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Load-Induced Cardiomyocyte Apoptosis in Cultured Multicellular Myocardial Preparations Is Unaltered in Presence of the β-Adrenoceptor Antagonist Nebivolol

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Key Words
Preload · Afterload · Calcium · Trabeculae · Stretch · β-blocker · Myocyte

Abstract
Overload-induced heart failure is associated with enhanced apoptosis of cardiomyocytes, and increased mechanical load is an inductor of this apoptosis. It is unknown whether nebivolol, a third generation β_1-adrenoceptor antagonist, possesses properties that can attenuate this apoptosis. Multicellular preparations from rabbit hearts were mounted in a culture system that allows for measurement of contractile parameters over several days. Culturing these muscles on a constant high preload induces apoptosis of the cardiomyocytes. Of each heart, 1 preloaded muscle preparation was treated with nebivolol (10^{-6} mol/l), 1 preloaded without continuous exposure to nebivolol (positive control) and 1 unloaded (negative control). After 48 h of continuous loaded contractions, apoptosis was assessed by TUNEL-assay to confirm that nuclei of myocytes were affected, or by DNA-ladder intensity analysis for semiquantification. Maximal twitch force development was slightly, but significantly, lower in preparations contracting in presence of nebivolol (compared to solvent) while twitch-timing parameters were similar. After 48 h of continuous contractions, no additional differences were observed between the groups regarding contractile parameters. DNA-ladder analysis showed a similar rate of apoptosis in presence of nebivolol. Nebivolol does not increase, nor decrease, the rate of load-induced cardiomyocyte apoptosis.

Introduction

The rate of myocytes undergoing apoptosis is enhanced in terminal failing myocardium [1–4]. This load-induced apoptosis may contribute to a progressive decline in left ventricular function and heart failure [5, 6]. We observed that preload is a major determinant of cardiomyocyte apoptosis [7]. This observation is in line with the finding that application of a ventricular assist device [8, 9] can induce a reverse remodeling; the amount of apoptotic cells decrease and the phenotype returns towards a more antiapoptotic and healthy one [10].

It has been shown that the third generation β-adrenoceptor antagonist carvedilol can attenuate apoptosis considerably [11]. Nebivolol is another third generation β-adrenoceptor antagonist which, unlike carvedilol, is highly β_1-selective. Similar to carvedilol [12], nebivolol has also been shown to act protectively in hydroxyl-radical medi-

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ated injury [13] albeit possibly via a different mechanism [14]. However, the impact of nebivolol on the apoptotic process is largely unknown: only 1 recent study has investigated this, and then only in the context of myocardial infarction [15]. It is unknown if nebivolol can alter load-induced apoptotic properties, and given the success of β-blockade therapy in heart failure, it is important to understand the apoptotic implications of treatment drugs such as nebivolol.

Many studies in the field of cardiomyocyte apoptosis are performed on isolated myocytes in culture [16–18]; however, multicellular preparations are potentially better suited to study specific load-dependent induction of apoptosis [7] because these preparations can contract under defined physiological conditions [19–21], whereas isolated myocytes lack cell-to-cell connections and generally contract unloaded.

Accordingly, we have studied cardiomyocyte apoptosis in the presence and absence of nebivolol in multicellular myocardial preparations from the rabbit. The results indicate that increased mechanical stretch in a pathophysiological relevant manner can induce apoptosis of cardiomyocytes and that nebivolol does not promote nor attenuate this process.

