Increased peripheral vascular resistance is a hallmark of congestive heart failure (CHF) (1). The impaired functional capacity of peripheral blood vessels to dilate in response to increased flow is a major determinant of the degree of exercise intolerance, one of the most important clinical features of patients with heart failure (2,3). The deficit in peripheral vasodilator capacity results from attenuated vasodilator capacity of peripheral blood vessels to dilate in response to physiologic stimuli (5,6). Immunologic and inflammatory responses may play a pathogenetic role in the development of CHF (7). Congestive heart failure is associated with elevated circulatory levels of pro-inflammatory cytokines, such as interleukin-1, interleukin-6 and tumor necrosis factor-alpha (TNF-alpha) (8–10), as well as C-C chemokines (11). All these cytokines cause endothelial cell dysfunction either directly or through the generation of reactive oxygen species (12,13), providing a potential pathophysiologic link between the loss of normal endothelial function and CHF.

Apoptosis or programmed cell death is a characteristic feature of endothelial cell activation after stimulation of systemic inflammatory responses in vivo (14). In addition, incubation with pro-inflammatory cytokines and reactive oxygen species induces endothelial cell apoptosis in vitro (12,13,15). Apoptosis is morphologically characterized by cell shrinkage, membrane blebbing, chromatin condensation and deoxyribonucleic acid (DNA) fragmentation (16). These stereotypical changes are accomplished by complex biochemical events involving the activation of cysteine proteases called “caspases” (17). The activation of initiator caspases by various stimuli triggers the release of mitochondrial cytochrome c, which in turn activates the multiprotein “poptosome” ensemble, composed of Apaf-1, cytochrome c and pro-caspase-9 (18,19). Activation of pro-caspase-9 then processes and activates other caspases to orchestrate the biochemical execution of cell death (20).

A very recent study suggested that the serum of patients with CHF induces endothelial cell apoptosis in vitro (21). Therefore, we investigated the signaling cascade involved in heart failure serum-induced apoptosis of endothelial cells.

Congestive Heart Failure Induces Endothelial Cell Apoptosis: Protective Role of Carvedilol

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Objectives The purposes of this study were to determine whether the serum of patients with congestive heart failure (CHF) can induce apoptosis of endothelial cells and to elucidate the underlying mechanisms. Moreover, the effect of the beta-blocker carvedilol was investigated.

Background Congestive heart failure is associated with impaired endothelial function in the peripheral systemic vasculature and with systemic release of inflammatory cytokines. Pro-inflammatory cytokines have been shown to induce endothelial cell apoptosis in vitro. Therefore, we hypothesized that CHF is associated with enhanced apoptosis of endothelial cells.

Methods Human umbilical vein endothelial cells were exposed to the serum of patients with CHF (n = 15) or healthy volunteers (n = 11), and apoptosis was determined by fluorescence staining of the nuclei and demonstration of deoxyribonucleic acid laddering. Moreover, apoptotic membrane particles were detected in plasma samples of patients with CHF.

Results The serum of patients with CHF revealed a significantly enhanced pro-apoptotic activity as compared with age- and gender-matched healthy volunteers (p < 0.001). Furthermore, patients with CHF revealed significantly elevated plasma concentrations of apoptotic membrane particles. Apoptosis of endothelial cells correlated with elevated tumor necrosis factor-alpha (TNF-alpha) (r = 0.585, p = 0.002) and soluble TNF receptor serum levels (r = 0.517, p = 0.007). Carvedilol completely suppressed the increase in apoptosis induced by the serum of patients with CHF. Moreover, carvedilol dose-dependently inhibited TNF-alpha-induced apoptosis. The antipapoptotic activity of carvedilol was mediated by reduced activation of the caspase cascade through inhibition of mitochondrial cytochrome c release. The suppression of apoptosis by carvedilol was due to its antioxidative rather than beta-blocking effects, as the analogue BM91.0228, which has no beta-blocking activity, exerted similar effects.

