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Cardiovascular Research 45 (2000) 788–794

Cardiovascular Research

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# Carvedilol prevents epinephrine-induced apoptosis in human coronary artery endothelial cells: modulation of Fas/Fas ligand and caspase-3 pathway☆

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Received 20 July 1999; accepted 22 September 1999

**Abstract**

**Background:** Several studies have shown that carvedilol, a multiple action neurohumoral antagonist, reduces mortality in patients with congestive heart failure (CHF). In addition to being a β-adrenoceptor antagonist, carvedilol is a potent antioxidant. Since there is evidence for elevation of catecholamine levels in plasma and coronary artery endothelial cell injury in CHF, the present study was designed to test the hypothesis that carvedilol inhibits epinephrine-induced apoptosis, and the inhibitory effect is mediated by modulation of Fas, Fas ligand (FasL) and caspase-3 pathway, in cultured human coronary artery endothelial cells (HCAECs). **Methods and results:** HCAECs were exposed to epinephrine alone, carvedilol+epinephrine, or atenolol+epinephrine for 24 h. Epinephrine increased the number of apoptotic cells, measured by in situ nick end-labeling staining (from 4.2±1.3% to 28.6±6.0%, $P<0.01$, $n=6$) and by DNA laddering on agarose gel electrophoresis. Epinephrine also increased Fas and FasL protein expression ($P<0.01$ vs. control, $n=6$), and activated intracellular protease caspase-3 ($P<0.01$ vs. control, $n=6$). These effects of epinephrine were completely inhibited by carvedilol. Atenolol in equimolar concentration also attenuated epinephrine-mediated effects, but the effects of atenolol were less marked than those of carvedilol ($P<0.01$). To explore the basis of differential effects of carvedilol and atenolol, effects of these agents on epinephrine-induced lipid peroxidation was measured. Lipid peroxidation was completely blocked by carvedilol, whereas equimolar concentration of atenolol had much less ($P<0.05$) effect. **Conclusion:** Epinephrine induces apoptosis in HCAECs, and this effect is associated with activation of Fas–FasL and caspase-3 signal transduction pathway. Carvedilol can, more effectively than atenolol, inhibit these effects of epinephrine. The potent antioxidant effect of carvedilol is probably responsible for the superior effect. 

*Keywords:* Adrenergic (ant)agonists; Apoptosis; Endothelial function; Free radicals

## 1. Introduction

Endothelial cells have an important role in the regulation of vascular tone. Endothelial dysfunction and injury have been shown to be associated with atherosclerosis [1], congestive heart failure (CHF) [2], and ischemia–reperfusion injury [3,4]. Therefore, strategies have been developed to protect endothelial function in these disease states. Endothelial cells are vulnerable to attack by a variety of factors, such as inflammatory cytokines [5], oxidized low-density-lipoprotein (ox-LDL) [6] and angiotensin II [7]. There is a large body of data on neurohumoral activation in CHF [8]. Elevated plasma levels of catecholamines may have adverse effect on endothelial function and integrity by inducing membrane lipid peroxidation [9].

Carvedilol is a novel β-adrenergic receptor blocker with antioxidant effect [10]. It has been evaluated in patients

☆Supported by a Merit Review Award from the VA Central Office and funds from Smith Kline Beecham, Philadelphia, PA, USA.

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**Time for primary review 20 days.**

0008-6363/00/$ – see front matter 
PII: S0008-6363(99)00369-7

with mild to moderate CHF [10]. Carvedilol has also been shown to protect cardiac tissues against oxygen free radical-mediated injury (apoptosis and necrosis) [11]. More recently, this agent has been shown to reduce infarct size in different models of acute myocardial infarction [12].

