ELSEVIER

European Journal of Pharmacology 366 (1999) 223–232

ejp

Transient and sustained impacts of hydroxyl radicals on sarcoplasmic reticulum function: protective effects of nebivolol

Paul M.L. Janssen *, Oliver Zeitz, Gerd Hasenfuss

Medizinische Klinik III, Universität Freiburg, Freiburg, Germany
Abt. Kardiologie und Pneumologie, Universität Göttingen, Göttingen, Germany

Received 10 August 1998; revised 23 November 1998; accepted 4 December 1998

Abstract

The hydroxyl radical ($^{\cdot}OH$) is a very reactive oxygen-free radical species that has profound effects on myocardial contractility. We investigated the impact of $^{\cdot}OH$ on free radical induced injury in right ventricular rabbit cardiac trabeculae. Additionally, we investigated the protective properties of the β-adrenoceptor antagonist nebivolol. The contractile response to a brief, 2 min exposure to $^{\cdot}OH$ consisted of a severe but transient rigor-like contracture, followed by a new steady state in which diastolic force (F_{dia}) remained increased and developed force (F_{dev}) remained decreased. In the new steady state sarcoplasmic reticulum function only partly recovered, reflected by a $>50\%$ blunted force–frequency relationship. In the presence of nebivolol (10^{-6} M), during the early phase the increase in F_{dia} was significantly smaller, and recovered better while F_{dev} was higher during peak. Moreover, nebivolol completely abolished blunting of the force–frequency relationship, which was observed in the sustained $^{\cdot}OH$ injury phase. The results indicate that hydroxyl radical injury induces systolic and diastolic dysfunction, and that nebivolol can effectively prevent a large part of this $^{\cdot}OH$ injury.

Keywords: Trabecula; β-adrenoceptor antagonist; Propranolol; (Rabbit); Myocardium; Oxygen-free radical

1. Introduction

Myocardial damage as a result of ischemia-reperfusion is thought to involve the destructive effect of oxygen-free radical species. The hydroxyl radical ($^{\cdot}OH$) is reported to be one of the most destructive of these oxygen-free radical species (Josephson et al., 1991; Gao et al., 1996). The mechanism via which hydroxyl radicals impact on contractile properties of myocardium is poorly understood. Oxygen-free radicals have a broad array of effects and multiple sites of interaction in the myocardium (Bolli, 1990; Takemura et al., 1993; Mekfi et al., 1996) and have been shown to depress contractility in the skinned muscle preparation (MacFarlane and Miller, 1992; Lowe et al., 1994), indicating a direct interaction with contractile proteins. More specifically, a proteolytic effect of TnI has recently been indicated as a major site of action of oxygen-free radicals (Gao et al., 1997; Van Eyk et al., 1998). The hydroxyl radical has been shown to affect the amount of calcium set free by the sarcoplasmic reticulum, indicating direct effects on sarcoplasmic reticulum calcium handling and/or sarcolemmal calcium channels (Gao et al., 1996; Xu et al., 1997). These effects even persist after removing hydroxyl radicals, indicating a more sustained damage. Although the hydroxyl radical injury pattern may not mimic all the features of stunned myocardium, its transient effects reflect a likely role in this type of myocardial injury.

The severe injury effects of oxygen-free radicals can be quite effectively attenuated by oxygen-free radical scavengers, such as the endogenous system of catalase plus superoxide dismutase (Pryzklenk and Kloner, 1986), or compounds like *n*-2-mercaptopropionylglycine (Myers et al., 1986) and dimethylthiourea (Bolli et al., 1987). Recently it has been reported that β-adrenoceptor antagonists like carvedilol might possess antioxidant properties (Yue et al., 1992a,b; Ma et al., 1996; Feuerstein et al., 1997) which might be useful to combat oxygen-radical induced injuries in myocardium. Other β-adrenoceptor antagonists, like nebivolol or propranolol, might also possess effective scavenging properties, although these have not yet been investigated.

* Corresponding author. Abt. Kardiologie und Pneumologie, Universität Göttingen, Robert-Koch Strasse 40, 37075 Göttingen, Germany. Tel.: +49-551-396380; Fax: +49-551-392953; E-mail: janssenp@med.uni-goettingen.de

0014-2999/99/$ - see front matter
PII: S0014-2999(98)00907-8

In this study we investigated the effects of exogenously generated hydroxyl radicals on myocardial contractility in rabbit cardiac trabeculae, and evaluated sarcoplasmic reticulum function by measurement of the force frequency relationship before and after exposure to $^{\cdot}OH$. Secondly, we investigated the β-adrenoceptor antagonistic capacity of nebivolol on a multicellular myocardial muscle preparation, and studied its impact on the $^{\cdot}OH$ radical induced injury in our experimental settings. Our results indicate that the hydroxyl radical severely impacts on the calcium handling ability of the sarcoplasmic reticulum in the early injury response, while a mild but sustained effect persists hours after OH-radical generation. In addition, we show that nebivolol is a potent protector of the hydroxyl radical induced injury, both during the transient and the sustained phase.

2. Material and methods

2.1. Muscle preparation, solutions, and apparatus

New Zealand White Star rabbits of either gender (1.5–2.5 kg) were fed ad lib. Prior to removal of the heart the rabbits were anaesthetized with sodium pentobarbital (60 mg/kg) after heparinization. Hearts were rapidly excised after mid-sternal thoracotomy and immediately washed in a modified Krebs–Henseleit solution at room temperature, containing (in mM): 120 NaCl, 5 KCl, 2.0 $MgSO_4$, 1.2 NaH_2PO_4, 20 $NaHCO_3$, 10 Glucose, 0.2 $CaCl_2$, and 20 2,3-butanedione monoxime (BDM, Sigma). This last compound was added to prevent the heart from beating and to minimize cutting injury during dissection (Mulieri et al., 1989). This solution was in equilibrium with a gas mixture of 95% O_2/5% CO_2 at all times, resulting in a pH of 7.4. The design of the experiments was approved by the animal use committee of the University of Freiburg.

Thin, unbranched trabeculae were carefully dissected from the free right ventricular wall. In addition to the trabeculae, a small block of tissue from the free wall at one end, and a piece of the valve or (depending on location in the ventricle) a second block of tissue of the free wall on the other end of the preparation were dissected and were used to mount the trabeculae in the muscle bath. The dimensions of the trabeculae were estimated using a calibrated reticule that was mounted in one of the oculars of the stereo dissection microscope ($\times 40$ magnification, ~ 10 μm resolution). On average, dimensions of all preparations were 274 ± 14 μm wide, 227 ± 8 μm thick, and 1.99 ± 0.15 mm long ($n = 35$). Cross-sectional area was calculated by assuming an ellipsoid shape (average 0.050 ± 0.004 mm^2).

