Carvedilol Protects Myocardial Cytoskeleton During Hypoxia in the Rat Heart

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ABSTRACT

Background: The cardiomyocyte cytoskeleton may be altered in chronic ischemia as well as in dilated cardiomyopathy. Carvedilol, a beta-blocking agent with alpha-blocking properties, is widely used for the treatment of heart failure. In addition to its beta-adrenergic and alpha-receptor blockade, additional cardioprotective, antioxidant and antiapoptotic effects have been demonstrated in experimental models. Whether carvedilol exhibits additional effects on the cytoskeleton in myocardial ischemia is unknown. We hypothesized that cytoskeleton stabilization is cardioprotective while cytoskeleton destabilization increases myocardial injury. Further, we hypothesized that carvedilol has a cytoskeleton stabilizing effect as one of its cardioprotective mechanisms.

Methods: The effects of carvedilol, propranolol, and BM-91.0228 (a vascularly inactive metabolite of carvedilol), with and without cytoskeleton-modulating agents taxol (cytoskeleton stabilizer) and vinblastin (cytoskeleton destabilizer) were evaluated with regard to myocardial enzyme release (creatine kinase, CK) and cellular apoptosis (TUNEL method) during hypoxia in isolated perfused rat hearts in the following groups (n = 10 per group): group 1, controls (normoxia followed by hypoxia); group 2, carvedilol; group 3, propranolol; group 4, BM-91.0228; group 5, taxol; group 6, vinblastin; group 7, taxol plus carvedilol; group 8, vinblastin plus carvedilol; group 9, taxol plus propranolol; group 10, vinblastin plus propranolol; group 11, taxol plus BM-91.0228; group 12, vinblastin plus BM-91.0228.

Results. Hypoxia-induced CK release was reduced by carvedilol (group 2, 4,817 ± 968 mU/g wwt), propranolol

(group 3, 4,513 ± 464 mU/g wwt) and taxol (group 5, 2,860 ± 1524 mU/g wwt; $P < 0.05$ versus controls), but not BM-91.0228, compared with controls (group 1, 16,747 ± 3026; $P < 0.05$). Vinblastin (group 6) increased CK release during hypoxia (28,626 ± 9700 mU/g wwt; $P < 0.05$ versus controls). Addition of carvedilol to vinblastin (group 8) ameliorated the increased CK release (8,353 ± 2230 mU/g wwt; $P < 0.05$), whereas propranolol (group 10) and BM-91.0228 (group 12) added to vinblastin had no effect during hypoxia. Treatment with carvedilol (group 2), BM-91.0228 (group 4) and taxol (group 5) resulted in reduced apoptosis (up to 50%), whereas vinblastin (group 6) or propranolol (group 3) showed no effect compared with controls. Carvedilol and taxol in combination (group 7) resulted in significant reduced CK release and reduced apoptosis compared with controls (9%±2% vs. 59%±12%, $P < 0.005$).

Conclusion: Modulating the stability of the cytoskeleton affects the degree of necrosis as defined by enzyme (CK) release. Carvedilol appears to exert a cytoskeleton stabilizing action, which may be involved in its cardioprotective effects. Both cytoskeleton stabilizing agents taxol and carvedilol also appear to demonstrate apoptosis sparing effects during hypoxia, which may be related to the beneficial effect on the cytoskeleton.

INTRODUCTION

Carvedilol is a non-selective □-adrenoceptor antagonist with vasodilating properties due to □-adrenoceptor antagonism.[1,2] It has become standard therapy in chronic heart failure patients.[3] Several experimental models of ischemia-reperfusion have demonstrated the potential cardioprotective effects of carvedilol.[4] Recent studies have shown that carvedilol also inhibits cardiomyocyte apoptosis.[5,6] Whether the antiapoptotic effects of carvedilol are caused by its beta-adrenoreceptor antagonism or other mechanisms remains a subject of controversy. BM-91.0228 is a metabolite of carvedilol with a hydroxyl group introduced at the third position of the carbazole moiety which has half-fold □-adrenoceptor antagonistic effects compared with carvedilol and lacks significant □-adrenoceptor antagonism.[4] Moreover, BM-91.0228 has a much stronger antioxidant activity than its parent compound.[5] Both compounds might exhibit potential cardioprotective effects due to different pharmacologic properties and pathways.[6]