Conclusions These findings indicate that endothelial cell apoptosis may play a role in the pathophysiology of heart failure. Inhibition of endothelial cell apoptosis by carvedilol may contribute to the beneficial effects of carvedilol in patients with heart failure. (J Am Coll Cardiol 2000;36:2081–9) © 2000 by the American College of Cardiology.
In addition, we examined whether apoptosis also occurs in vivo, thus providing a method to mechanistic link for the impaired endothelial function in patients with CHF. Moreover, we examined the effects of the neurohormonal antagonist carvedilol, which has been shown to exert beneficial effects in patients with CHF (22). The results of our studies demonstrate that the serum of patients with CHF induces apoptosis of endothelial cells through activation of the caspase cascade. Carvedilol suppresses activation of the caspase cascade by inhibiting mitochondrial cytochrome c release and significantly reduces endothelial cell apoptosis induced by the serum of patients with CHF.

**METHODS**

**Patients and control subjects.** Fifteen patients with CHF were studied (Table 1). Patients received standard treatment for heart failure, consisting of angiotensin-converting enzyme inhibitors, diuretics and digitalis. Patients with elevated serum creatinine levels (>1.4 mg/dl), infectious or pulmonary disease or myocardial infarction within the last three months were not included. In addition, six patients with valvular heart disease or myocardial infarction within the last three months were not included. In addition, six patients receiving carvedilol treatment (6.25 to 50 mg/day; plasma levels 14.99 ± 7.4 μg/liter) were studied. Eleven healthy age-matched volunteers (six men and five women; mean age 50 ± 11.6 years) with no history of cardiac disease served as the control group. Venous blood was drawn into 10-ml serum tubes without additives. After centrifugation, serum aliquots were frozen at −80°C. The Ethics Committee of the University of Frankfurt approved the study protocols, and written, informed consent was obtained from all study participants.

**Table 1. Clinical Characteristics of Patients With Congestive Heart Failure (n = 15)**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>56 ± 9</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>12/3</td>
</tr>
<tr>
<td>NYHA class (II/III/IV)</td>
<td>7/5/3</td>
</tr>
<tr>
<td>Cause of heart failure (NYHA class II/III/IV)</td>
<td></td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>2/0</td>
</tr>
<tr>
<td>Dilated cardiomyopathy</td>
<td>6/3</td>
</tr>
<tr>
<td>Valvular heart disease</td>
<td>0/0/1</td>
</tr>
<tr>
<td>Left ventricular ejection fraction (%)</td>
<td>23 ± 8</td>
</tr>
</tbody>
</table>

Data are given as the mean value ± SD or number of patients. NYHA = New York Heart Association.

**Reagents.** Carvedilol (1-[9H-carbazol-4-ylx]-3-[[2-methoxyphenoxo][ethyl] amino]-2-propanol) and its analog BM91.0228 (4[2-hydroxy-3-][2-(2-methoxyphenoxo)ethyl amino-propoxy]-9H-carbazol-3-ol) were kindly provided by Dr. Spener (Boehringer Mannheim, Germany). Propranolol hydrochloride was from Zeneca (Plankstadt, Germany); Z-Val-Ala-Asp-fluoromethylketone (ZVADA-fmk) was from Bachem (Heidelberg, Germany); and human recombinant TNF-alpha and anisomycin were from Sigma (Munich, Germany).

**Cell culture.** The human umbilical vein endothelial cells (HUVECs) (Cell Systems/Clonetics, Solingen, Germany) were cultured in endothelial basal medium supplemented with hydrocortisone, bovine brain extract, gentamicin, amphotericin B, epidermal growth factor and 10% fetal calf serum until the third passage before experiments were performed.