Fas (CD95/Apo-1) is a type I transmembrane protein belonging to the tumor necrosis factor receptor family [13]. Fas triggers an apoptosis-inducing signal when bound by its ligand, FasL [13]. FasL can induce apoptosis in Fas-bearing cells [14]. Many cells expressing both Fas and FasL do not undergo apoptosis under normal conditions [15], but can become significantly sensitized to the Fas-mediated apoptosis in response to specific stimuli [16]. Caspases are cysteine proteases that play a central role in Fas-mediated apoptosis signaling pathway [17]. Fas is oligomerized [18] and recruits Fas-associated death domain protein (FADD) and proFADD-like interleukin-1 beta converting enzyme, resulting in proteolytic activation of FADD-like interleukin-1 beta converting enzyme [19,20]. Active FADD-like interleukin-1 beta converting enzyme is released into the cytosol and triggers a cascade of caspases [17].

Recent studies show that neurotransmitters (norepinephrine and epinephrine) induce apoptosis in cardiomyocytes [21] and neurons [22]. However, it is not known if these neurotransmitters induce apoptosis in human coronary artery endothelial cells (HCAECs).

In the present study, we show that epinephrine induces apoptosis in HCAECs, which is associated with modulation of Fas–FasL and activation of caspase-3 pathway. We also show that the more potent effect of carvedilol than other $\beta_1$-adrenergic blocking agent may relate to its antioxidant effect.

## 2. Methods

### *2.1. Cell culture*

The methodology for culture of HCAECs has been described previously [5–7]. In brief, initial batch of HCAECs was purchased from Clonetics. HCAECs used in this study were passage from a 51-year-old female. The endothelial cells were pure based on morphology and staining for factor VIII-related antigen and acetylated LDL. These cells were 100% negative for alpha actin smooth muscle expression. These cells were sterile and negative for myoplasma and viral contamination. Microvascular endothelium growth medium consisted of 500 ml of endothelial cell basal medium, 5 ng of human recombinant epidermal growth factor, 5 mg of hydrocortisone, 25 mg of gentamycin and 25 μg of amphotericin B, 6 mg of bovine brain extract, and 25 ml of fetal bovine serum. Fifth generation HCAECs were used in this study.

HCAECs were incubated with epinephrine (10 μM) alone or with carvedilol (10 μM) or atenolol (10 μM) for 24 h to determine apoptosis and caspase-3 activity, Fas and FasL protein expression, and lipid peroxidation. The concentration of epinephrine, carvedilol and atenolol was chosen on the basis of previous studies [21].

### *2.2. Caspase-3 activity in HCAECs*

CPP32/caspase-3 activity was measured by a caspase-3 colorimetric protease assay kit (Chemicon), which provides a simple and convenient means for assaying the activity of caspase-3 that recognize the sequence Asp–Glu–Val–Asp (DEVD). HCAECs treated with epinephrine (±carvedilol or atenolol) were harvested and centrifuged and then washed twice. The cells were resuspended in 200 μl of chilled cell lysis buffer and incubated on ice for 10 min. The supernatant (cytosolic extract) was transferred to a fresh tube after centrifugation 10 000 *g* for 1 min and put on ice. Protein concentration was quantified for each sample. Protein concentration of the lysate was adjusted to 1–2 μg protein/μl. Reaction mixture consisted of 50 μl of cell lysate, 50 μl of 2×reaction buffer (containing 10 mM dithiothreitol (DTT)) and 5 μl of the 4 mM DEVD-*p*-nitroanilide (*p*NA) substrate (200 μM final concentration). Reaction mixture was incubated at 37°C for 2 h. Samples were read at 405 nm in a microtiter plate reader. Caspase-3 activity was determined by comparing the results of the epinephrine (±carvedilol or atenolol)-treated sample with the level of the control sample [23].

### *2.3. Western blot for Fas and FasL protein*

HCAEC lysates from each experiment (20 μg per lane) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to nitrocellulose membranes. After incubation in blocking solution (4% nonfat milk, Sigma), membranes were incubated with 1:1000 dilution of mouse monoclonal antibody to Fas or FasL (Santa Cruz Biotechnology) in Tris-buffered saline (TBS) (25 mM Tris, pH 7.4, 137 mM NaCl, 2.7 mM KCl) with 0.1% Tween 20. Excess primary antibody was removed by washing the membranes in TBS with 0.1% Tween 20. The membranes were incubated with 1:3000 dilution second antibody (Santa Cruz Biotechnology) for 1 h. The protein band of interest was detected by enhanced chemiluminescence (ECL) system, and relative intensities of protein bands were analyzed by MSF-300G scanner (Microtek) [6,7].