Previous studies have demonstrated that changes of the cytoskeleton—ie, the three-dimensional intracellular structure consisting of polymers of subunits of microfilaments, microtubules, and intermediate filaments responsible for movement and stability—are involved in the progression of chronic heart failure, ie, increased density of the cytoskeletal microtubule network with augmented tubulin synthesis may be involved in the progression of contractile dysfunction.[7,8] In the present study the effects of carvedilol, BM-91.0228, and the beta blocker propranolol were tested in an experimental rat model of hypoxia, with and without combination with microtubuli stabilizing or destabilizing agents.

MATERIALS AND METHODS

The experiments were performed following the national and the institutional guidelines for experimental animal use. The studies were carried out in isolated perfused rat hearts (female Sprague Dawley rats, body weight 200-260 g; perfusate: Krebs-Ringer; Bicarbonate (KHB) 2 mmol Ca^{++}, supplemented with 5.6 mmol glucose and 1.2 mmol pyruvate). Animals were anaesthetized with 1 mL/kg ketamine and 10 mg/kg xylazine intraperitoneally and euthanized by abdominal aorta dissection.

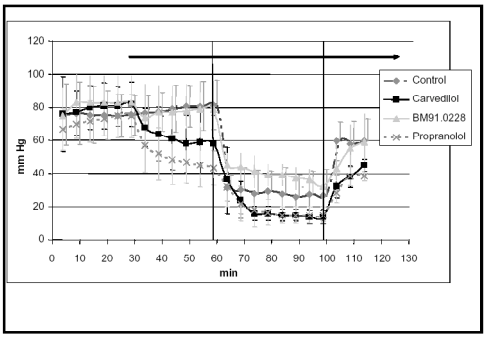

Figure 1. Peak systolic pressure during administration of propranolol, carvedilol, and BM-91.0228. Arrow shows the application of the drugs. After an equilibration phase of 30 minutes, the hearts were perfused for 30 minutes with a buffer containing the agents (arrow) to be tested and subsequently for another 40 minutes with the hypoxic medium equilibrated with 10% O_2. The hearts were then reoxygenated for 15 minutes with the original O_2 (5% CO_2) saturated medium (*X* ± SEM).

Hearts were excised as soon as possible after thoracotomy. Heart perfusion (38°C) was performed in a non-recirculating system according to the Langendorff technique (constant pressure, 65 mm Hg). A thin-walled latex balloon, formed according to an intraventricular cast of hearts of animals of the same size and mounted on a rigid catheter, was inserted into the left ventricular cavity via the mitral valve. Left ventricular pressure and heart rate were monitored with a chart recorder. Initial balloon volume was adjusted to an end-diastolic pressure of zero by using a microliter syringe. The hearts were placed with the apex uppermost in a water-jacketed, thermostated and moistened chamber and the ventricles were covered with a thin-walled floppy latex cap under which the interstitial transudate emerging on the surface of the heart was sampled by slight suction. The total amount of creatine kinase (CK) released was estimated from the entire perfusate passing the heart during the experimental period, ie, the collection period was 30 minutes for normoxic perfusion and 40 minutes for hypoxic perfusion. Perfusate collection was started immediately after completed preparation, ie, at 3 minutes following the beginning of preparation.