Trabeculae were mounted between the basket shaped extension (after Ter Keurs et al., 1980) of a force transducer (KG4, Scientific Instruments, Germany) and a hook-like extension of a micro manipulator that was used to adjust overall muscle length. This attachment system was chosen because it has proven to minimize damaged-end compliance that is unavoidable in intact multicellular preparations (Ter Keurs et al., 1980; De Tombe and Ter Keurs, 1991; Janssen and De Tombe, 1997). A heat conducting teflon muscle bath was tightly placed in an aluminum block that was kept at 38°C, while all solutions were also heated and kept at 37°C. Temperature during the experiments in the muscle bath could thus be maintained at 36.5 ± 0.2°C throughout the entire experiment. After mounting the trabeculae in the setup in the 2,3-butanedione monoxime containing solution, the solution was switched to a Krebs–Henseleit solution containing 0.25 mM $CaCl_2$. Under recirculating flow, Ca^{2+} concentration was raised stepwise (0.5, 1.0, and 2.0 mM, in 10 min intervals) to a final concentration of 2.5 mM. Stimulation was started when the Ca^{2+} concentration of 1.0 mM was reached. The trabeculae were stimulated at 20–30% over threshold level (2–3 V typically) using symmetric pulses of 5 ms duration at a frequency of 1 Hz. The preparations were then slowly stretched until passive force amounted to L_{max} (muscle length where developed force is maximal). After L_{max} was adjusted several times and had stabilized, the preparation was left to equilibrate for at least 45 min at L_{max} and at 2.5 mM Ca^{2+} concentration, this length was then maintained throughout the experiment. Solutions were superfused at a rate such that the entire bath volume was exchanged every 2 s.

2.2. Generation of hydroxyl radicals

Hydroxyl radicals were specifically generated with the H_2O_2–Fe^{3+}–nitrilotriacetic acid system, it has been shown that by the use of this system a reproducible amount of $^{\cdot}OH$ radicals is generated (Zweier et al., 1989; Corretti et al., 1991; Gao et al., 1996). Under catalyzation of the Fe^{3+}–nitrilotriacetic acid complex the net reaction that forms the highly reactive $^{\cdot}OH$ is:

$$3\,H_2O_2 \rightarrow 2 \cdot OH + O_2 + 2\,H_2O$$

Fe^{3+}–nitrilotriacetic acid (10 μM) was added to the solution at the beginning of the protocol and did not influence the outcome of the data: pilot experiments showed no effect on Fe^{3+}–nitrilotriacetic acid alone on the force–frequency relationship nor on any contractile parameter. Because the hydroxyl radicals have an extreme short half-life, to ensure we produced reproducible amounts of OH-radicals we took the following steps, (1) to prevent photobleaching we kept the H_2O_2-stock solution (16.5 mM, made fresh just prior to use in regular Krebs–Henseleit solution) in a aluminum foil covered perfusor apparatus and darkened the room during preparation and infusion, (2) we infused H_2O_2 directly (ratio H_2O_2–regular perfusate 1:21) into the muscle bath rather than in the perfusate container, (3) except from the thin tungsten basket shaped extension of the force transducer (isolated from the muscle

attachment to the actual force transducer by silicone tubing) no parts upstream of the muscle preparation contained metal that might influence the amount of hydroxyl radicals formed in the bath. Using this system, a 2 min infusion of 0.75 mM H_2O_2 (which reflects the concentration in the muscle bath) was sufficient to evoke a serious, highly reproducible injury response. The effective amount of specific $^{\cdot}OH$-radicals generated during this period in the muscle bath is comparable or even slightly higher as reported to occur during ischemia/reperfusion (Zweier et al., 1989; Corretti et al., 1991) and in previous studies using similar preparations (Gao et al., 1996). From start of H_2O_2 infusion until 15 to 20 min after the end of infusion the solution was thrown away; after this period the solution was recirculated again.

2.3. Experimental protocols

After the contractile parameters of the trabecula had stabilized, a standard protocol consisted of the measurement of a force–frequency relationship. Stimulation frequency was switched from 1 Hz to 0.25 Hz, and contractile response was assessed when force had stabilized at this new frequency. The frequency was then switched back to 1 Hz, ensuring that all frequency changes were initiated from the same baseline conditions (1 Hz). This protocol was then repeated for 0.5, 1, 2, 3, and 4 Hz. After the 4 Hz step the frequency was also switched back to 1 Hz, and the entire protocol was then repeated after a resting period of at least 1.5 h. Interventions were applied during this period, or at the start of the protocol when applicable.

With this basic protocol, the following interventional protocols were performed. First a protocol was applied where in the period between 2 force–frequency protocols no intervention was applied. This group ($n = 7$) served as a control protocol to assess the change of the force–frequency relationship over time per se, and potential effects of rundown of F_{dev} over time. In the second group we studied the effects of hydroxyl radicals; after the first force–frequency a 2 min infusion of H_2O_2 was given to generate the hydroxyl radicals in the muscle bath ($^{\cdot}OH$-group, $n = 7$).

To study the effects of nebivolol (a racemic mixture of the R,S,S,S and S,R,R,R-enantiomers, Berlin-Chemie, Germany) 3 additional protocols were performed. (1) in a separate protocol ($n = 7$) we studied the effect of 10^{-6} M nebivolol (made fresh from powder) per se on the alteration of the force–frequency relationship, (2) we studied the effects of hydroxyl radicals under 10^{-6} ($^{\cdot}OH$ and nebivolol group, $n = 7$) and 10^{-7} M nebivolol ($n = 3$), and (3) we studied the effects of hydroxyl radicals under 10^{-6} M propranolol ($^{\cdot}OH$ and propranolol group, $n = 7$) to compare as a commonly used β-adrenoceptor antagonist.

In several additional experiments we studied the β-adrenoceptor antagonistic capacity of nebivolol under our experimental conditions. We studied the isoproterenol (±-isoproterenol, Sigma) dose–response under different concentrations of nebivolol. In the presence of nebivolol (10^{-8} ($n = 3$), 10^{-7} ($n = 2$) or 10^{-6} M ($n = 2$)), a cumulative dose-response curve was measured. In these experiments, ascorbic acid (0.25 mM) was added to the solution at the start of the experiment to prevent isoproterenol degradation.

2.4. Data analysis and statistics

Data were collected and analyzed with custom-designed data-acquisition and analysis programs written in LabView (National Instruments). Two identical setups were used in parallel, while protocols were distributed among muscles and setups in a randomized way. From start of stimulation until the end of the experiment, from every twitch the minimal and maximal value (F_{dia} and F_{dev} respectively) were recorded on disk (i.e., a digital chart recording). Additionally, after every intervention (frequency change, infusion of compounds, etc.) an entire twitch was recorded on disk (1 kHz/channel sample frequency). Data could be optionally analyzed on-line to monitor all contractile parameters during the experiment. The following parameters were analyzed from the twitch recordings: active developed force (F_{dev}, in mN/mm^2), diastolic force (F_{dia}, in mN/mm^2), time to peak force (in ms), time from peak force to 50% relaxation ($RT_{50\%}$, in ms), and time from peak force to 90% relaxation ($RT_{90\%}$, in ms).