### *2.4. Determination of apoptosis*

Two different methods were utilized for assessment of apoptosis.

#### *2.4.1. In situ nick end-labeling (TUNEL) and propidium iodide (PI) staining*

To detect fragmented DNA, TUNEL was performed by

the method described by Gavrieli et al. [24] and by us [6,7]. Briefly, the cells plated on slides were fixed with 4% methanol-free formaldehyde, pH 7.4 for 25 min at 4°C and washed with phosphate-buffered saline (PBS). The slides were incubated with 3% $H_2O_2$ for 5 min for inactivation of endogenous peroxidase and equilibrated with terminal deoxynucleotidyl transferase (TDT) buffer (Promega) for 10 min at room temperature (RT). The slides were covered with 0.3 U/μl TDT and 0.04 nmol/μl fluorescein–12-dUTP (Promega) in TDT buffer for 60 min at 37°C. Unincorporated fluorescein–dUTP was removed, and the slides were immersed in 1 μg/ml of PI in PBS for 15 min at RT and washed, and analyzed under a fluorescence microscope (green fluorescence at 520 nm and red fluorescence of PI at >620 nm). The negative controls were performed without TDT enzyme. The positive controls were performed in samples pretreated with DNase I.

#### *2.4.2. DNA gel electrophoresis (DNA ladder)*

HCAECs were lysed with buffer (1% Nonidet-P40 (NP-40) in 20 mM EDTA, 50 mM Tris–HCl, pH 7.5), and the supernatants were treated for 2 h with RNase A (final concentration 5 μg/μl) at 56°C followed by digestion with proteinase K (final concentration 2.5 μg/μl) for 2 h at 37°C. After addition of 1/2 volume of 10 M ammonium acetate, DNA was precipitated with 2.5 volume of absolute ethanol. DNA was recovered by centrifugation at 12 000 *g* for 10 min and dissolved in gel loading buffer. DNA was separated by electrophoresis in 1.6% agarose gel with ethidium bromide [25].

### *2.5. Malondialdehyde (MDA) in cultured HCAECs*

MDA was measured in duplicate in cell lysates, as an index of lipid peroxidation, by a modification of the method of Ohkawa et al. [26]. The assay mixture consisted of 0.1 ml of the medium, 0.4 ml of 0.9% NaCl, 0.5 ml of 3% SDS, 3 ml of thiobarbituric acid reagent (containing equal parts of 0.8% aqueous thiobarbituric acid and acetic acid) and was heated for 75 min at 95°C. Thereafter, 1 ml cold 0.9% NaCl was added to the mixture, which was cooled and extracted with 5 ml *n*-butanol. After centrifugation at 3000 rpm for 15 min, the butanol phase was assayed spectrophotometrically at 532 nm. Tetramethoxypropane (in amounts of 0, 0.1, 0.2, 0.4, 0.8, 1.0 nmol) served as external standard. MDA content in the HCAEcs was expressed as nmol/mg protein.

### *2.6. Data analysis*

All data represent mean of duplicate samples from six independently performed experiments. Data are presented as mean±S.D.. Statistical significance was determined in multiple comparisons among independent groups of data in which analysis of variance and the *F*-test indicated the presence of significant differences. A *P*-value <0.05 was considered significant.

## 3. Results

### *3.1. Epinephrine-induced apoptosis of HCAECs and its inhibition by carvedilol and atenolol*

Since a small number of cells normally die during culture or are damaged during processing, 1 to 5% (3.8±1.9%) of control cells stained positive on TUNEL staining. Treatment of HCAECs with epinephrine (10 μM) for 24 h caused a marked increase in apoptosis compared with control ($P<0.01$). The presence of carvedilol (10 μM) or atenolol (10 μM) markedly reduced epinephrine-induced apoptosis ($P<0.01$). Carvedilol was more effective than atenolol ($P<0.05$) in this effect. Data from six experiments are summarized in Fig. 1.