Experimental Protocol

After a 30-minute equilibration phase the hearts were perfused for 30 minutes under normoxic conditions with a buffer containing the agents to be tested and subsequently for another 40 minutes with the hypoxic medium equilibrated with 10% O_2 (Figure 1). Hearts were reoxygenated for 15 minutes with the original O_2 (5% CO_2) saturated medium and fixed and stained as described above. Twelve experimental groups were analyzed, each containing 10 rat hearts: group 1, normoxically perfused control hearts, followed by hypoxia, without drug addition; group 2, carvedilol (0.05 µmol, Boehringer Ingelheim, Germany); group 3, propranolol (0.05 µmol, Sigma, Deisenhofen, Germany); group 4, BM-91.0228 (0.05 µmol, Boehringer Ingelheim, Germany); group 5, taxol (1*10-6 mol) Sigma, Deisenhofen, Germany); group 6, vinblastin (1*10-6 mol, Sigma, Deisenhofen, Germany); group 7, taxol plus carvedilol; group 8, vinblastin plus carvedilol; group 9, taxol plus propranolol; group 10, vinblastin plus propranolol; group 11, taxol plus BM-91.0228; group 12, vinblastin plus BM-91.0228. Vinblastin was dissolved in Krebs-Henseleit Buffer (10^{-6} mol). Taxol was first dissolved in 17% propanediol/water. These stock solutions were kept in the dark and added to Krebs-Henseleit buffer before the experiments to a final concentration of 10^{-6} mol. As reported previously, propanediol (0.1 mol) alone had no detectable effect on any of the evaluated parameters when used in the corresponding dilution.[9]

TUNEL Method

Paraffin-embedded myocardial sections (4-5 µm) were mounted on silanized

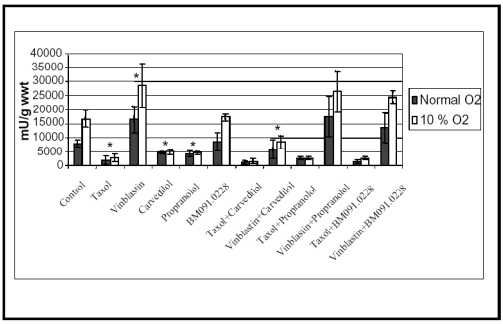

Figure 2. Total release of creatine kinase. In controls (group 1), CK release is shown after 30 minutes of normoxic perfusion (left bar) and after 40 minutes of hypoxia (right bar); CK release all other groups is shown after 40 minutes of hypoxic perfusion; $X \pm$ SEM.

slides and dried at 37˚C overnight. Immunohistochemical procedures for detecting apoptotic cardiomyocytes were performed by fluoresceinisothiocyanate (FITC) detection of digoxigenin-labeled genomic DNA. ApopTag (Oncor) was used according to the manufacturer's (Intergen Company, Oxford, UK) instructions. The percentage of positively stained immunolabeled nuclei of myocytes was determined by randomly counting six fields (320 x 320 µm) out of each ring. The apoptosis index (number of apoptotic myocytes/total number of myocytes counted x 100) was determined.[10]

Statistical Analysis

All values were expressed as means ± standard error of means (SEM). For comparisons between groups, all data were compared by analysis of variance (ANOVA). A P value < .05 was considered statistically significant.

RESULTS

Effect of Taxol and Vinblastin on CK-release

CK release in normal perfused isolate rat hearts was 7916 ± 1300 mU/g wwt (assessed after 30 minutes of normal perfusion). In contrast, hypoxic perfusion for 40 minutes resulted in a CK release of 16747 ± 3,026 mU/g wwt in controls (group 1, $P < 0.05$ versus normoxic conditions in the same hearts).

Taxol (group 5) reduced hypoxia-induced total CK release (2860 ± 1,524 mU/g wwt, $P < 0.05$ compared with both hypoxic and normal perfused controls). Addition of vinblastin (group 6), a microtubuli-destabilizing agent, resulted in increased CK release during hypoxia (28,626 ± 7,700 mU/g wwt, $P < 0.05$ compared with controls, Figure 2).

Effect of Carvedilol, BM-91.0228 and Propranolol on CK-release

After adding carvedilol (group 2) and propanolol (group 3) but not BM-91.0228 (group 4) the total CK release during hypoxia was reduced (carvedilol 4817 ± 968 mU/g wwt, propranolol 4513 ± 464 mU/g wwt, $P < 0.05$ compared with controls, 16747 ± 3,026 mU/g wwt, Figure 2).