**Detection of apoptosis.** Assessment of apoptotic nuclei and internucleosomal DNA laddering were performed as described previously (12,23). The amount of apoptotic particles in human plasma samples was measured according to Aupeix et al. (24). Results were expressed as nanomolar phosphatidylyserine normalized by a standard curve using liposomes of a defined composition. In addition to apoptosis assessment, cell viability was determined with the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay, as previously published (12).

**Cytokine measurements.** Tumor necrosis factor-alpha and soluble TNF-receptor 1 (sTNF-R1) serum levels were measured by commercially available enzyme-linked immunosorbent assay kits (R & D Systems, Wiesbaden, Germany).

**Mitogen-activated protein kinase (MAPK) assays.** The activity of c-Jun NH2-terminal kinase (JNK) was detected by using a nonradioactive assay (New England BioLabs, Frankfurt am Main, Germany). Phosphorylation of p38 was assayed by Western blotting using a phosho-specific antibody against phosphorylated Thr-180 and Tyr-182 of p38 MAPK (New England Biolabs).

**Plasmids and transfection.** Caspase-8 was cloned into the pcDNA3.1 vector, as previously described (25). The HUVECs were transiently co-transfected with 3-μg plasmids using Superfect (Qiagen, Hilden, Germany) (23). Transfected cells were identified by beta-galactosidase staining. Stained cells were analyzed by two investigators in a blinded manner, and the results were expressed as dead/visible cells × 100.

**Caspase-3-like enzyme activity.** Caspase-3-like activity was detected by measuring cleavage of the fluorogenic substrate 7-amino-4-coumarin-Asp-Glu-Val-Asp, as previously published (12).

**Cytochrome c release.** The mitochondrial versus cytosolic fractions were separated, Western blotted and probed with anti-cytochrome c antibody (PharMingen, San Diego, California), as described (26).
Statistics. Data are expressed as the mean value ± SD. Continuous variables were tested for normal distribution with the Kolmogorov-Smirnov test. Differences between two treatment groups were analyzed by use of the independent samples *t* test. Three or more groups were compared by means of one-way analysis of variance followed by post-hoc analysis with the Bonferroni correction for multiple comparisons. Categorical variables were compared by means of the chi-square test and the Fisher exact test. Apoptosis rates of subgroups of patients with CHF, according to New York Heart Association (NYHA) functional classes, and apoptotic membrane particles are presented as the mean value ± SEM. Linear regression analysis and nonparametric bivariate correlation (Spearman rank correlation coefficient \( r_s \)) were used to compare apoptosis rates with TNF-alpha serum levels. Statistical significance was assumed if a null hypothesis could be rejected at *p* < 0.05.

RESULTS

Serum of patients with heart failure induces endothelial cell apoptosis. Exposure of HUVECs to the serum of patients with CHF led to a significant increase in apoptosis compared with the serum of healthy volunteers (4.3 ± 0.7% Figure 1. Induction of apoptosis in HUVECs by the serum of patients with CHF. A, Apoptosis rates of HUVECs after exposure to 10% serum of patients with CHF as compared with healthy volunteers (control group) for 18 h. Box plots show median, 75% quartile and total range of values (*p* < 0.001 vs. control group). B, Deoxyribonucleic acid fragmentation in HUVECs after 18-h incubation with the serum of a representative patient with CHF. Left lane, Serum of a healthy volunteer (control). Upper panel, Ethidium bromide staining of the gel to demonstrate equal loading of DNA. C, Cell viability after incubation with CHF or control serum for 24 h (*p* = 0.002 vs. control group). D, Correlation of apoptosis induction by the serum of patients with CHF symptoms according to the NYHA classification (*p* < 0.05 vs. NYHA class II; **p** < 0.001 vs. control group). Data are presented as the mean value ± SEM. E, Shed membrane particles detected in human plasma derived from control subjects as compared with patients with CHF (mean ± SEM) (*p* < 0.001).
as demonstrated by morphological analysis of cell nuclei (Fig. 1A), as well as by DNA laddering (Fig. 1B). Moreover, CHF serum significantly reduced cell viability (Fig. 1C). The extent of serum-induced endothelial cell apoptosis correlated with the functional status of the patients (Fig. 1D). The serum of patients with severe CHF (NYHA class III and IV) revealed a significantly enhanced pro-apoptotic activity compared with the serum of patients in class II (p < 0.05). To test the in vivo relevance of these in vitro findings, apoptotic particles were measured in human plasma. As shown in Figure 1E, plasma levels of apoptotic particles were significantly increased in patients with CHF compared with healthy control subjects.

