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# Effects of nitric oxide donors on cardiac contractility in wild-type and myoglobin-deficient mice

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**1** The effects of the nitric oxide (NO) donors S-nitroso-N-acetylpenicillamine (SNAP), sodium(*Z*)-1-(*N,N*-diethylamino)diazen-1-ium-1,2-diolate (DEA-NONOate), and (*Z*)-1-[*N*-(2-Aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate) on force of contraction ($F_c$) were studied in atrial and ventricular muscle strips obtained from wild-type (WT) and myoglobin-deficient (myo$^{-/-}$) mice.

**2** SNAP slightly reduced $F_c$ in preparations from WT mice at concentrations above 100 $\mu$M; this effect was more pronounced in myo$^{-/-}$ mice.

**3** DEA-NONOate reduced $F_c$ in preparations from myo$^{-/-}$ mice to a larger extent than those from WT mice.

**4** DETA-NONOate reduced $F_c$ in preparations from myo$^{-/-}$ but not from WT mice.

**5** Pre-incubation with an inhibitor of the soluble guanylyl cyclase (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; 100 $\mu$M) prevented the effects of SNAP, DEA-NONOate and DETA-NONOate on $F_c$ in myo$^{-/-}$ mice.

**6** It is suggested that, in physiological conditions, myoglobin acts as intracellular scavenger preventing NO from reaching its intracellular receptors in cardiomyocytes, whereas, in myoglobin-deficient conditions, NO is able to reduce contractility *via* activation of the soluble guanylyl cyclase/cyclic GMP pathway.

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**Keywords:** Nitric oxide; force of contraction; myoglobin; heart muscle; guanylyl cyclase

**Abbreviations:** DEA-NONOate, sodium(Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate; DETA-NONOate, (Z)-1-[N-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2diolate; DMSO, dimethylsulphoxide; eNOS, endothelial NO synthase; $F_c$, force of contraction; myo$^{-/-}$, myoglobin-deficient; NO, nitric oxide; NS, not statistically significant; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; sGC, soluble guanylyl cyclase; SNAP, S-nitroso-N-acetylpenicillamine; WT, wild-type

## Introduction

It is well established that nitric oxide (NO) represents an important signal molecule involved in several physiological processes including regulation of vascular tone and platelet aggregation (Moncada *et al*., 1991). However, the effects of NO on myocardial contractility are still discussed controversially. For instance, NO or related substances have been reported either to reduce (Flesch *et al*., 1997; Smith *et al*., 1991), to increase (Kojda *et al*., 1996) or not to affect cardiac contractility (Nawrath *et al*., 1995; Weyrich *et al*., 1994). Several possibilities have been suggested to account for this discrepancy. Developmental changes in the expression of the NO/cGMP signalling pathway may induce different responses to NO at different ages of the preparations used (Ji *et al*., 1999; Vulcu *et al*., 2000). Alternatively, the myoglobin content in cardiac muscle, which is different among species (O'Brien *et al*., 1992) and even between cardiac muscle preparations of one species (Ishibashi *et al*., 1993) may account for the differences in NO actions on the heart since myoglobin acts as an NO scavenger (Flögel *et al*., 2001; Mittal *et al*., 1978). In the present study, the latter hypothesis was tested by investigating the effects of different NO donors on force of contraction ($F_c$) in cardiac muscle preparations from wild-type mice (WT) and myoglobin-deficient (myo$^{-/-}$) mice.

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## Methods

Myo$^{-/-}$ mice were obtained from the Dept. of Cardiovascular Physiology, Düsseldorf, at which they were generated as described (Gödecke *et al*., 1999). Briefly, myoglobin-specific genomic clones were isolated from a genomic library of mouse strain 129Sv by using a 266-bp, PCR-amplified cDNA fragment of the murine myoglobin gene spanning exon 2. A targeting vector containing the neomycin resistance gene instead of exon 2 was used to transfect the embryonic stem cell line R1. Transfected stem cells were aggregated with murine embryos isolated from NMRI mice.