Combination of Carvedilol, BM-91.0228 and Propranolol with Cytoskeleton Affecting Agents

Taxol decreased the hypoxia-induced and reoxygenation-induced interstitial CK release. Co-adminstration with carvedilol (group 8), but not with propranolol (group 10) or BM-91.0228 (group 12), resulted in further reduction of CK-release compared with controls. Co-administration of carvedilol to taxol resulted in CK of 1564 ± 880 mU/g wwt (group 7). Co-administration of propranolol to taxol resulted in CK of 2517 ± 464 (group 9) and co-administration of BM-91.0228 to taxol resulted in CK of 2630 ± 450 mU/g wwt (group 11) compared with taxol alone (2860 ± 1500 mU/g wwt, $P < 0.05$, Figure 2).

Effects on Global Hemodynamics

Peak isovolumic systolic pressure and

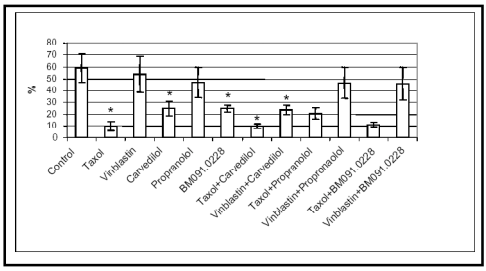

Figure 3. Index of Apoptosis (number of apoptotic myocytes/total number of myocytes x 100 in percent) ± SEM.

heart rate were not detectably altered by taxol or vinblastin in the normoxic state (peak systolic pressure: control [group 1] 75 ± 15 mm Hg, vinblastin [group 6] 68 ± 12 mm Hg, taxol [group 5] 70 ± 12 mm Hg). During hypoxia (60%–70% reduction of systolic pressure and heart rate with full recovery in reoxygenation), no effects of taxol or vinblastin on systolic pressure were detectable (Figure 1). Administration of carvedilol and propranolol, but not of BM-91.0228 (Figure 1) resulted in a comparable reduction of peak systolic pressure (Figure 1).

In Situ Determination of Apoptosis

At forty minutes of hypoxia (in non-treated hearts), 59% ± 12% of the cardiomyocytes stained positive for TUNEL (group 1 = controls). Carvedilol or BM-91.0228 pretreatment resulted in reduced apoptosis (carvedilol: 24.6% ± 6%, BM 91.0228: 24% ± 3%, $P < 0.05$), whereas propranolol administration did not change the percentage of apoptotic cells (propranolol: 47% ± 13%). Taxol decreased apoptotic cardiomyocytes (10% ± 4%), whereas vinblastin did not (53.6% ± 15%). Combination of vinblastin and carvedilol (group 8) decreased apoptotic cell percentage (23.6% ± 4%, $P < 0.05$ versus controls and vinblastin alone). Combination of vinblastin and propranolol did not change apoptosis percentage (46.5% ± 13%). Carvedilol with taxol did not show additive effects on apoptosis (9% ± 2%). Propranolol with taxol did not decrease apoptosis percentage (20.6% ± 5%) compared with taxol alone (Figures 3 and 4).

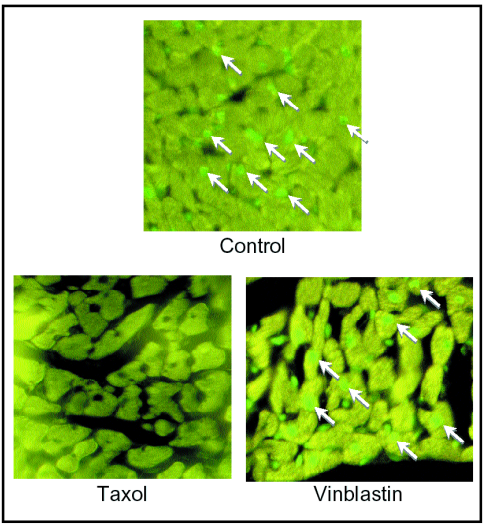

Figure 4. Histologic studies of paraffin-embedded myocardial sections (4-5 µm) representative for controls (group 1), after taxol treatment (group 5) and after vinblastin (group 6) treatment. Apoptotic cells (arrows) were detected by fluoresceinisothiocyanate (FITC) detection of digoxigenin-labeled genomic DNA (Magnification X 40).

