclerotic progression, IL-10 expression undoubtedly has its important role in limiting atherosclerotic change. Here, we emphasize that short-term carvedilol effect might bypass IL-10 expression on monocytic cells to modulate the immune response for achieving the clinically effective responses in treating coronary heart

disease, heart failure or hypertension. Upregulation of IL-10 expression might partially explain one of the causes of long-term beneficial effects of carvedilol on cardiovascular diseases.

The limitation of this study is that we only show in-vitro carvedilol effects on LPS-induced IL-10 production in U937 cells and human monocytic cells but do not further evaluate whether carvedilol could regulate other inflammatory and/or proinflammatory cytokines on monocytes or T cells. However, in another study of our laboratory, we have found that carvedilol could modulate the function of activated human T cells. Subsequently, the patients with carvedilol treatment can be enrolled to study the relationship between carvedilol effects and cytokines and how they could modulate atherosclerosis.

In conclusion, one-time carvedilol treatment, if acting as an immune modulatory role on U937 cells and human monocytic cells, produced neither inhibition nor upregulation on IL-10 expression. Contrarily, long-term carvedilol treatment could upregulate IL-10 expression on activated U937 cells. These suggest that carvedilol, with its special antioxidant activity, might modulate IL-10 expression on activated U937 cells, depending on the time course and its concentrations.

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Carvedilol 調控體外脂多醣體誘發單核球細胞產生第十介白質的作用

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背景 第十介白質是調節發炎反應很重要的媒介物。本篇文章想要了解 Carvedilol 是否可以調控脂多醣體誘發單核球細胞對於產生第十介白質的效應。

方法 正常健康人類單核球細胞及 U937 細胞株，先接受不同濃度的 Carvedilol 來培養兩個小時，然後給予脂多醣體刺激 3 到 24 小時後，收集上清液，來測試第十介白質的表現。同時，另一組 U937 細胞株接受不同濃度的 Carvedilol 或 Labetalol 來培養，約 1 到 2 個月後，再給予脂多醣體刺激 24 小時，然後測試第十介白質的表現。

結果 本實驗發現人類單核球細胞及 U937 細胞株，若僅接受一次 Carvedilol 的培養，並無法提高因脂多醣體誘發單核球細胞而產生的第十介白質的濃度。但是，利用 U937 細胞株接受不同濃度的 Carvedilol 來培養至 1 到 2 個月後，再給予脂多醣體刺激，則可以發現第十介白質的濃度與 Carvedilol 的濃度呈現正相關的現象；但是，這種現像在 Labetalol 的實驗中卻沒有發現。

結論 本實驗的觀察，或許可以解釋 Carvedilol 經由抗氧化的作用，在臨床上可以調節有關發炎反應導致動脈硬化之一部份原因。

關鍵詞：Carvedilol、U937 細胞株、第十介白質。

Carvedilol : An Anti-inflammatory and Anti-atherogenic β-blocker?

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Human IL-10 is a protein of 160 amino acids, with a molecular mass of 18.5 κda, which contains two intra-molecular disulphide bonds. The gene encoding IL-10 is located on chromosome 1 and contains several non-coding sequences which are involved in the regulation of its transcription and of the stability of the corresponding mRNA. IL-10 was characterized initially as a factor generated by mouse T-helper 2 (TH2) cells which prevent cytokine production by TH1 cells. However, several other cell types have been further identified as a source of this cytokine, including $CD4^+$ and $CD8^+$ T lymphocytes, monocytes/macrophages, mast cells, keratinocytes, eosinophils, epithelial cells and various tumor cells.[1] In the majority of inflammatory disorders, cells of the monocytic lineage represent the major source of IL-10. IL-10 exerts its biological effects on cells by interacting with a specific cell-surface receptor. Functionally, active IL-10 receptor is composed of two distinct subunits. Both subunits belong to the class cytokine receptor family. The IL-10 receptor α chain (or IL-10R1) plays the dominant role in mediating high- affinity ligand binding and signal transduction. The IL-10 receptor β subunit (IL-10R2, also known as the orphan receptor CRF2-4) serves as an accessory chain essential for the active IL-10 receptor complex and to initiate IL-10-induced signal transduction events.[2] IL-10 functions to block NF-κB activity through both the suppression of IκB kinase activity, preventing IκBα degradation, and the suppression of NF-κB DNA-binding activity.[3]