Apoptosis, measured as DNA laddering on gel electrophoresis, was also induced by incubation of HCAECs with epinephrine. Again, carvedilol markedly reduced epinephrine-induced DNA laddering, and was more effective than atenolol. The pattern of DNA laddering in a representative experiment is shown in Fig. 2. These data complemented the TUNEL staining data.

### *3.2. Epinephrine-induced activation of caspase-3 and its inhibition by carvedilol and atenolol*

Incubation of HCAECs with epinephrine for 24 h activated caspase-3 ($P<0.05$ compared with control, $n=6$).

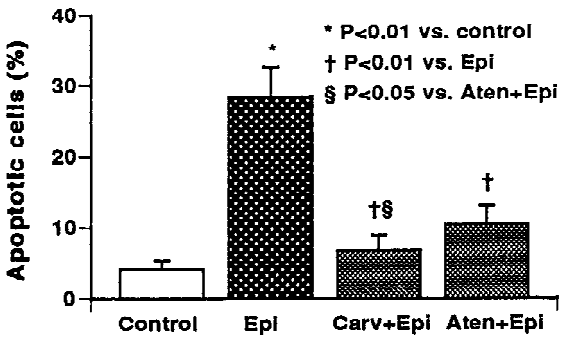


Fig. 1. Induction of apoptosis in cultured HCAECs in response to epinephrine (10 μM), as assessed by TUNEL under fluorescence microscopy. Since a small number of cells normally die during culture or are damaged during processing, 1 to 5% of control cells stained positive on TUNEL staining. The number of apoptotic cells was significantly increased in HCAECs treated with epinephrine. Carvedilol completely inhibited the proapoptotic effect of epinephrine. Atenolol also inhibited, but to a lesser degree than carvedilol, epinephrine-induced apoptosis. These data are a mean (±S.D.) of six independently performed experiments. Abbreviations: Epi=epinephrine, Carv=carvedilol, Aten=atenolol.

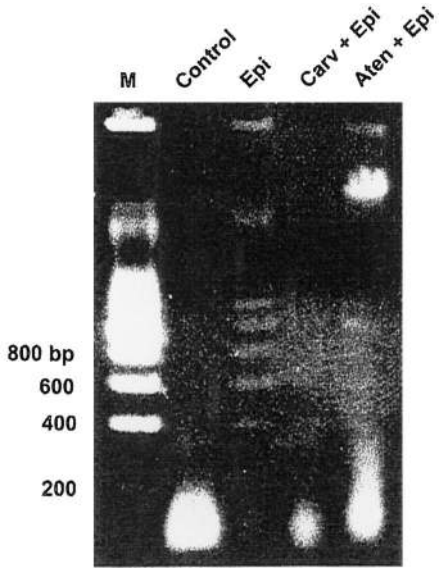


Fig. 2. DNA laddering on gel electrophoresis. The typical laddering pattern was observed in epinephrine-treated HCAECs, suggestive of apoptosis. Carvedilol completely inhibited appearance of DNA laddering in HCAECs treated with epinephrine. Under similar conditions, atenolol in equimolar concentration, reduced DNA laddering, but did not eliminate it. This result is representative of six separate experiments.

Carvedilol as well as atenolol significantly reduced epinephrine-induced activation of caspase-3 (Fig. 3).

### *3.3. Epinephrine-induced upregulation of Fas and FasL proteins and the inhibitory effect of carvedilol and atenolol*

Incubation of HCAECs with epinephrine for 24 h significantly increased expression of both Fas and FasL

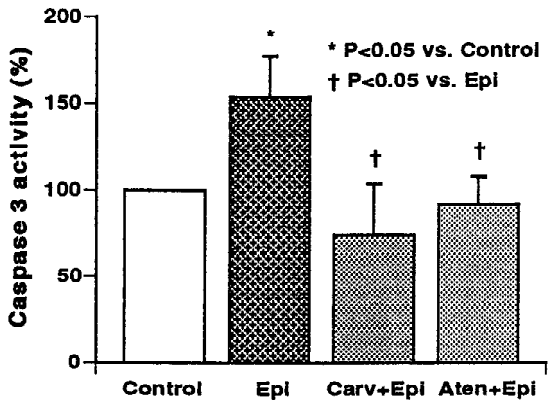


Fig. 3. Caspase-3 activity in HCAECs. Incubation of HCAECs with epinephrine (10 μM) for 24 h markedly activated caspase-3 compared with control ($P<0.05$). Carvedilol (10 μM) and atenolol (10 μM) completely inhibited epinephrine-induced activation of caspase-3. Data from six experiments in mean±S.D..