Muscles had to meet several pre-determined criteria to be included in the data analysis; trabeculae were discarded during the experiment or excluded from analysis when either (1) cross-sectional area of the trabeculae exceeded 0.10 mm^2, (2) F_{dev} was less than 5 mN/mm^2 or 10 mN/mm^2 at 1 or 4 Hz respectively, (3) trabeculae of which F_{dia} exceeded 25% of F_{max} at the physiological stimulation frequency of 4 Hz (indicating damaged preparations), or (4) more than 15% rundown for F_{dev} per hour during steady state conditions was observed.

The following parameters were used to quantify the early response (a transient rigor-like contracture) to $^{\cdot}OH$ induces injury: level of F_{dia} at peak contracture compared to pre-intervention value, time to peak contracture, measured from end of $^{\cdot}OH$ infusion, F_{dev} at peak contracture compared to pre-intervention value. Additionally, F_{dev} and F_{dia} after establishment of a new steady state baseline were determined.

Force–frequency relationships are analyzed as percentage of F_{dev} and F_{dia} at the lowest frequency (= 0.25 Hz) within a series. This was done to exclude differential effects of rundown of the preparation over time, and to make pre- and post-intervention data comparable. Both Student's *t*-test for paired data and for non-paired data are used where applicable. Analysis of variance (ANOVA) followed by Bonferroni was used to determine differences between multiple groups. Dose–response curves are fitted

with the hill-equation, and the half maximal dose ($EC_{50\%}$) is compared using the *t*-tests for non-paired data between groups. All data are expressed as mean ± S.E. unless mentioned otherwise. Values (doublesided) for $P < 0.05$ were considered significant.

3. Results

The average developed force (without intervention) of the trabeculae used in this study after 1 h of equilibration was 14.7 ± 1.9 and 48.8 ± 6.3 mN/mm^2 at a stimulation frequency of 1 and 4 Hz respectively. Diastolic tension amounted to 4.9 ± 0.7 mN/mm^2 (measured at 4 Hz) on average ($n = 14$, reflecting the groups without intervention). These data were not significantly different between any of the intervention groups ($P = 0.63$), nor was cross-sectional area of the preparations ($P = 0.87$), indicating a normal distribution of quality of preparations among the different interventions.

3.1. Contractile response to hydroxyl radicals in trabeculae

A typical chart recording of the response to the infusion of hydroxyl radicals is given in Fig. 1a. About 10 min after the end of the 2 min hydroxyl radical superfusion, diastolic force slowly rises, while developed force slowly decays. Over the course of several minutes, this contracture increases to the point that diastolic force rises well above previous developed force. F_{dia} during peak contracture was $723 \pm 151\%$, compared to pre-infusion values of F_{dia} ($P < 0.05$). Meanwhile developed force decreases to $56 \pm 18\%$, in 2 preparations at peak contracture the preparation even ceased to develop active force. During this early transient period, oscillations in force were observed in between stimulated contractions in 19 out of 21 trabeculae. Twitch timecourses during the intervention as well as pre- and post-intervention baselines are given in the bottom panel. Several minutes after peak contracture, diastolic force slowly decays, and the preparations that stopped contracting actively recovered and redeveloped force. Diastolic force then continues to decay, and eventually the contractions stabilize on a new baseline. This new baseline displays a sustained increase in diastolic force ($295 \pm 77\%$, $P < 0.05$) and a loss of active developed force to about $80 \pm 12\%$ of pre-radical values ($P < 0.05$).

Nebivolol (10^{-6} mol/l) could reduce this rigor-like contracture, as can be seen in the example in Fig. 1b. During peak contracture F_{dia} rose less (to $329 \pm 73\%$) compared to both the $^{\cdot}OH$ and propranolol (Fig. 1c) groups ($P < 0.05$), while F_{dev} only decreased to $74 \pm 16\%$. Also, in 3 additional experiments we tested a lower dose of nebivolol (10^{-7} M). In these experiments the induced early injury (increase in F_{dia} accompanied by a decrease in F_{dev}) was less than without nebivolol, but more severe than

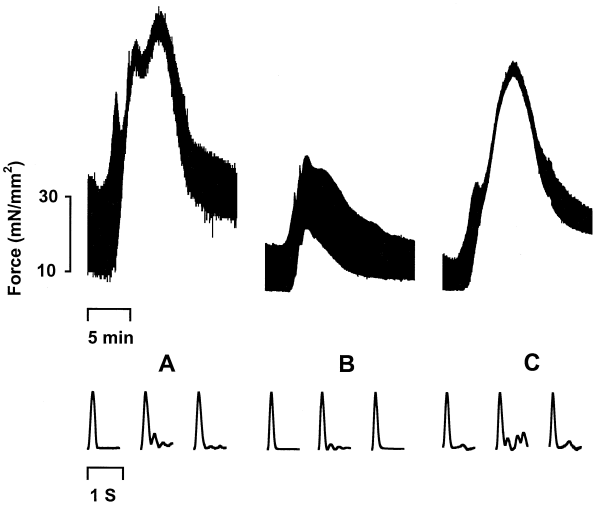

Fig. 1. Examples of chart-recordings of diastolic and developed force after a 2 minute superfusion with hydroxyl radicals only (A), and in the presence of 10^{-6} M nebivolol (B) or 10^{-6} M propranolol (C). Fe^{3+}–NTA was present from the start of the experiment, 0.75 mM H_2O_2 was infused directly into the bath for a duration of 2 min. The bottom panel shows the twitch time courses (represented as fraction of individual peak force) from a twitch as indicated immediately after generation of $^{\cdot}OH$ (twitch 1), near peak contracture (twitch 2) and about 10 min after peak contracture (twitch 3), similar for nebivolol (twitches 4, 5 and 6) and propranolol (twitches 7, 8 and 9). Spontaneous contractile oscillations (as can be seen in twitch 2) occurred in all 7 trabeculae in this protocol. In the presence of nebivolol contractile oscillations were less severe and only present in 5 out of 7 trabeculae; in the propranolol series these oscillations were stronger and observed in all trabeculae.

with 10^{-6} M nebivolol, indicating a dose dependency of the protective effect. In sharp contrast, propranolol (right panel) did not show any protective effect to early hydroxyl radical induced injury; in this group the contracture was even more severe than in the $^{\cdot}OH$ group, although not significant. In the bottom panels of Fig. 1 individual twitches of pre- and post-intervention baselines are given, as well as twitches taken during the rigor-like contraction. Mean data of the response to the radical induced injury are given in Fig. 2: the percentile changes in F_{dia} (right panel) and F_{dev} (left panel) are given for pre-, peak-, and post-contracture. We compared 3 groups that were treated with hydroxyl radicals between two force–frequency relationships: $^{\cdot}OH$ only, $^{\cdot}OH$ in the presence of 10^{-6} M nebivolol, and $^{\cdot}OH$ in the presence of 10^{-6} M propranolol. It is clear that under nebivolol at the 10^{-6} M dose a large portion of the rigor-like contraction is prevented ($P < 0.05$, compared to $^{\cdot}OH$). In the presence of propranolol (10^{-6} M) no protective effect was observed. Furthermore, the timecourses of the development of contracture were also affected by nebivolol. Peak of contracture occurred earlier (811 ± 102 vs. 1475 ± 215 s (propranolol), $P < 0.05$), while the baseline after contracture was established earlier (1655 ± 254 vs. 2718 ± 317 s (propranolol), $P < 0.05$).