DISCUSSION

The cytoskeleton, possibly from microtubule disassembly, may play a role in injury elicited by hypoxic perfusion. We have previously demonstrated that taxol abolished the hypoxia/reoxygenation induced interstitial CK release without altering contractile activity, indicating that cytoskeleton modifying agents may alter cell injury.[9]

The effects of taxol reflect an apparent protection of cardiomyocytes, showing the involvement of the cytoskeleton in both necrosis and apoptosis and enzyme release during hypoxia and also during the natural cell deterioration from the preparation itself (normoxic controls). Stabilizing the cytoskeleton exhibit cytoprotective effects, whereas

destabilization produces increase in necrosis as reflected in higher CK-release, which has been shown by our group before.[9]

In the present study the effects of carvedilol, its metabolite BM-91.0228 with much stronger antioxidant activity, and the non selective beta blocker propranolol were tested in an experimental rat model of hypoxia in combination with cytoskeleton stabilizing or destabilizing agents.

Effect of Cytoskeleton-modulating Agents and Combination Treatment on CK Release

The main findings of the study are as follows: 1) Taxol diminished hypoxia-induced interstitial CK release. 2) Vinblastin resulted in increased CK release. 3) These effects were ameliorated by addition of carvedilol, but not by either BM-91.0228 or propranolol. Thus, cytoskeletal elements apparently participate in the hypoxia-induced enzyme release and irreversible structural injury. Only carvedilol exhibited protective effects on presumed microtubuli disruption during hypoxic conditions in the rat heart.

Carvedilol's Effects Compared with Other Beta-Blockers

Carvedilol has been shown to be highly effective in improving left ventricular performance and ejection fraction in patients with heart failure from systolic dysfunction.[12,13] Beta-blockers are implemented in the treatment guidelines for the management of patients with heart failure.[14] However, there is still controversy whether the cardioprotective effects of carvedilol are due to its beta-blocking properties alone or are enhanced by its antioxidant or other unknown properties. By application of cytoskeleton-stabilizing agents, the possible effects of carvedilol as well as propranolol and BM-91.0228 were studied.

In the present study decreased CK-release during hypoxia with carvedilol and propranolol occurred. These data confirm previous data regarding the effect of carvedilol and propranolol on infarct size reduction[15-21] and delay of cell death (but not complete inhibition) after carvedilol administration.[22,23] Such decrease may be due to reduction of peak systolic pressure. This mechanism may explain why carvedilol's metabolite BM-91.0228 did not show any effect on CK-release as systolic pressure was not altered. It has previously been shown also that BM-91.0228 does not reduce infarct size in the rat heart if administered five minutes prior to 30 or 60 minutes of coronary occlusion.[10]

Classically, CK release is associated with the necrosis mode of cell death. However, it is possible in the current preparation part of the CK rise was related to apoptosis. In this regard it is interesting that the effects of the various drugs on CK release was not directly correlated with the degree of inhibition of apoptosis. Carvedilol, BM-91.0228 and taxol but not propranolol reduced significantly the percentage of apoptotic myocytes after 40 minutes of hypoxia, indicating that these effects are probably independent of the adrenoceptor blocking properties. Interestingly, these effects were not accompanied by any changes of global hemodynamics by taxol or BM-91.0228-application. A reduction of ischemia reperfusion-induced apoptosis was seen after administration of carvedilol directly (5 minutes) prior to reperfusion according to Yue et al.[23] The present data demonstrate that BM-91.0228 reduces the number of apoptotic cardiomyocytes in hypoxic rat heart similar to carvedilol, which may be due to its antioxidant property.