IL-10 is a pleiotropic cytokine that inhibits a broad array of immune parameters, including TH1 lymphocyte cytokine production, antigen presentation, and antigen specific T-cell proliferation. IL-10 also has potent anti-inflammatory properties on macrophages. The concept that IL-10 acts as an anti-inflammatory molecule was suggested primarily by studies showing inhibition of the synthesis of a large spectrum of pro-inflammatory cytokines by different cells, particularly of the monocytic lineage. Thus, IL-10 inhibits the production of IL-1α, IL-1β, IL-3, IL-6, IL-8, TNFα, G-CSF and GM-CSF and chemokines, including IL-8 and macrophage inflammatory protein (MIP)-α from lipopolysaccharide (LPS)-activated human monocytes. These cytokines and chemokines play a critical role in the activation of granulocytes, monocytes/macrophages, NK cells, T and B cells and in their recruitment to the sites of inflammation. In vivo, IL-10 most likely exerts its anti-inflammatory effects on the vascular system through inhibition of leukocyte-EC interaction[4-6] and inhibition of pro-inflammatory cytokine and chemokine production by macrophages or lymphocytes. The expression of IL-10 in LPS-stimulated monocytes is delayed relative to that of other proinflammatory cytokines (TNF-α and IL-1) and coincides with their downregulation. Moreover, in vivo studies showed that plasma TNF-α levels are higher and remained elevated for a much longer period of time in IL-10-deficient ($IL\text{-}10^{-/-}$) mice injected with LPS than in $IL\text{-}10^{+/+}$ mice.[7] It therefore appears that IL-10 acts by feedback to inhibit continued proinflammatory cytokine production.

Atherosclerosis is a vascular pathology in which inflammation plays a major role at every stage of the disease.[8] The inflammatory process develops in response to abnormal cholesterol deposits in the intima of large arteries. The inflammatory reaction is initiated by a phase of endothelial activation induced by cytokines, oxidized low-density lipoprotein and/or changes in endothelial shear stress. This leads to the primary expression of the endothelial adhesion molecules, intercelluar adhesion molecule-1 and vascular cell adhesion molecule-1, and the chemokine, monocyte chemotactic protein-1 (MCP-1), followed by the recruitment and activation of circulating monocytes and lymphocytes. The clinical manifestations of atherosclerosis, including acute coronary syndromes,

are the consequences of atherosclerotic plaque rupture/ erosion that triggers thrombus formation, leading to the occlusion of the vessel lumen. The local inflammatory process at the level of the atherosclerotic plaque might influence the stability of the plaque through its potential effects on the extracellular matrix, and on plaque thrombogenicity.[9] In humans, systemic inflammation has been recognized as a major risk factor for the occurrence of acute coronary syndromes.[10] IL-10 is capable of modulating numerous cellular pathways that may play an important role in the development, progression and stability of atherosclerotic plaque, including nuclear factor-κB activation, tissue factor and cyclo-oxygenase-2 expression, metalloproteinase production and cell death.[11] Interestingly, IL-10 is expressed in both early and advanced human atherosclerotic plaques and limits the local inflammatory process.[12] Moreover, IL-$10^{-/-}$ mice fed an atherogenic diet and raised under specific pathogen-free conditions exhibit increased atherosclerotic lesion formation in comparison with wild-type mice.[13,14]

Carvedilol is a nonselective β-adrenergic antagonist with vasodilating activity caused by a concomitant α_1-antagonist action. Although the benefits of carvedilol in heart failure may be due primarily to its β-adrenergic effects, it also has α_1-adrenergic blocking effects. The antioxidant properties of carvedilol may also contribute to its efficacy in heart failure. It has been suggested that heart failure progression is accompanied by activation of neurohumoral and cytokine systems. Recent studies have highlighted the importance of cytokines in the pathogenesis of heart failure. Cytokines exert similar toxic effects on the heart as do angiotensin II and norepinephrine. This forms the logical basis for antagonizing these molecules in patients with heart failure. In this issue of *ACTA Cardiologica Sinica*, Cheng et al.[15] report carvedilol increased LPS-induced IL-10 release in cultured monocytic cells. These results implicate that carvedilol may also have a protective role in atherosclerosis in addition to its cardiac protection. It would therefore be of great interest to determine in the future whether carvedilol is really an anti-inflammatory and anti-atherogenic β-blocker.

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