Material and Methods

Muscle Preparation and Mechanical Measurements

Female chinchilla bastard rabbits (1.5–2.5 kg) were anesthetized with sodium thiopental (50 mg/kg i.v.) after heparinization (1,000 IU). Hearts were rapidly dissected and retrogradely perfused through the aorta with a Krebs-Henseleit solution containing (in mmol/l): 120 NaCl, 5.0 KCl, 2.0 $MgSO_4$, 1.2 NaH_2PO_4, 20 $NaHCO_3$, 10 glucose, 0.25 $CaCl_2$, 20 2,3-butanedione monoxime. During dissection and the experiments, the solutions were kept at equilibrium with 95% O_2/5% CO_2, pH 7.4, at 37°C. Under conditions as sterile as possible, right ventricular trabeculae were dissected under a stereo microscope and dimensions were measured at ×40 magnification [19]. Dimensions of the preparations were (average ± SEM): 469 ± 29 μm in width, 398 ± 27 μm in thickness and 2.87 ± 0.23 mm in length (n = 38). Outlines of the study were designed and carried out in accordance with institutional guidelines.

From each heart, 3 or 4 preparations were dissected and each individually mounted in experimental chambers (Scientific Instruments, Heidelberg, Germany) that allow for multiday cardiac trabecula culture [19–21]. At least 1 of the muscles was assigned to each of 3 groups. In the first group, muscles were mounted in absence of any load. Thus, the muscle was buckled between the basket-shaped extension of the force transducer and the length-altering device, and remained buckled during isotonic contractions at 0 load. This muscle served as negative control. The muscles in the other groups were mounted and stretched to a resting tension of about 4–5 mN/mm^2. This level of resting tension reflects a sarcomere length of around 2.2 μm [22, 23], reflecting the maximal length a sarcomere reaches in an in situ ejecting beat [24]. Of these muscles, 1 group was continuously exposed to 10^{-6} mol/l nebivolol, the other group was not exposed. All muscles were mounted using 2 blocks of tissue at the ends of the preparation in order to minimize damage and to facilitate mounting of the muscles in the chamber [19, 22, 23, 25]. The calcium concentration of the Krebs-Henseleit solution was raised stepwise to 1.0 mmol/l and the K-H solution was exchanged for modified M-199 culture media [19], containing 1.75 mmol/l calcium. At this calcium concentration, muscles were stimulated electrically at a frequency of 1 Hz. After 48 h, the muscles were taken out of the setup, and the center part of the muscle was immediately frozen in liquid nitrogen and stored at –80°C for DNA analysis. Alternatively, muscles were immediately fixed in 4% buffered formaldehyde and embedded in paraffin for histological analysis. At the time of dissection of the heart, additional preparations were taken and served as baseline values.

Detection of Apoptosis on Tissue Sections

Apoptotic nuclei were detected in situ using the TUNEL assay [26]. Tissue sections (5 μm) were deparaffinized, rehydrated in descending concentrations of alcohol and incubated with 20-μg/ml proteinase K (Roche Diagnostics, Mannheim, Germany) in 10 mmol/l Tris/HCl pH 8.0, 5 mmol/l Na-EDTA, 0.5% SDS for 15 min. Endogenous peroxidase was inactivated by 3% H_2O_2 in PBS. The tissue sections were stained with the peroxidase ApopTag® Plus in situ apoptosis detection kit (Oncor Appligene, Heidelberg, Germany) according to the standard protocol. For detection of the peroxidase, DAKO® Liquid DAB (DAKO Diagnostika GmbH, Hamburg, Germany) was used. The sections were stained with Meyer's Haemalaun solution for microscopical analysis. A negative control was performed without the enzyme TdT, and for a positive control the sections were incubated with DNase I (Roche Diagnostics). At high magnification, evidence of morphological changes and/or myocyte damage (other than apoptotic nuclei) due to the culture of these muscles was not detected when compared to freshly isolated specimens.

Detection and Quantification of Apoptotic DNA Fragmentation

DNA of apoptotic cells shows a typical internucleosomal fragmentation [27]. The genomic DNA of the trabeculae was prepared using the Puregene DNA isolation kit (Biozym, Hessisch Oldendorf, Germany). 400 ng of the DNA were separated by agarose gel electrophoresis using SYBR®Green (Biozym) for DNA detection. The characteristic apoptotic DNA fragments (of nucleosome size or multiples thereof) were determined by scanning Polaroid negatives using a laser densitometer with an evaluation system (Molecular Dynamics, Krefeld, Germany). Using NIH image software, the average pixel intensity of each individual lane was calculated after background (the control lane) was subtracted. DNA ladder intensity is expressed as the average value of darkness of pixels minus the average value for background of the control lane.