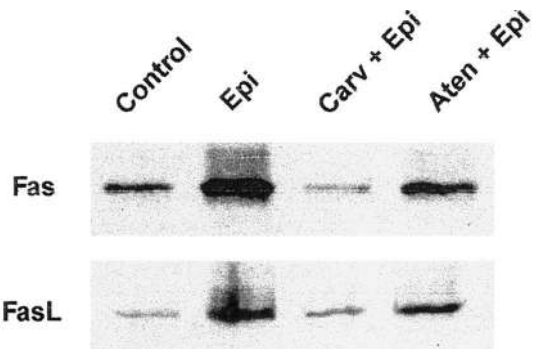


Fig. 4. Fas and FasL protein expression in HCAECs as determined by Western analysis. For immunoblotting, HCAEC lysates were subjected to SDS–PAGE, electroblotted onto nitrocellulose membranes and incubated with rabbit monoclonal Fas or FasL antibody followed by incubation with secondary antirabbit antibodies. Incubation of HCAECs with epinephrine significantly upregulated expression of Fas and FasL protein ($P<0.01$ vs. control). Carvedilol completely inhibited epinephrine-induced Fas and FasL protein expression. Atenolol also inhibited, but to a lesser degree than carvedilol, epinephrine-induced Fas and FasL protein expression. These Western blots are representative of six independently performed experiments.

($P<0.01$ vs. control, $n=6$). Carvedilol and atenolol blocked epinephrine-induced upregulation of Fas and FasL expression. Carvedilol was more effective than atenolol in this effect ($P<0.05$). Representative Western blots are shown in Fig. 4.

### *3.4. Epinephrine induced lipid peroxidation generation*

To explore if the more potent effect of carvedilol (vs. atenolol) on apoptosis of HCAECs and the underlying signal conduction pathway is related to its potent antioxidant activity, MDA was measured in HCAECs homogenate. Incubation of HCAECs with epinephrine for significantly increased lipid peroxidation product MDA ($P<0.05$ vs. control, $n=6$). Carvedilol and atenolol both markedly reduced epinephrine-induced lipid peroxidation ($P<0.05$); however, carvedilol had more potent inhibitory effect than atenolol in equimolar concentration ($P<0.05$) (Fig. 5).

## 4. Discussion

This study demonstrates that epinephrine induces apoptosis of HCAECs. This study also shows that the effect of epinephrine is associated with an increase in expression of both Fas and FasL proteins and activation of caspase-3. Lastly, this study shows that carvedilol can, more effectively than equimolar concentrations of atenolol, inhibit these effects of epinephrine. The more potent antioxidant

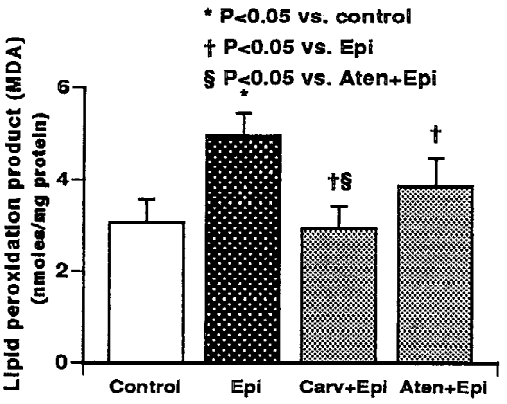


Fig. 5. Epinephrine-induced lipid peroxidation in HCAECs. Incubation of HCAECs with epinephrine markedly increased lipid peroxidation product MDA compared with control ($P<0.05$). Carvedilol completely inhibited epinephrine-induced lipid peroxidation. The effect of atenolol on epinephrine-induced lipid peroxidation was less than that of carvedilol ($P<0.05$). Data from six experiments in mean±S.D..

effect is perhaps one mechanism responsible for this superior effect of carvedilol.