To get more insight into the transient effects of hydroxyl radical induced injury, an abbreviated force–

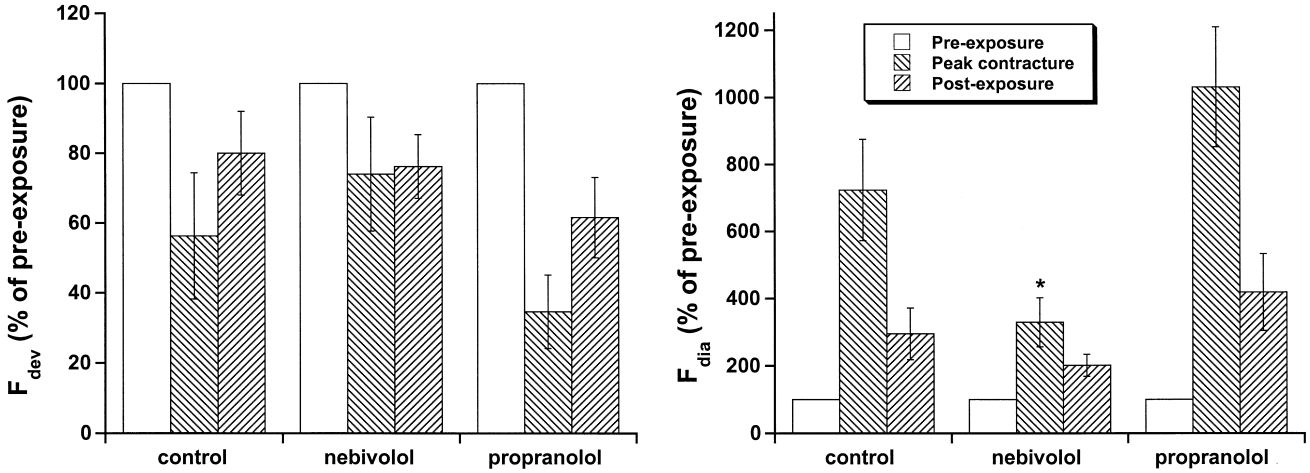

Fig. 2. Quantification of rigor-like contractures. Percentile change in F_{dev} (left panel), and F_{dia} (right panel) during peak contracture and post-contracture normalized to pre-intervention steady state levels. Interventions: Control ($^{\cdot}$OH only, $n = 7$), nebivolol ($^{\cdot}$OH in the presence of 10^{-6} M nebivolol, $n = 7$), and propranolol ($^{\cdot}$OH in the presence of 10^{-6} M propranolol, $n = 7$). $*$ Denotes that in the presence of nebivolol a significant attenuated rise in diastolic tension during peak contracture was observed compared to the other groups.

frequency-protocol was measured. During the diastolic rise stimulation frequency was briefly switched from 1 to 2 Hz, and the contractile response was followed for several beats only because no steady state conditions are present at this time. The 2 Hz frequency was chosen for this protocol because within a force–frequency relationship the largest jump increase in F_{dev} is usually observed between 1 and 2 Hz, and thus impacts of change in frequency are more apparent. During these transients effects of hydroxyl radicals, this rise in stimulation frequency showed an absence of increase in force on average, compared to the switch from 1 to 2 Hz during the first force–frequency relationship (Fig. 3). In the presence of nebivolol the increase in

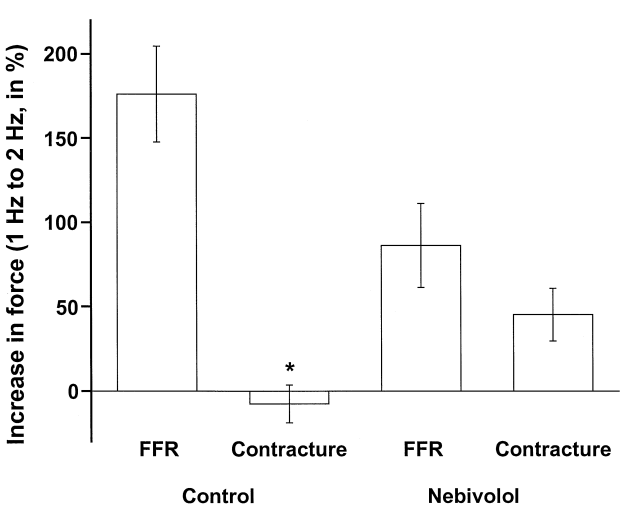

Fig. 3. Contractile response to change in stimulation frequency during the transient contracture. Percentile increase in developed force after a switch in stimulation frequency of 1 to 2 Hz. Left bars: Control ($^{\cdot}$OH only). The response during the contracture is significantly lower from the response observed before infusion (FFR-bar) of hydroxyl radicals ($P < 0.05$). Right bars: impact of 10^{-6} M nebivolol. The contractile response is preserved although slightly attenuated (not significant).

F_{dev} upon stimulation frequency increase to 2 Hz was lower (not significant) but preserved compared to the switch during the first force–frequency relationship. The effect of propranolol was similar to the effect of the $^{\cdot}$OH group, the switch from 1 to 2 Hz did not show any marked changes in F_{dev}.

Roughly 25 min after end of hydroxyl radical injury, contractile parameters stabilized and returned to a new steady baseline. For all groups contractile parameters of pre- and post-intervention steady states are summarized in Table 1. In both the $^{\cdot}$OH and the $^{\cdot}$OH plus propranolol group we observed a significantly increased $RT_{90\%}$, indicating that in these groups an impaired relaxation persists in the new steady state condition. In the nebivolol group, this parameter has not changed from pre $^{\cdot}$OH infusion values.