Mice of either sex were killed by cervical dislocation at the age of 4–12 weeks. The heart was quickly removed and immersed in warmed (37°C) and oxygenated buffer solution (containing in mM: NaCl 137, KCl 5.4, $CaCl_2$ 3.6, $MgCl_2$ 1, $NaHCO_3$ 12, $NaH_2PO_4$ 0.42, glucose 5.6; aerated with 95%

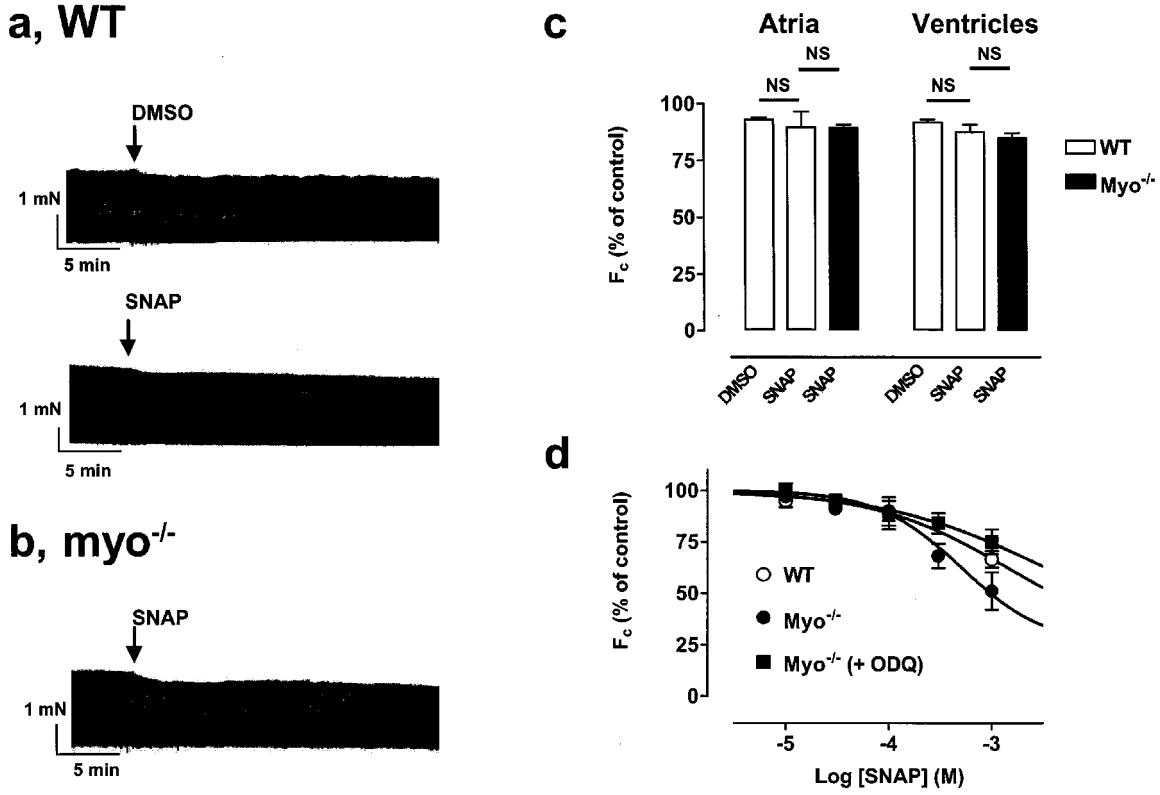


**Figure 1** Effects of SNAP on $F_C$ in cardiac muscle from WT and myo$^{-/-}$ mice. (a, b) Original recordings of $F_C$ from atrial preparations of WT (a) and myo$^{-/-}$ mice (b). Arrows indicate the times at which SNAP (100 $\mu$M) or the solvent DMSO (0.5%, v v$^{-1}$) was added. (c) Bars represent means$\pm$s.e.mean ($n=6-19$). Data were obtained 5 min after the addition of SNAP (100 $\mu$M) or DMSO. NS indicates the absence of a statistically significant difference. (d) Concentration-response curves of SNAP in atria from WT mice and from myo$^{-/-}$ mice in the absence and presence of ODQ (100 $\mu$M). Symbols represent means$\pm$s.e.mean ($n=3-4$).

$O_2+5\%$ $CO_2$; pH 7.4). The left and right atria were supplied at either end with silk ligatures as well as ventricular muscle strips as described (Wegener & Nawrath, 1997).

The cardiac preparations were mounted vertically in organ baths (5 ml) containing oxygenated buffer solution at $36\pm1$°C. One end was fixed to a hook of a muscle holder while the other end was connected to an inductive force-displacement transducer whose output was fed to a carrier frequency preamplifier (TA2000, Gould, www.gouldis.de). The preparations were stretched to the apex of the preload active tension curve, mounted next to two platinum electrodes built in a muscle holder, and electrically stimulated by square-wave voltage pulses at 3 Hz (1 ms duration, voltage 20% above threshold; Grass S4, www.astro-med.com). Drugs were applied as single dose or cumulatively to achieve the concentrations indicated.