Data Analysis and Statistics

Muscle contraction data were collected with custom-designed data-acquisition programs and commercially available software.

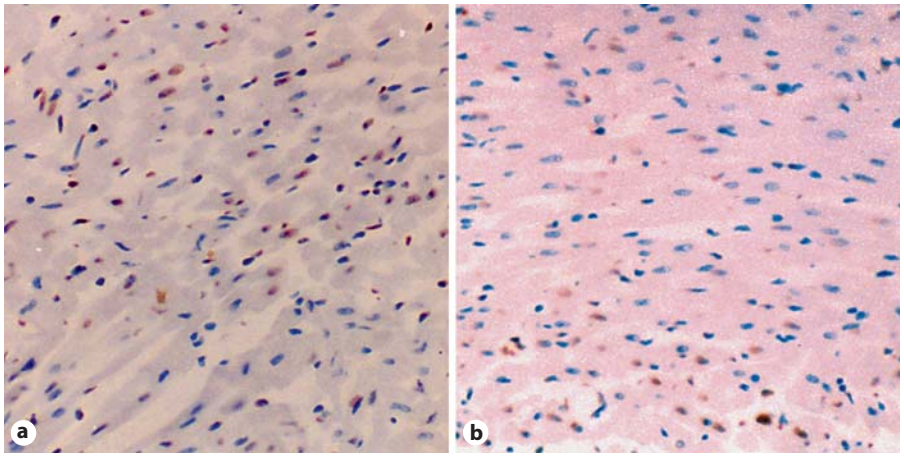

Fig. 1. TUNEL histology. **a** Detection of apoptosis in a muscle that had contracted for 48 h in presence of preload under isometric conditions. Nuclei of the cardiomyocytes clearly show TUNEL-positive staining. **b** In an unloaded control, fewer apoptotic nuclei are detected.

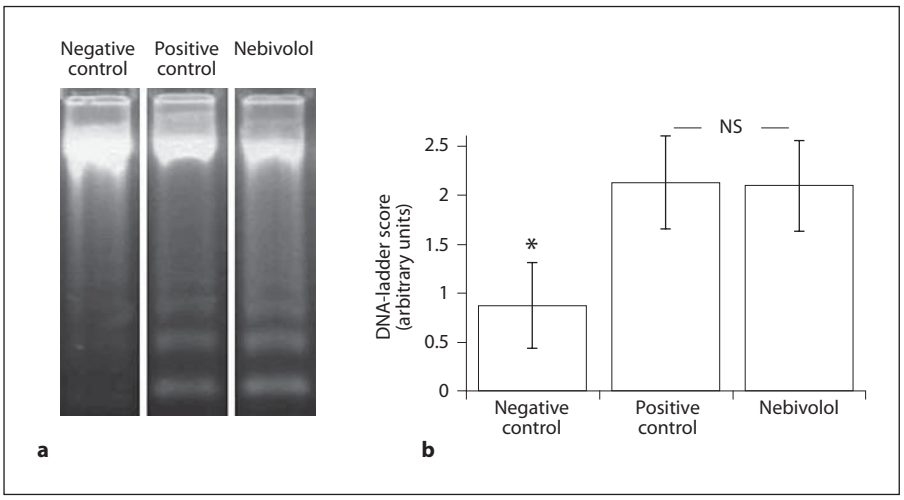

Fig. 2. DNA-ladder analysis. **a** Typical example of DNA-laddering in a muscle that contracted for 48 h in presence of preload under isometric conditions. This muscle showed significantly more apoptosis than preparations contracting in absence of load. However, no differences were observed between loaded muscle in the absence and in presence of 10^{-6} mol/l nebivolol. **b** Average values of DNA-ladder analysis. * $p < 0.05$ between 2 groups (unpaired t test); NS = not significant.