3.2. Effects of hydroxyl radicals on the force–frequency relationship

Measurements of force–frequency relationships were used to get insight into the injuries that had occurred during the hydroxyl radical generation period. In the control experiment it was found that although a limited run-down of force was observed over time, the shape of the force frequency relationship did not change over time. In the second force–frequency relationship none of the frequencies showed significant lower or higher response than in the first one (results not shown). Thus, changes would be solely contributable to the intervention between 2 force–frequency relationships. All force frequency relationships were shown as percentage of developed force at 0.25 Hz. In absolute values, developed force amounted to 6.5 ± 0.6 mN/mm^2, and no differences were observed between any two groups during the first force–frequency

Table 1
Contractile parameters before-, during-, and after the transient injury phase

	Group	F_{dev} (mN/mm^2)	F_{dia} (mN/mm^2)	TTP (ms)	$RT_{50\%}$ (ms)	$RT_{90\%}$ (ms)
Pre-intervention	Control	13.1 ± 2.2	4.1 ± 0.7	117 ± 6	54 ± 4	94 ± 6
	Nebivolol	20.6 ± 3.0	5.9 ± 0.8	144 ± 8	79 ± 2	132 ± 7
	$^{\cdot}$OH	13.6 ± 3.1	8.4 ± 1.5	129 ± 7	62 ± 4	109 ± 10
	$^{\cdot}$OH and nebivolol	18.8 ± 3.3	5.1 ± 0.9	152 ± 5	77 ± 4	129 ± 6
	$^{\cdot}$OH and propranolol	17.7 ± 2.5	5.5 ± 1.2	131 ± 4	63 ± 2	112 ± 4
During contracture	$^{\cdot}$OH	6.5 ± 1.7[a]	54.6 ± 8.7[a]	119 ± 7	75 ± 6	ND
	$^{\cdot}$OH and nebivolol	13.2 ± 3.5[a]	20.7 ± 5.6[a,b]	141 ± 12	79 ± 5	ND
	$^{\cdot}$OH and propranolol	6.4 ± 1.7[a]	54.0 ± 6.0[a]	128 ± 4	68 ± 2	ND
Post-intervention	Control	12.0 ± 2.0	4.4 ± 0.6	121 ± 8	55 ± 4	99 ± 6
	Nebivolol	15.1 ± 2.8	5.3 ± 0.9	142 ± 9	80 ± 2	142 ± 9
	$^{\cdot}$OH	9.3 ± 1.5[a]	21.6 ± 3.9[a]	125 ± 6	72 ± 4	145 ± 6[a]
	$^{\cdot}$OH and nebivolol	15.2 ± 3.2[a]	10.1 ± 1.9[a,b]	148 ± 6	75 ± 5	135 ± 5
	$^{\cdot}$OH and propranolol	11.1 ± 2.6[a]	22.4 ± 5.3[a]	125 ± 6	62 ± 2	137 ± 6[a]

All data reflect the average values of 7 experiments. During the contracture the $RT_{90\%}$ parameter could not be reliably quantified because of the contractile oscillations.
[a]Significant difference compared to pre-intervention value.
[b]Significant difference compared to $^{\cdot}$OH and $^{\cdot}$OH and propranolol.

relationship. In the experiments where nebivolol was tested, we performed a similar series to serve as the appropriate control by eliminating effects that might arise from the addition of 10^{-6} M nebivolol per se to the superfusate.

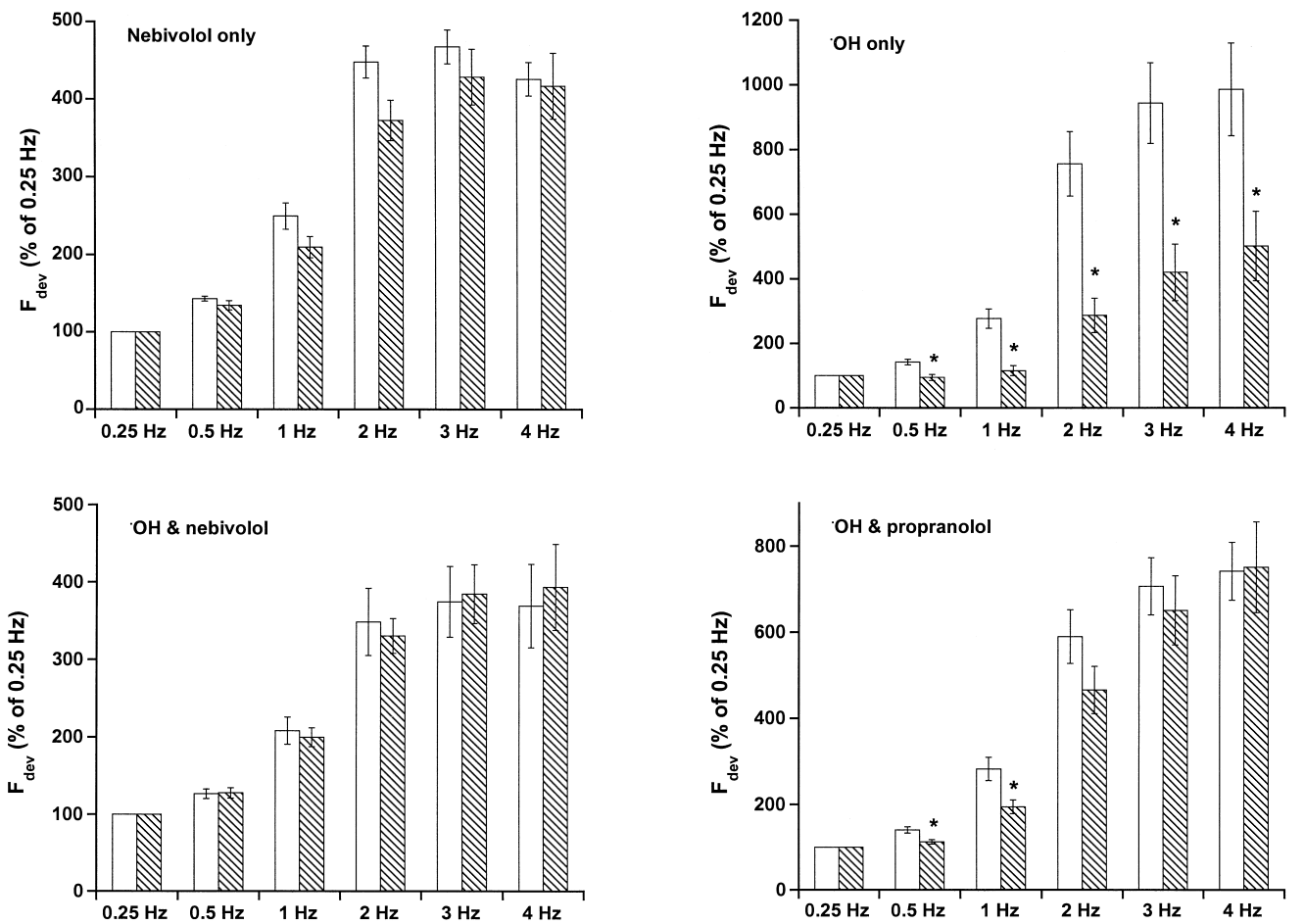

Fig. 4. Effect of $^{\cdot}$OH on the force–frequency relationships. Interval between 2 measurements is 90 min, infusion of $^{\cdot}$OH radicals was done after the first FFR where applicable. All forces are normalized to the developed force at the lowest frequency (0.25 Hz). Top left panel: Control (no intervention, 10^{-6} M nebivolol present from start of experiment). Top right panel: $^{\cdot}$OH only. Bottom left panel: $^{\cdot}$OH in the presence of 10^{-6} M nebivolol. Bottom right panel: $^{\cdot}$OH in the presence of 10^{-6} M propranolol.