Anti-oxidant Effects of Carvedilol and BM-91.0228

Recently it has been shown that

carvedilol inhibits norepinephrine release during ischemia.[24] Alpha-adrenoceptor-mediated vasodilation does not explain the reduction of infarct size by carvedilol, which has been more pronounced compared with other β-blockers[25,26] and confirms our data since the alpha1-adrenoceptor antagonist BM-91.0228 did not reduce infarct size.[10]

Carvedilol posesses antioxidant and radical-scavenging properties and decreases neutrophil infiltration, which might play a role in ischemia/reperfusion-induced myocardial damage.[25] Although the mechanism is not yet entirely understood this phenomenon might be caused by the ability of carvedilol to suppress the expression of intercellular adhesion molecule-1 (ICAM-1). However, as BM-91.0228, an even stronger antioxidant, did not inhibit CK release, the antioxidant property cannot explain carvedilol's effect on CK release.

Carvedilol, but not its metabolite BM-91.0228, reduced CK release with vinblastin co-administration. Although BM-91.0228 has known antioxidant properties,[25] its administration has not resulted in infarct size reduction.[10] One explanation is that carvedilol, but not BM-91.0228, prevents cytoskeleton destabilization induced by ischemia, as shown in the present study.

Remarkably, both carvedilol and BM-91.0228 significantly reduced apoptosis after 40 minutes hypoxia compared with controls. Interestingly, BM-91.0228 inhibits apoptosis equipotentially to its parent compound. Since BM-91.0228 lacks beta-adrenoreceptor antagonism, properties other than beta-blockade appear to be responsible for the anti-apoptotic effects. Galang et al[11] showed an attenuation of apoptosis by antioxidant therapy. Antioxidant and radical scavenging action are shared by both compounds and may explain similar effects on anti-apoptosis. Moreover, carvedilol has been shown to modulate the signaling pathway of the active gene-directed process of cell suicide through suppression of the SAPK (stress-activated-protein-kinase—a pro-apoptotic kinase),[6] suppression of caspase 3 (pro-apoptotic protease),[27] as well as down-regulation of the Fas expression (proapoptotic gene).[11]

It remains unclear, however, whether BM-91.0228 has any influence on modulation of the signaling pathway, but since the SAPK is activated by free radicals and oxidative stress, BM-91.0228 may prevent this activation by its antioxidant and radical-scavenging properties. This effect might provide a future therapeutic option in acute and chronic myocardial ischemia including hibernating myocardium, congestive heart failure, and ventricular arrhythmias, in which ongoing apoptosis has been demonstrated.[28] Yaoita et al[29] showed that carvedilol but not propranolol, metoprolol or bunazosin decreased ascorbyl free radical in ischemic myocardium. In addition, carvedilol, but not bisoprolol, markedly decreased cardiac membrane lipid peroxidation measured by thiobarbituric acid formation. These data suggest that the cardioprotection of carvedilol (compared with bisoprolol) is possibly the result of its antioxidant and anti-neutrophil effects, but not to beta-blockade induced hemodynamic changes.[30]

While the present results suggest that β-blocking-effects play a major role in necrosis prevention, antioxidant and radical scavenging properties as well as modulation of the signaling pathway might strongly contribute to prevention of apoptosis. The effect of carvedilol on destabilizing the cytoskeleton might help understanding the pathophysiologic changes in ischemic or non-ischemic heart failure with possible therapeutic changes of the cytoskeleton.[31,32]

The exact role of the cytoskeleton

and its potential therapeutic alteration has not to date gained widespread attention. However, our previous data[9] as well as the present data indicate that the cytoskeleton-stabilizing effects of taxol potentially exert cardioprotection in the setting of myocardial ischemia.

CONCLUSION

In conclusion, cytoskeletal elements participate in hypoxia induced release of enzymes (CK) and irreversible injury in a different way and extent. Taxol and carvedilol exhibit protective effects during hypoxic conditions in the rat heart, which may be one mechanism of carvedilol's cardioprotective effect.

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