Data are expressed as means ± SEM, statistical significance was determined by paired or unpaired Student's t tests where applicable, and 2-tailed values of $p < 0.05$ were accepted as significant.

Results

Detection and Quantification of Apoptotic Myocytes

Using the TUNEL method, apoptotic nuclei of cardiomyocytes were detected in paraffin sections of muscles that contracted in the presence of pre- and afterload after 48 h. It can be seen in figure 1 that many nuclei of cardiomyocytes were positive for TUNEL staining. Similar results were obtained for muscles in the presence of nebivolol.

To quantify and compare the rate of apoptosis in these multicellular preparations, we performed an apoptotic DNA ladder score as an assessment of myocardial apoptosis. The intensity of the DNA ladder was assessed as described in the methods section. Typical examples are shown in figure 2a and average values in figure 2b. Control trabeculae (taken at time of dissection) had no signs of an apoptotic DNA ladder. Muscles that contracted in the absence of load (i.e. slack) showed a low-level apoptosis (5.8 ± 3.4 arbitrary units, AU). In sharp contrast, muscles that contracted in presence of both pre- and afterload showed an intense fragmentation of the genomic DNA (29.1 ± 8.5 AU, $p < 0.05$ vs. slack). When muscles were kept contracting under the same loading conditions but now in presence of 10^{-6} mol/l nebivolol, the intensity of DNA-laddering was similar (28.9 ± 7.6 AU, p = not significant vs. absence of nebivolol). The values for the positive and negative controls are nearly identical to the ones observed in a previous study under similar conditions [28].

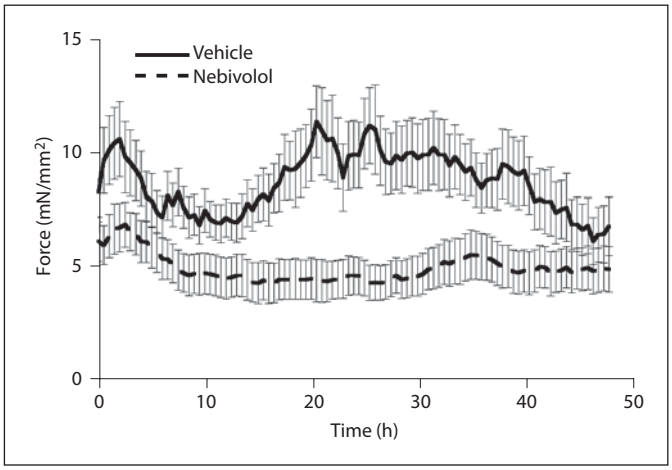

Fig. 3. Contractile developed force of preloaded isometrically contracting preparations. Maximal twitch force development (F_{dev}) displays a slight transient behavior over time, while diastolic tension remains unchanged over 48 h in both groups (not shown). No significant differences (unpaired t test) other than a lower initial F_{dev} were observed between preparations in the presence of 10^{-6} mol/l nebivolol (n = 11) and in the solvent group (n = 14).

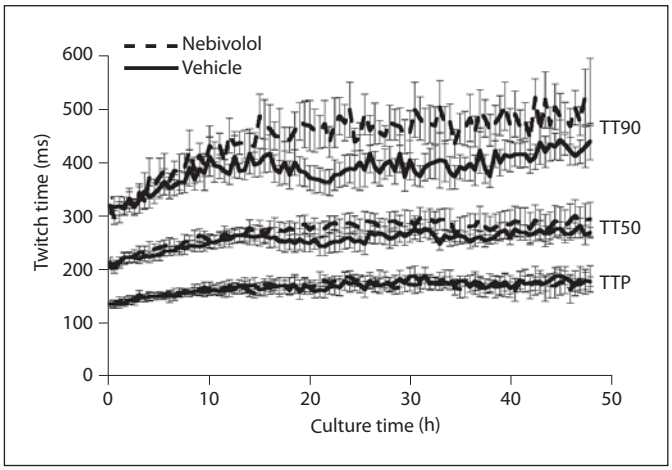

Fig. 4. Twitch timing parameters. In both groups, twitch timing is slightly prolonged as culture time increases. No significant differences were observed between the 2 groups (repeated measures ANOVA, followed by unpaired t test). Same data pool as figure 3. TTP = Time to peak tension; TT50 = time to 50% relaxation; TT90 = time to 90% relaxation.