We observed that nebivolol alone slightly (~10–15%, not significantly) blunted the force–frequency relationship (vs. control, i.e., the force–frequency measured prior to addition of nebivolol). Accordingly, in the experiments where nebivolol was used the drug was added at the beginning of the experiment to exclude effects of nebivolol itself (Fig. 4, top left panel). Thus, changes in the absolute amplitude of the force–frequency response arise from variability of response of the individual muscles, and are not caused by an effect of nebivolol or propranolol per se. The effects of a 2 min exposure to hydroxyl radicals on the force–frequency relationship are given in the top right panel. As can be clearly seen, the post-intervention force–frequency relationship is still positive, but severely blunted compared to the first one. The inotropic response to an increase in stimulation frequency was significantly lower than the pre-interventional ($P < 0.05$, all frequencies, pre- vs. post-interventional). In the bottom panel of Fig. 4 the data of similar protocols with nebivolol (left) and propranolol (right) are given. Nebivolol completely prevented blunting of the force–frequency relationship, while propranolol showed lower increases in force at 0.5 and 1 Hz ($P < 0.05$). In 3 additional experiments we tested a lower dose of nebivolol (10^{-7} M) and observed that the prevention of sustained injury was dose dependent, i.e., the bluntage of the force–frequency relationship was significantly larger compared to 10^{-6} M ($P < 0.05$), but significantly less than without nebivolol ($\cdot$OH-only group, $P < 0.05$). In none of the groups were significant changes observed in values for F_{dia} during the second force–frequency relationship compared to the first. In all groups F_{dia} on average decreases by about 5 and 10% at stimulation frequencies of 3 and 4 Hz, respectively, compared to the value at 0.25 Hz (results not shown).

For an additional 1 to 2 h after the second force–frequency measurement contractile parameters were followed. We observed a sustained increase of F_{dia} on this steady state level. Prior to the end of the experiment, the muscle was released to slack length to test whether diastolic tension measurements (balance of the force transducer) were correct. Drift of zero-tension was less than 5% of diastolic tension on average, indicating that the increase in diastolic tension was sustained even after several hours after hydroxyl radical insult.

3.3. β-adrenoceptor antagonistic properties of nebivolol

To test the β-adrenoceptor antagonism of nebivolol on cardiac muscle preparations, we performed a series of dose response curves. In dose response curves to increasing concentrations of isoproterenol, different concentrations of nebivolol (10^{-6} ($n = 3$), 10^{-7} ($n = 2$), and 10^{-8} M ($n = 2$)) were used to (partly) block the β-adrenoceptor agonistic response (Fig. 5). These were compared to the values obtained in a dose response curve in absence of nebivolol ($n = 7$). The $EC_{50\%}$ value of the isoproterenol dose–response curve were 0.010 ± 0.006 μM, 2.0 ± 0.4 μM, and 13.8 ± 14.8 μM for zero, 10^{-8}, and 10^{-7} M nebivolol response. The $EC_{50\%}$ value for 10^{-6} M nebivolol could not be determined.

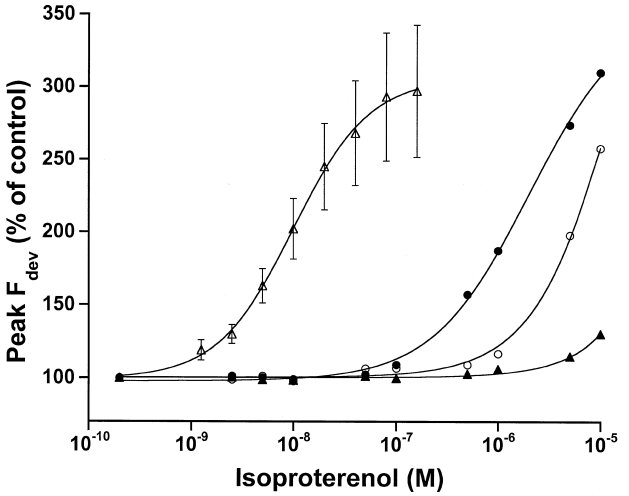

Fig. 5. Dose–response curves of isoproterenol in the presence of zero ($n = 7$, open triangles), 10^{-8} ($n = 2$, closed circles), 10^{-7} ($n = 2$, open circles), and 10^{-6} ($n = 3$, closed triangles) M nebivolol. $EC_{50\%}$ values were calculated using the Hill-equation from each individual experiment and then averaged.

4. Discussion

In the present study we present evidence that hydroxyl radical-mediated myocardial injury severely impacts on the force–frequency relationship, indicating a change in the calcium handling ability of the sarcoplasmic reticulum. In the early, transient phase of injury diastolic force rises considerably while developed force is reduced. After a short period diastolic force declines, while developed force redevelops and a new contractile steady state is reached. In this sustained injury phase, diastolic force remains increased and developed force is still decreased compared to pre-injury values. Nebivolol both reduces the contractile rigor-like transient in the early phase, and the force–frequency relation remains completely preserved in the sustained injury phase.

4.1. Impact of hydroxyl radical injury on sarcoplasmic reticulum calcium handling

In cardiac myocytes a continuous infusion of hydroxyl radicals induces a contracture; after several minutes, resting length decreases until the cell is in rigor, while calcium transients showed an increase in diastolic calcium before becoming severely deregulated (Josephson et al., 1991). A limited exposure to hydroxyl radicals induces a transient rigor-like contraction in rabbit cardiac trabeculae that is only partially reversible: after the transient effect diastolic