### *4.1. Catecholamines and apoptosis*

It has long been postulated that exposure to high levels of catecholamines is toxic to cardiac myocytes and endothelial cells. Although in a previous study [21], proapoptotic effect of norepinephrine on cardiac myocytes was shown and attributed to its β-adrenergic activity, it is noteworthy that HCAECs possess abundant β-adrenoceptors [27]. Accordingly, the use of epinephrine, which has more potent β-adrenergic activity than norepinephrine, may be considered more physiologic than norepinephrine to study the phenomenon of injury to HCAECs.

Several mechanisms have been proposed for the toxic effects of catecholamines, and these include activation of calcium overload, release of free radicals, hypoxia, increased sarcolemmal permeability and elevation of cAMP [28–30]. Notably, plasma catecholamines are markedly elevated during myocardial ischemia and contribute to the propagation of myocardial injury. Hence, β-adrenergic blocking agents alleviate myocardial injury, and have become the cornerstone of therapy of myocardial ischemia. Yue et al. [11] showed that the β-blocker carvedilol reduces the number of apoptotic myocytes in rabbit ischemic/reperfused hearts in concert with a downregulation of stress-activated protein kinase signaling pathway and inhibition of Fas receptor expression. The authors of these studies [11] observed that carvedilol was more potent than an equimolar concentration of another β-blocker, propranolol. In the present study, we demonstrate for the first time that epinephrine induces apoptosis in HCAECs, and carvedilol completely inhibits apoptosis. Importantly, we observed that atenolol, at equimolar concentration, was less potent than carvedilol in inhibiting epinephrine-induced apoptosis. The process of apoptosis was measured by TUNEL staining and DNA laddering, and the results of the two different methods were complementary [5–7].

### *4.2. Signal conduction pathway of epinephrine-induced apoptosis*

Many injurious stimuli, such as cytokines [5], ox-LDL [6] and angiotensin II [7] induce apoptosis in HCAECs. Apoptosis is an active gene-directed process of cell suicide controlled by proapoptotic genes such as Fas [13] and antiapoptotic genes such as bcl-2 [31]. These injurious stimuli induce changes in apoptosis-related gene expression. A recent study from our laboratory [6] demonstrated that ox-LDL causes apoptosis in HCAECs in association with upregulation of Fas and downregulation of bcl-2 protein. Fas is a member of the tumor necrosis factor receptor family, and Fas-related apoptosis has been demonstrated in different cell types [13]. Vascular endothelial cells express functional FasL and detectable Fas on their cell surface, but these are resistant to Fas-mediated apoptosis under normal conditions [15,32]. Sata and Walsh [33] showed that injurious stimuli induce apoptosis through Fas–FasL interaction by sensitizing endothelial cells to Fas-mediated apoptosis. A recent study [11] suggests that carvedilol prevents myocardial ischemia/reperfusion-induced apoptosis in cardiomyocytes by inhibition of Fas receptor expression. We extend these observations and show that epinephrine increases Fas and FasL expression and probably subsequently induces apoptosis in HCAECs. These observations indicate that Fas–FasL interaction may be an important signal conduction pathway in epinephrine-induced apoptosis of HCAECs.

Another mechanism involved in apoptosis is the activation of caspase proteases [18,34]. Typically, proapoptotic stimuli activate caspase-1 and caspase-3 sequentially. However, there are many alternative caspases to cleave the death substrate [18]. Caspases play a central role in Fas-mediated apoptosis signaling pathway [17]. Upon ligand activation, Fas is oligomerized [18] and proFADD-interleukin-1 beta converting enzyme formed, resulting in proteolytic activation of a cascade of caspases. In the present study, we examined if epinephrine-induced apoptosis of HCAECs is associated with activation of caspase-3, and found that epinephrine indeed increases caspase-3 activity by about 1.5-fold. Carvedilol completely blocked epinephrine-induced activation of caspase-3 and simultaneously totally inhibited apoptosis. Together, these observations show that the interaction of Fas and FasL is associated with the activation of caspases. This process may be a critical signal transduction pathway in catecholamine-mediated apoptosis in HCAECs.