The pro-apoptotic cytokine TNF-alpha has been shown to be elevated in CHF (10). Indeed, serum levels of TNF-alpha and sTNF-R1 were significantly increased in patients with CHF compared with healthy volunteers (Fig. 2A). Apoptosis induction in vitro correlated with TNF-alpha serum levels (Fig. 2B). A similar correlation was observed between apoptosis rates and sTNF-R1 serum levels (r = 0.517, p = 0.007), suggesting a link between pro-inflammatory cytokine levels and endothelial cell apoptosis in CHF.

Because activation of the caspase cascade is central to apoptosis signaling, we investigated whether serum-induced apoptosis of endothelial cells involves caspase activation. Complete inhibition of caspases by ZVAD-fmk abolished serum-induced endothelial cell apoptosis (Fig. 3A). Thus, apoptosis of endothelial cells induced by the serum of patients with CHF depends on activation of the caspase cascade.

Carvedilol suppresses endothelial cell apoptosis induced by serum of patients with CHF. Carvedilol has been shown to be effective in the management of CHF (22). Therefore, we tested the effect of carvedilol on endothelial cell apoptosis. The addition of carvedilol suppressed the
induction of apoptosis by the serum of patients with CHF to control levels (Fig. 3, A and B). Moreover, carvedilol slightly but not significantly decreased apoptosis of HUVECs in the presence of control serum (20.6 ± 15.2% inhibition). Next, we tested the in vivo relevance of the apoptosis-suppressive effect of carvedilol. Apoptosis induction by the serum of patients with CHF receiving carvedilol treatment (n = 6) was significantly lower compared with the serum of patients without treatment (3.5 ± 0.8 vs. 4.5 ± 0.7%, p < 0.05).

**Carvedilol inhibits apoptosis by means of its antioxidative properties.** To characterize the mechanisms by which carvedilol prevents apoptosis, the effects of carvedilol on stimulus-induced apoptosis were determined in vitro. Coincubation with carvedilol dose-dependently reduced TNF-alpha–induced apoptosis in endothelial cells (Fig. 3, C and D). Therefore, we tested whether the beta-adrenoreceptor antagonistic or the antioxidative effect accounts for the antiapoptotic action of carvedilol. The hydroxylated analogue BM91.0228, which is a potent antioxidant but lacks beta-blocking activity, led to a similar suppression of apoptosis, whereas both propranolol, a beta-blocker without antioxidative properties, and the alpha-blocker prazosin had no significant effect on TNF-alpha–induced apoptosis (Fig. 3D; data not shown). Thus, inhibition of endothelial cell apoptosis by carvedilol appears to be due to its antioxidative properties.

**Carvedilol inhibits apoptosis signaling by suppressing cytochrome c release.** Previous studies using cardiac myocytes have suggested that the antiapoptotic effect of carve-