S-nitroso-N-acetylpenicillamine (SNAP), sodium(*Z*)-1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate (DEA-NONOate), (*Z*)-1-[*N*-(2-Aminoethyl)-*N*-(2-ammonioethyl)amino]-diazen-1-ium-1,2-diolate (DETA-NONOate), and 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) were purchased from Alexis (www.alexis-corp.com). Stock solutions of SNAP and ODQ were prepared in dimethylsulphoxide (DMSO) and further diluted to the concentrations indicated. The DMSO content in the test solutions did not exceed 0.5% v v$^{-1}$. All other chemicals used were as pure as commercially available and purchased from Sigma (www.sigma-aldrich.com).

Data are presented as original recordings or expressed as means$\pm$s.e.means. The magnitude of $F_c$ was measured as the difference between resting and peak tension. Statistical analysis was performed using either paired or unpaired Student's *t*-test. The present study conforms with the Guide for the Care and Use of Laboratory Animals (U.S. National Institute of Health, publication 8523, revised 1985).

## Results

The effects of three NO donors on myocardial contractility were investigated in WT and myo$^{-/-}$ mice. The NO donor SNAP, at a concentration of 100 $\mu$M, fully effective in smooth muscle (Henry *et al.*, 1989), did not change significantly force of contraction ($F_c$) in atrial and ventricular preparations from both groups of animals (Figure 1a, b). The minor decrease of $F_c$ after addition of the drug was not statistically different from that observed if the solvent (DMSO) was applied without drug (Figure 1c). However, concentration of SNAP above 100 $\mu$M slightly reduced $F_c$ in atrial preparations from WT mice; this effect was larger in preparations from myo$^{-/-}$ mice (Figure 1d). In the presence of ODQ (100 $\mu$M), the effects of SNAP in myo$^{-/-}$ mice were not statistically different from those obtained in WT mice.

The NO donor DEA-NONOate (100 $\mu$M) transiently reduced $F_c$ in atrial and ventricular preparations from both