tension remains elevated. In our setup these transient injury phases were more severe than previously reported for rat cardiac trabeculae by Gao et al. (1996). These differences are likely due to the following experimental differences/factors: (1) although we used lower doses of H_2O_2 and a shorter exposure time, we very likely had higher level of hydroxyl radicals, because we generated the radicals directly in the muscle bath, rather than in the solution container, (2) our experiments were performed at 37°C, rather than at room temperature, (3) effects of other reactive oxygen radicals have been shown to be animal-species dependent (De Jong et al., 1990), thereby making it plausible that $^{\cdot}OH$ radicals might also display an effect that is species dependent. The transient-like effects, however, are remarkably similar to these other studies (Josephson et al., 1991; Gao et al., 1996). The absence of a strong contractile response on increase of stimulation frequency is very likely due to altered calcium handling, indicating a dysfunction of the sarcoplasmic reticulum. The contractile response to higher extracellular calcium concentrations was blunted after hydroxyl radical injury, indicating alterations in calcium handling (Gao et al., 1996). In addition to sarcoplasmic reticulum dysfunction, it has been shown that the calcium current is reduced in the presence of oxygen-free radicals (Goldhaber et al., 1989; Coetzee and Opie, 1992). Furthermore, it has been shown in vitro that hydroxyl radicals directly attack the ATP binding site of the sarcoplasmic calcium ATPase (Xu et al., 1997). The combined effects of sarcoplasmic reticulum dysfunction and reduced calcium current are therefore most likely responsible for the altered calcium handling of the myocardium. The strong rise in F_{dia} is most likely due to a calcium overload of the myocytes (Josephson et al., 1991), although short, rather mild exposures to hydroxyl radicals (Gao et al., 1996) did not reveal this diastolic tension increase. Near the peak of the transient phase calcium handling becomes severe deregulated, we observed large oscillations in twitch force in between two stimulated contractions. These are similar to oscillations observed both in calcium transients and cell shortening in isolated myocytes (Josephson et al., 1991). This deregulation of calcium handling would explain the increase in diastolic tension and decrease in F_{dev}.

After a new baseline had been reached following the transient contracture, measurements of force–frequency revealed that an effect of hydroxyl radical injury persisted. In control experiments we showed that over time either the force–frequency relationship remains unchanged, or even increases in amplitude. In sharp contrast, in the second force–frequency relationship of the $^{\cdot}OH$ group a bluntage was observed compared to the force–frequency relationship measured before $^{\cdot}OH$ insult: upon increase in stimulation frequency the contractile response (increase in F_{dev}) was significantly smaller. Diastolic tension however did not significantly change compared to the first force–frequency relationship. Because the second force–frequency relationship was measured about 1 h after end of hydroxyl radical infusion when baseline values had stabilized on the new level indicates that a sustained damage had occurred hours after the brief exposure to hydroxyl radicals. In our type of experiments with isolated crystalline-perfused muscle preparations we could not measure long-term effects of this sustained damage, and could not confirm whether these changes were actually permanent and irreversible. However, for an additional 1 to 2 h after the second force–frequency relationship contractile parameters were followed and we observed that diastolic tension remained increased on this steady state level, most likely due to persisting high levels of diastolic calcium. Although upon stretching the muscle slowly at the end of the experiment did not reveal an increased stiffness of the preparation, we did not quantify stiffness and thus cannot completely exclude that a part of increasing diastolic tension is due to rigor of a part of the preparation.

4.2. Protective effects of nebivolol

We observed that nebivolol alone had a slight, but not significant blunting effect on the FFR compared to control. To exclude a possible effect nebivolol was added at the beginning of the entire protocol. In the presence of nebivolol (10^{-6} M) the contracture during the transient injury phase was greatly attenuated. Diastolic tension rose significantly less, as was the decrease in F_{dev}. After the transient injury phase, the permanent damage was also less, indicated by a lower F_{dia}, and larger F_{dev} compared to either $^{\cdot}OH$ alone or $^{\cdot}OH$ in the presence of propranolol. Moreover, a switch in frequency from 1 to 2 Hz resulted in a much less-attenuated response than in either of the control groups. This protective effect of nebivolol was dose dependent: a smaller protective effect (although significant) was observed in the presence of 10^{-7} M nebivolol. Other novel β-adrenoceptor antagonists like carvedilol have also been reported to possess antioxidant effects via direct free radical scavenger properties (Yue et al., 1992a,b), and attenuates myocardial ischemia-reperfusion injury (Ma et al., 1996). Thus, the protective effects of nebivolol are likely to be a direct free-radical scavenging property. Propranolol did not attenuate the transient injury response, which is in close agreement with the study of Yue et al. (1992a,b) who indicated that propranolol, in contrast to carvedilol, did not have direct scavenging properties nearly as potent. NO has been shown to interact with oxygen-free radicals, thus changes in NO synthase as have been observed to play a role during vasodilation by nebivolol (Cockroft et al., 1995), might take part in the protective effect, possibly due to the stimulation of the L-arginine/nitric oxide pathway. We therefore cannot exclude that at least a part of the protective effect of nebivolol might be mediated via this mechanism. However, regarding the high amount of OH-radicals used, and their short half-life, it is unlikely that this is the only pathway via

which nebivolol protects from $^{\cdot}OH$-injury. In this regard the profile of nebivolol is similar to that of carvedilol which has been shown to improve clinical status and survival of patients with heart failure (Packer et al., 1996). Unlike carvedilol, nebivolol has a high β_1-adrenoceptor selectivity which may be favorable regarding metabolic side effects (Van de Water et al., 1988). Additionally, the long biological half-life of elimination allows once a day oral application, hinting that nebivolol may be a promising β-adrenoceptor antagonist for the treatment of patients with heart failure. A previous study in a small number of patients with moderate congestive heart failure show beneficial hemodynamic effects after 3 months of nebivolol treatment (Stoleru et al., 1993).

In conclusion, hydroxyl radical injury is characterized (at least in part) by a severely deregulated calcium handling ability. In the early injury response a rigor-like contracture, most likely due to diastolic calcium overload was observed, while calcium handling as probed by changes in stimulation frequency and observation of contractile oscillations was severely deregulated. In the sustained-damage phase, developed force remains attenuated while diastolic tension remains elevated, while calcium handling remains to be effected. Nebivolol significantly reduces the impact of hydroxyl radicals in both the transient injury phase as in the permanent injury phase. This protection is dose-dependent and this radical scavenging effect might be an important implication for the clinical use of this β-adrenoceptor antagonist.

Acknowledgements

Part of this research was supported by a grant of DFG to G. Hasenfuss. We would like to thank Sabine Pollex for assisting in some of the experiments.

References

Bolli, R., 1990. Mechanism of myocardial stunning. Circulation 82, 723–738.

Bolli, R., Zhu, W.X., Hartley, C.J., Michael, L.H., Repine, J.E., Hess, M.L., Kukreja, R.C., Roberts, R., 1987. Attenuation of dysfunction in the postischemic 'stunned' myocardium by dimethylthiorea. Circulation 76, 458–468.

Cockroft, J.R., Chowienczyk, P.J., Brett, S.E., Chen, C.P., Dupont, A.G., Van Nueten, L., Wooding, S.J., Ritter, J.M., 1995. Nebivolol vasodilates human forearm vasculature: evidence for an L-arginine/NO-dependent mechanism. J. Pharmacol. Exp. Ther. 274, 1067–1071.

Coetzee, W.A., Opie, L.H., 1992. Effects of oxygen free radicals in isolated cardiac myocytes from guinea-pig ventricle: electrophysiological studies. J. Mol. Cell. Cardiol. 24, 651–663.

Corretti, M.C., Koretsune, Y., Kusuoka, H., Chacko, V.P., Zweier, J.L., Marban, E., 1991. Glycolytic inhibition and calcium overload as consequences of exogenously generated free radicals in rabbit hearts. J. Clin. Invest. 88, 1014–1025.