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**Figure 3.** Involvement of caspases in apoptosis induction in CHF and apoptosis suppression by carvedilol. **A,** Effect of carvedilol (5 μmol/liter) and the caspase inhibitor ZVAD-fmk (100 μmol/liter) on HUVEC apoptosis induced by the serum of control subjects (n = 7) or patients with CHF (n = 10). *p < 0.001 vs. CHF serum in the absence of the compounds; **p < 0.001 vs. control group. **B,** Suppression of CHF serum-induced DNA fragmentation by carvedilol (5 μmol/liter). **C,** Effect of carvedilol (5 μmol/liter) on DNA laddering induced by TNF-alpha (50 ng/ml; 18 h). **Upper panel,** amounts of DNA loaded. A representative autoradiographic study, out of three experiments, is shown. **D,** Dose-dependent inhibition of TNF-alpha–induced apoptosis by carvedilol, its analogue BM91.0228 and propranolol. 100% HUVEC apoptosis induced by TNF-alpha (50 ng/ml; 18 h). *p < 0.001 vs. carvedilol or BM91.0228 at the respective concentration (n = 4 to 6).
dilol is mediated through inhibition of the stress-activated JNK pathway (27). In endothelial cells, TNF-alpha–induced activation of JNK was not prevented by co-incubation with carvedilol (Fig. 4A). Carvedilol also had no effect on TNF-alpha–induced activation of p38 MAPK (Fig. 4B). Thus, the antiapoptotic effect of carvedilol on endothelial cells appears to be mediated by other mechanisms. Therefore, we determined the effect of carvedilol on the distal pro-apoptotic cysteine protease caspase-3 (28). Both carvedilol and its antioxidative analogue BM91.0228, but not propranolol, significantly inhibited TNF-alpha–induced caspase-3 activation (Fig. 5A). Moreover, carvedilol and BM91.0228 significantly suppressed apoptosis induction by overexpression of caspase-8, which links cytokine receptor stimulation to the caspase cascade. (Fig. 5B). Next, we tested whether interference with the mitochondrial cytochrome c release may account for the antiapoptotic effect of carvedilol. Co-incubation with carvedilol significantly reduced the TNF-alpha–triggered release of cytochrome c into the cytosol (Fig. 5C). These results suggest that carvedilol stabilizes the mitochondrial membrane in endothelial cells and thus prevents TNF-alpha–mediated caspase-3 activation.

**DISCUSSION**

The activation of endothelial cells, which results in impaired peripheral vasoactivity, may play an important role in the pathophysiology of CHF. The results of the present study confirm recently published data (21) showing that the serum of patients with CHF induces endothelial cell apoptosis in vitro. More importantly, the increase of plasma-shed membrane particles in patients with CHF indicates that apoptotic cell death also occurs in vivo. The pro-apoptotic serum effects correlate with the systemic inflammation indicated by increased levels of TNF-alpha and sTNF-R1 and suggest that circulating factors may directly damage endothelial cells in CHF. Mechanistically, our data demonstrate that the injury to endothelial cells is mediated by activation of the caspase cascade. Carvedilol, which has been shown to have...
beneficial effects in patients with CHF, inhibits activation of the caspase cascade by blocking mitochondrial cytochrome c release and completely suppresses apoptosis induction in endothelial cells by the serum of patients with CHF. These findings may provide novel and important insights into the potential mechanisms underlying the beneficial effects of carvedilol.

Involvement of apoptosis in cytokine-mediated endothelial injury in CHF. Congestive heart failure is associated with increased circulating levels of pro-inflammatory cytokines (7–9). The inflammatory response correlates with the severity of CHF symptoms (10,11) and has been suggested to play a pathogenetic role in the development of CHF (29). The results of the present study considerably extend these findings by demonstrating that increased circulating levels of TNF-alpha and sTNF-R1 in patients with CHF are associated with the induction of endothelial cell apoptosis in vitro. This could provide a mechanistic link between CHF and endothelial dysfunction.

Mechanisms of endothelial cell apoptosis induction in CHF. The precise serum factor(s) responsible for activation of the caspase cascade in endothelial cells remain to be determined. We have previously shown that TNF-alpha (12), reactive oxygen species (13,30) and angiotensin II (31) all represent potent activators of the caspase cascade, leading to endothelial cell apoptosis. All these factors are elevated in serum of patients with CHF (10,32,33). Serum activation of endothelial cells was followed by apoptosis induction, even in the presence of neutralizing antibodies against TNF-alpha (data not shown) (21). Thus, mediators other than TNF-alpha contribute to the activation of caspases in endothelial cells. However, regardless of the identity of the caspase activator(s), apoptosis induction was directly related to the severity of symptoms in patients with CHF, reflecting the relevance of endothelial cell apoptosis as a pathogenetic mechanism in this disease.