Baseline Contractile Characteristics

In total, 38 muscle preparations met the required preset parameters and were included in the study. At t = 0 h, preload (diastolic force, F_{dia}) was similar in both groups, and amounted to 3.1 ± 0.4 mN/mm^2 (n = 11) in preparations in the presence of 10^{-6} mol/l nebivolol, and to 3.4 ± 0.5 mN/mm^2 (n = 14) in preparations in the absence of nebivolol (fig. 3). Maximal twitch force development (F_{dev}) was significantly lower in preparations contracting in presence of nebivolol (6.1 ± 0.9 mN/mm^2) compared to those in the absence of nebivolol (8.7 ± 1.1 mN/mm^2, $p < 0.05$). Over time, fluctuations in F_{dev} were observed in both groups, but these were not significant.

Initial (t = 0 h) twitch timing parameters were similar in both groups (fig. 4). Twitch timing parameters increased slightly over time in the pre- and afterload groups. Times to peak tension, to 50% relaxation and to 90% relaxation increased over time (fig. 4). However, unlike the force data, these timing parameters continued to increase, even after peak force development had been reached. No statistically significant differences were observed between the 2 groups of muscles contracting under loaded conditions.

Discussion

The results of the present study indicate that nebivolol does not promote or attenuate load-induced cardiac myocyte apoptosis. In muscles contracting isometrically at or even below a sarcomere length that is achieved during the end of diastole in the in situ beating heart, apoptosis is induced. In the presence of nebivolol, the amount of apoptosis was unaltered under the same loading conditions.

Load-induced apoptosis has been shown to occur in end-stage heart failure [1–4]. In end-stage failing hearts, removal of the excess loading stress on the heart by a ventricular assist reversed myocyte apoptosis, most likely as a result of improved loading conditions [10]. Using a multiday culture system for multicellular muscle preparations [19, 20], we showed that mechanical load within the physiological range of achievable sarcomere lengths can induce apoptosis of cardiomyocytes [7]. The levels of preload we used in this study (3–5 mN/mm^2) can be achieved in the healthy beating heart at the end of diastole [24, 29]. The mechanism of load-induced apoptosis has to be sought in the direction of the inhibitory pathway of apoptosis. In muscle that contracted in the presence of high loads, the anti-apoptotic bcl-x_L was significantly reduced [7], indicating that the bcl-2 pathway [30, 31] is an important factor in load-induced cardiomyocyte apoptosis. However, a difference with the in situ cardiac contractions and our in vitro muscle contractions is the sarcomeres of the heart are allowed to shorten to accomplish

ejection. In vitro these sarcomeres are kept isometric, increasing the afterload. Thus, it is likely that also the afterload of the heart may play a role in induction of apoptosis. Unfortunately, current technical limitations prevent a controlled (partial) unloading of the preparation as would occur in the in situ beating heart during ejection.

Nebivolol did not reduce nor promote load-induced cardiomyocyte apoptosis. Interestingly, the third-generation β-receptor antagonist carvedilol has been shown to attenuate apoptosis [12, 32]; however, this was assessed under different experimental conditions. The antiapoptotic effect of carvedilol has been shown in the setting of ischemia-reperfusion injury, whether carvedilol can attenuate load-induced apoptosis thus remains unclear. Both carvedilol [12, 32] and nebivolol [13] have been shown to attenuate hydroxyl-radical mediated injury in contracting myocardium, a model that mimics many aspects of ischemia-reperfusion injury. However, the reduction of injury was mediated via different pathways [14, 32]. Thus, further investigation would be required to assess whether reperfusion-injury-induced apoptosis, or apoptosis induced by other mechanisms, can be attenuated by nebivolol. A recent study has indeed suggested that nebivolol lowers the apoptosis rate in a rat infarct model [15].