### *4.3. Carvedilol and atenolol and epinephrine-induced lipid peroxidation*

Our studies demonstrate that while both carvedilol and

atenolol significantly inhibited apoptosis and associated signal conduction pathways in HCAECs, carvedilol was more potent than atenolol in these effects. Several potential mechanisms may explain the more potent effect of carvedilol, and these include blockade of $\beta_1$- and $\beta_2$-adrenoceptors as well as a potent antioxidant effect. Notably, coronary endothelial cells possess mostly $\beta_1$-adrenoceptors [27], which would be blocked similarly by carvedilol and atenolol. The antioxidant effect may play an important role, since epinephrine induces generation of free radicals and membrane lipid peroxidation [9]. A recent study from our laboratory showed that a potent antioxidant γ-tocopherol attenuates apoptosis in HCAECs [35]. Carvedilol has been shown to inhibit oxygen radical-induced lipid peroxidation and glutathione depletion in endothelial cells [36]. This agent in a dose-dependent fashion inhibits xanthine–xanthine oxidase-induced LDH release from endothelial cells and significantly reduces cell death in response to free radicals [37]. Carvedilol also prevents LDL-enhanced monocyte adhesion to endothelial cells by inhibition of oxidation of LDL [38]. Under similar conditions, other β-blockers such as, propranolol, labetalol, pindolol, atenolol and celiprolol, have only mild or no effect. Free radicals-induced lipid peroxidation is a major player in apoptosis of endothelial cells [39,40]. In the present study, we found that carvedilol completely blocked epinephrine-induced lipid peroxidation in human coronary endothelial cells, whereas atenolol had only a modest inhibitory effect. These findings are consistent with the concept that carvedilol has a more potent antioxidant effect than atenolol. In addition, there may well be some non-specific effects of carvedilol to account for the different effects of the two β-blockers.

### *4.4. Clinical application of this study*

Endothelial dysfunction [1–4] is associated with atherosclerosis, myocardial ischemia and heart failure. Many studies [8,9] have shown that elevated plasma levels of catecholamines adversely affect endothelial function and cause cardiac dysfunction. Many clinical studies [41,42] have demonstrated that β-adrenergic receptor blocking agents, particularly carvedilol, significantly improve outcome of patients with heart failure. Experimental studies have shown apoptosis of cardiac myocytes in association with CHF. Carvedilol [11,21] significantly inhibits norepinephrine- and ischemia–reperfusion-induced apoptosis of cardiac myocytes. Other studies have shown potent dose-dependent antioxidant effects of carvedilol [36,43–46]. Apoptosis of HCAECs may induce coronary artery constriction and enhance myocardial ischemia and cardiac dysfunction. In this study, we demonstrate that carvedilol inhibits epinephrine-induced apoptosis of HCAECs. These observations compliment previous observations and provide a molecular basis for the efficacy of carvedilol in patients with CHF.

### *4.5. Limitations of this study*

One of the limitations of the study is use of a single concentration of epinephrine. In preliminary studies, we conducted a dose–response to epinephrine and found that epinephrine does indeed exert a dose-dependent effect on lipid peroxidation. Hence, only a single concentration 10 μM was used to study the effect of carvedilol and atenolol. Use of a single concentration of the two β-blockers may also be considered a limitation. The concentration of the β-blockers used in this study represents a therapeutically achieved concentration (therapeutic concentration 3.5–20 μM). Further, the purpose of the study was to compare the effects of the two β-blockers rather than study dose–response. Hence we elected to study in detail the effects of the two agents albeit in a single concentration.

### *4.6. Summary*

The present study demonstrates that epinephrine induces apoptosis of HCAECs. The effect of epinephrine on apoptosis of HCAECs is associated with an increase in Fas and FasL protein expression and activation of caspase-3. Carvedilol can, more effectively than atenolol, inhibit these effects of epinephrine. The potent antioxidant action of carvedilol is most likely responsible for the observed superior effect of this agent in inhibiting apoptosis and the underlying signal conduction pathway.

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