Effect of carvedilol on endothelial cell apoptosis in CHF. Carvedilol, which reduces mortality and worsening of symptoms in patients with CHF (22,34), leads to peripheral vasodilation through blockade of alpha1 receptors and acts as an antioxidant, in addition to its nonselective beta-blocking activity (35). Recently, some of the beneficial effects of carvedilol have been suggested to rely on the inhibition of cardiac myocyte apoptosis (27), which may contribute to the progression of heart failure (36). The present study now provides evidence for an additional vasculoprotective effect of carvedilol—namely, the suppression of increased endothelial cell apoptosis induced by the serum of patients with CHF. In contrast with cardiac myocytes, the antiapoptotic effect of carvedilol in endothelial cells was not related to JNK or p38 inhibition, but involved the reduction of mitochondrial cytochrome c release. The mitochondrial membrane “stabilizing” effect of carvedilol is mediated through its antioxidative action, because the analogue BM91.0228, which has no beta-blocking activity, was equipotent to carvedilol in apoptosis inhibition. The antioxidative capacity of carvedilol in vivo has recently been documented by a reduction in the oxidative modification of lipid products in volunteers during a seven-day treatment period with carvedilol (37). However, the results of the present study do not discard the possibility that beta-blockers, with no direct in vitro antioxidant properties, might likewise reduce the incidence of endothelial cell apoptosis in vivo in patients with CHF. Indeed, it has recently been demonstrated that carvedilol and metoprolol treatment is associated with equipotent reductions in systemic oxidative stress, as measured by thiobarbituric acid

Figure 5. Effect of carvedilol on caspase-mediated apoptosis signal transduction in HUVECs. A, Caspase-3–like activity after treatment with TNF-alpha (50 ng/ml, 18 h) alone and in combination with carvedilol (5 μmol/liter), its analogue BM91.0228 (5 μmol/liter) or propranolol (5 μmol/liter). *p < 0.001 vs. TNF-alpha group (n = 4). B, Apoptosis rates of HUVECs cotransfected with pcDNA3.1 caspase-8 or pcDNA3.1 without insert (mock) and pCDNA3.1 LacZ in the presence or absence of carvedilol (5 μmol/liter), BM91.0228 (5 μmol/liter) or propranolol (5 μmol/liter). *p < 0.001 vs. caspase-8. C, Cytosolic cytochrome c levels after incubation with TNF-alpha (50 ng/ml) and carvedilol (5 μmol/liter) for 18 h. Equal loading of the blot was confirmed by reprobing against actin. A representative blot, out of three independent experiments, is shown.
reactive substances (TBARS) in patients with CHF (38). Importantly, TBARS formation directly correlates with the functional severity of CHF as assessed by the NYHA classification (32). Therefore, the functional improvement of patients with CHF receiving beta-blocker therapy might be associated with reduced oxidative stress, which will translate into a beneficial effect upstream of caspase activation and cytochrome c release, irrespective of the direct antioxidant properties of the beta-blocking agent.

**Conclusions.** The results of the present study demonstrate that the serum of patients with CHF potently induces apoptosis of endothelial cells, providing another possible mechanistic clue in the pathogenesis of endothelial dysfunction in patients with CHF. The inhibition of endothelial cell apoptosis by carvedilol through prevention of mitochondrial cytochrome c release discloses a novel mechanism, which might contribute to its beneficial effects in the management of patients with CHF. In general, interventions that interfere with the induction of apoptosis of endothelial cells may provide a novel therapeutic target in patients with CHF.

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