Unfortunately, a technical limitation of our study is that only 3 experimental groups can be compared. This is due to the fact that only a limited number of suitable trabeculae are present per heart, in combination with the restriction that these studies should be executed in a paired fashion. The success rate for completed experiments (i.e. all 3 muscles survive for 48 h) would drop drastically when introducing additional groups (i.e. another β-receptor antagonist).

Our results indicate that load-induced cardiomyocyte apoptosis does not depend on β-adrenoceptor availability. Antiapoptotic properties are therefore most likely mediated independent of β-adrenoceptor antagonism, possibly by the different structures of the individual molecules, thereby explaining the different effects of drugs in the third-generation β-adrenoceptor antagonist group.

It is well known that stretching a multicellular muscle preparation acutely changes the level of force development via several mechanisms, including differing the degree of myofilament overlap [22] and interfilament lattice spacing [33], an increase in transient intracellular calcium [34, 35] and an increase in pH [36, 37]. These factors can all contribute to an acute increase in the contractile force of cardiac muscle within the physiological range of sarcomere lengths. In our experiments we observed a very slow increase in contractile force in the control group. Either this is due to an entirely different process, or one or several of the above mentioned processes was gradually developing. Altered end-damage compliance may impact on contractile behavior [23], but the stable diastolic force indicates this process did not play a significant role in our experiments. Although twitch-timing parameters are codetermined by the level of developed force [23], upon the decline of force during the second day, times to peak tension and 50% relaxation did not decline. This likely indicates the involvement of multiple processes on the alteration of contractile behavior of preparations contracting under a high preload and afterload. The lower initial force of preparations contracting in presence of nebivolol is most likely related to the small negative inotropic effect of nebivolol described previously [14]. In addition, nebivolol to has a large number of active metabolites. Therefore, we cannot unambiguously determine whether the compound itself or one of its active metabolites mediated the observed effects.

Technical limitations prohibited quantification of shortening in the preparations that contracted fully unloaded. However, visual inspection at t = 0 and at t = 48 h was performed to ensure that these preparations contracted at these time-points. Extensive experience with the system has accumulated and has shown that in all previously measured preparations (well over 100 muscles, in total) when preloaded muscles are contracting at both t = 0 and at t = 48 h, they have been contracting continuously throughout the protocol [7, 19–21, 38]. It remains unanswered whether the preparations that contracted 'slack' displayed functional changes over this 48-hour period. We recognize that the TUNEL method may not produce unambiguous results. In overload-induced heart failure studies, the Didenko-method [39] has been used in addition to the TUNEL method under the assumption that this technique is more specific for apoptosis than the TUNEL technique. However, Guerra et al. [2] argue that there is no difference between the techniques. In addition, the DNA laddering we observed is reminiscent of apoptotic nuclei, where the DNA from merely necrotic or ischemic nuclei would 'smear' much more rather than showing the clear laddering pattern we observed.

The results obtained were gathered from multicellular preparations that may give a more realistic mechanical loading than the often-used isolated myocyte. However, the isometric nature of the experiments in this study likely does not fully reflect the loading conditions present in

vivo. Hence, the results need to be interpreted with due care. In addition, the changes in function and morphology that occur in this in vitro model are greatly accelerated compared to the in vivo situation, where changes in myocardial remodeling do not occur on the scale of hours or days, but rather months or years. Despite these limitations, we believe the sensitivity of the system would have discovered if a significant effect of nebivolol on cardiomyocyte apoptosis after mechanical loading would have occurred. Thus, in summary we show that in a multicellular, physiologically loaded contracting muscle, nebivolol does not increase nor decrease the rate of apoptosis when these muscles are subjected to high afterloading conditions.

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