De Jong, J.W., Van der Meer, P., Nieukoop, A.S., Huizer, T., Stroeve, R.J., Bos, E., 1990. Xanthine oxidoreductase activity in perfused hearts of various species, including humans. Circ. Res. 67, 770–773.

De Tombe, P.P., Ter Keurs, H.E.D.J., 1991. Lack of effect of isoproterenol on unloaded velocity of sarcomere shortening in rat cardiac trabeculae. Circ. Res. 68, 382–391.

Feuerstein, G.Z., Bril, A., Ruffolo, R.R. Jr., 1997. Protective effects of carvedilol in the myocardium. Am. J. Cardiol. 80, 41L–45L.

Gao, W.D., Liu, Y., Marban, E., 1996. Selective effects of oxygen free radicals on excitation–contraction coupling in ventricular muscle: implication for the mechanism of stunned myocardium. Circulation 94, 2597–2604.

Gao, W.D., Atar, D., Liu, Y., Perez, N.G., Murphy, A.M., Marban, E., 1997. Role of troponin-I proteolysis in the pathogenesis of stunned myocardium. Circ. Res. 80, 393–399.

Goldhaber, J.I., Ji, S., Lamp, S.T., Weiss, J.N., 1989. Effects of exogenous free radicals on electromechanical function and metabolism in isolated rabbit and guinea pig ventricle: implications for ischemia and reperfusion injury. J. Clin. Invest. 34, 267–274.

Janssen, P.M.L., De Tombe, P.P., 1997. Uncontrolled sarcomere shortening increases the intracellular Ca^{2+} transient in rat cardiac trabeculae. Am. J. Physiol. 272, H1892–H1897.

Josephson, R.A., Silverman, H.S., Lakatta, E.G., Stern, M.D., Zweier, J.L., 1991. Study of the mechanisms of hydrogen peroxide and hydroxyl free radical-induced cellular injury and calcium overload in cardiac myocytes. J. Biol. Chem. 266, 2354–2361.

Lowe, H., Baeger, I., Blasig, I.E., Haseloff, R.F., 1994. Oxygen radicals attenuate the contractility of skinned muscle fibers from the pig myocardium. Pharmazie 49, 845–849.

Ma, X.L., Yue, T.L., Lopez, B.L., Barone, F.C., Christopher, T.A., Ruffolo, R.R. Jr., Feuerstein, G.Z., 1996. Carvedilol, a new beta adrenoceptor blocker and free radical scavenger, attenuates myocardial ischemia-reperfusion injury in hypercholesterolemic rabbits. J. Pharmacol. Exp. Ther. 277, 128–136.

MacFarlane, N.G., Miller, D.G., 1992. Depression of peak twitch force without altering calcium sensitivity by the superoxide anion in chemically skinned cardiac muscle of rat. Circ. Res. 70, 1217–1224.

Mekfi, M., Veksler, V., Meteo, P., Maupoli, V., Rochette, L., Ventura-Clapier, R., 1996. Creatin kinase is the main target of reactive oxygen species in cardiac myofibrils. Circ. Res. 78, 1016–1027.

Mulieri, L.A., Hasenfuss, G., Ittleman, F., Blanchard, E.M., Alpert, N.R., 1989. Protection of human left ventricular myocardium from cutting injury with 2,3-butanedione monoxime. Circ. Res. 65, 1441–1444.

Myers, M.L., Bolli, R., Lekich, R.F., Hartley, C.J., Roberts, R., 1986. *N*-2-Mercaptopro-pionyglycine improves recovery of myocardial function after reversible regional ischemia. J. Am. Coll. Cardiol. 8, 1161–1168.

Packer, M., Bristow, M.R., Cohn, J.N., Fowler, M.B., Gilbert, E.M., Shusterman, N.H., 1996. The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. U.S. Carvedilol heart failure study group. N. Engl. J. Med. 334, 1349–1355.

Pryzklenk, K., Kloner, R.A., 1986. Superoxide dismutase plus catalase improve contractile function in the canine model of the 'stunned myocardium'. Circ. Res. 58, 148–156.

Stoleru, L., Wijns, W., van Eyll, C., Bouvy, T., Van Nueten, L., Pouleur, H., 1993. Effects of D-nebivolol and L-nebivolol on left ventricular systolic and diastolic function: comparison with D-L-nebivolol and atenolol. J. Cardiovasc. Pharmacol. 22, 183–190.

Takemura, G., Onodera, T., Millard, R.W., Ashraf, M., 1993. Demonstration of hydroxyl radical and its role in hydrogen peroxide-induced myocardial injury: hydroxyl radical dependent and independent mechanisms. Free Radic. Biol. Med. 15, 13–25.

Ter Keurs, H.E.D.J., Rijnsburger, W.H., Van Heuningen, R., Nagelsmit, M.J., 1980. Tension development and sarcomere length in rat cardiac trabeculae: evidence of length-dependent activation. Circ. Res. 46, 703–714.

Van de Water, A., Janssens, W., Van Neuten, J., Xhonneux, R., De Cree, J., Verhaegen, H., Reneman, R.S., Janssen, P.A., 1988. Pharmacolog-

ical and hemodynamic profile of nebivolol, a chemically novel, potent, and selective beta 1-adrenergic antagonist. J. Cardiovasc. Pharmacol. 11, 552–563.

Van Eyk, J.E., Powers, F., Law, W., Larue, C., Hodges, R.S., Solaro, R.J., 1998. Breakdown and release of myofilament proteins during ischemia and ischemia-reperfusion in rat hearts: identification of degradation products and effects on the pCa-force relation. Circ. Res. 82, 261–271.

Xu, K.Y., Zweier, J.L., Becker, L.C., 1997. Hydroxyl radical inhibits sarcoplasmic reticulum Ca^{2+}-ATPase function by direct attack on the ATP binding site. Circ. Res. 80, 76–81.

Yue, T.L., Cheng, H.Y., Lysko, P.G., McKenna, P.J., Feuerstein, R., Gu, J.L., Lysko, K.A., Davis, L.L., Feuerstein, G.Z., 1992a. Carvedilol, a new vasodilator and beta adrenoceptor antagonist, is an antioxidant and free radical scavenger. J. Pharmacol. Exp. Ther. 263, 92–98.

Yue, T.L., McKenna, P.J., Ruffolo, R.R. Jr., Feuerstein, G., 1992b. Carvedilol, a new beta-adrenoceptor antagonist and vasodilator antihypertensive drug, inhibits superoxide release from human neutrophils. Eur. J. Pharmacol. 214, 277–280.

Zweier, J.L., Kuppusamy, P., Williams, R., Rayburn, B.K., Smith, D., Weisfeldt, M.L., Flaherty, J.T., 1989. Measurement and characterization of post-ischemic free radical generation in the isolated perfused heart. J. Biol. Chem. 264, 18890–18895.