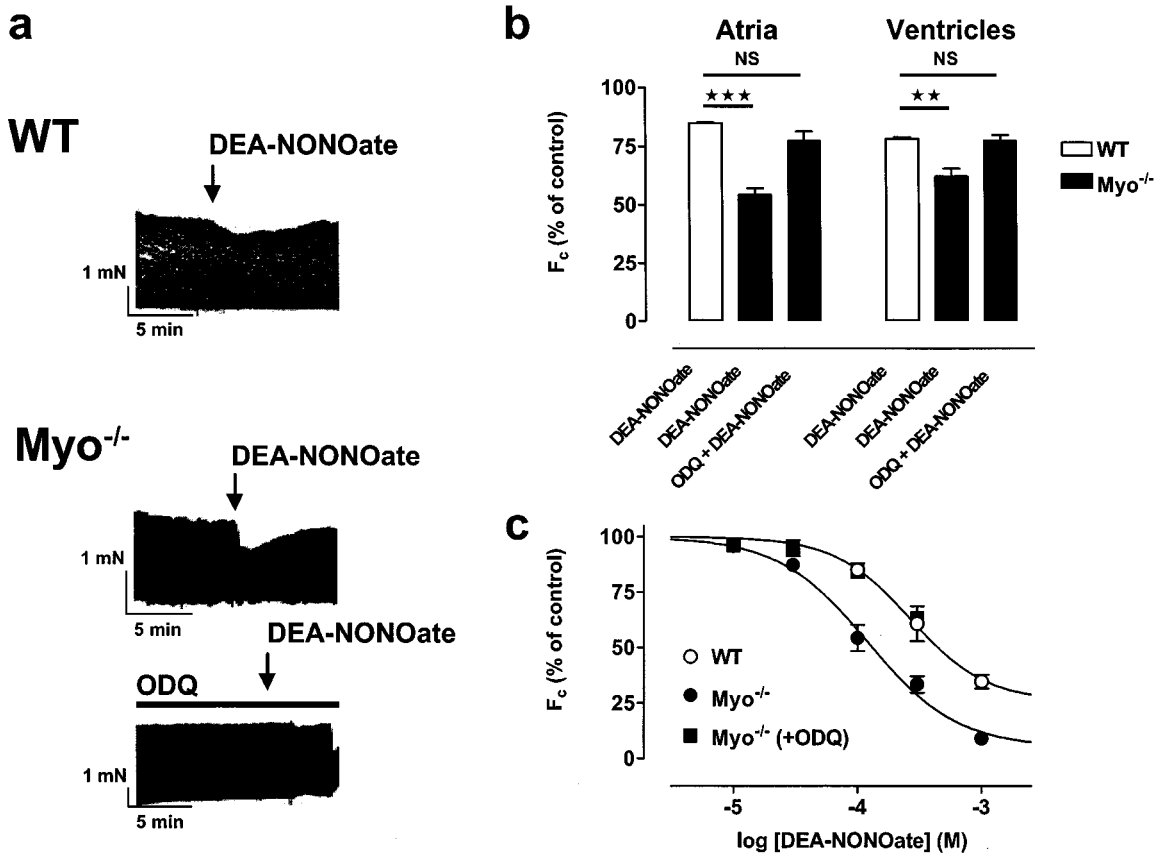


**Figure 2** Effects of DEA-NONOate on $F_C$ in cardiac muscle from WT and myo$^{-/-}$ mice. (a) Original recordings of $F_C$ from atrial preparations of WT (upper panel) and myo$^{-/-}$ mice (middle and lower panel). Arrows indicate the times at which DEA-NONOate (100 $\mu$M) was added. The bar indicates the presence of ODQ (100 $\mu$M). (b) Bars represent means$\pm$s.e.mean ($n=3-14$). Data represent the maximal effects of DEA-NONOate (100 $\mu$M) which occurred after 2 to 5 min. ODQ (100 $\mu$M) was applied 10 min before DEA-NONOate. NS and asterisks indicate the absence and presence of statistically significant differences, respectively. (c) Concentration-response curves of DEA-NONOate in atria from WT and myo$^{-/-}$ mice in the absence and presence of ODQ (100 $\mu$M). Symbols represent means$\pm$s.e.mean ($n=3-6$).

WT and myo$^{-/-}$ mice. The effect was more pronounced in preparations from myo$^{-/-}$ than in those from WT mice (Figure 2). $F_c$ was maximally reduced after 2–5 min and reached control values within 10 min. Pre-incubation of the preparations with ODQ (100 $\mu$M) for 10 min prevented the effects of DEA-NONOate on $F_c$ in myo$^{-/-}$ mice (Figure 2). The concentration-dependence of the effects was studied in atrial preparations: the $EC_{50}$ of DEA-NONOate was calculated to 0.3 and 0.1 mM in preparations from WT and myo$^{-/-}$ mice, respectively (Figure 2d). However, a major part of the preparations developed a contracture at concentrations higher than 1 mM and was rejected from the analysis.

The NO donor DETA-NONOate was without effect in atrial and ventricular preparations from WT mice but reduced $F_c$ in those from myo$^{-/-}$ mice (Figure 3). The effect of 100 $\mu$M DETA-NONOate developed slowly and reached a steady state within 30 min (Figure 3a). The effects of DETA-NONOate were absent, if the preparations were pre-incubated with ODQ (100 $\mu$M) for 10 min (Figure 3). The concentration-dependence of the effects were studied in atrial preparations from myo$^{-/-}$ mice: the $EC_{50}$ amounted to 0.1 mM (Figure 3c).

## Discussion

The present study has shown that NO donors of the NONOate group, DEA-NONOate and DETA-NONOate, were more effective in reducing contractility in cardiac preparations from myo$^{-/-}$ than from WT mice. The S-nitrosothiol-type NO donor SNAP, although effective in smooth muscle preparations (Henry *et al*., 1989), did barely change cardiac $F_c$ in WT mice but reduced weakly $F_c$ in myo$^{-/-}$ mice, albeit at concentrations greater than 100 $\mu$M. In line with these findings, DEA-NONOate but not SNAP have been reported to reduce $\beta$-adrenergic-stimulated $Ca^{2+}$ current in rat ventricular cardiomyocytes (Abi-Gerges *et al*., 2001). A possible explanation for this difference may be the way how the substances release NO. NONOates are relatively stable as the pH is raised but release NO spontaneously at physiological or low pH, albeit at different time constants (Keefer *et al*., 1996). S-nitrosothiols are believed to decompose non-enzymatically to give NO but also to transfer the nitroso group to other molecules (Williams, 1996). In addition, the generation of NO from S-nitrosothiols has been shown to depend on protein-bound $Cu^{2+}$ sources (Dicks & Williams, 1996). However, it remains

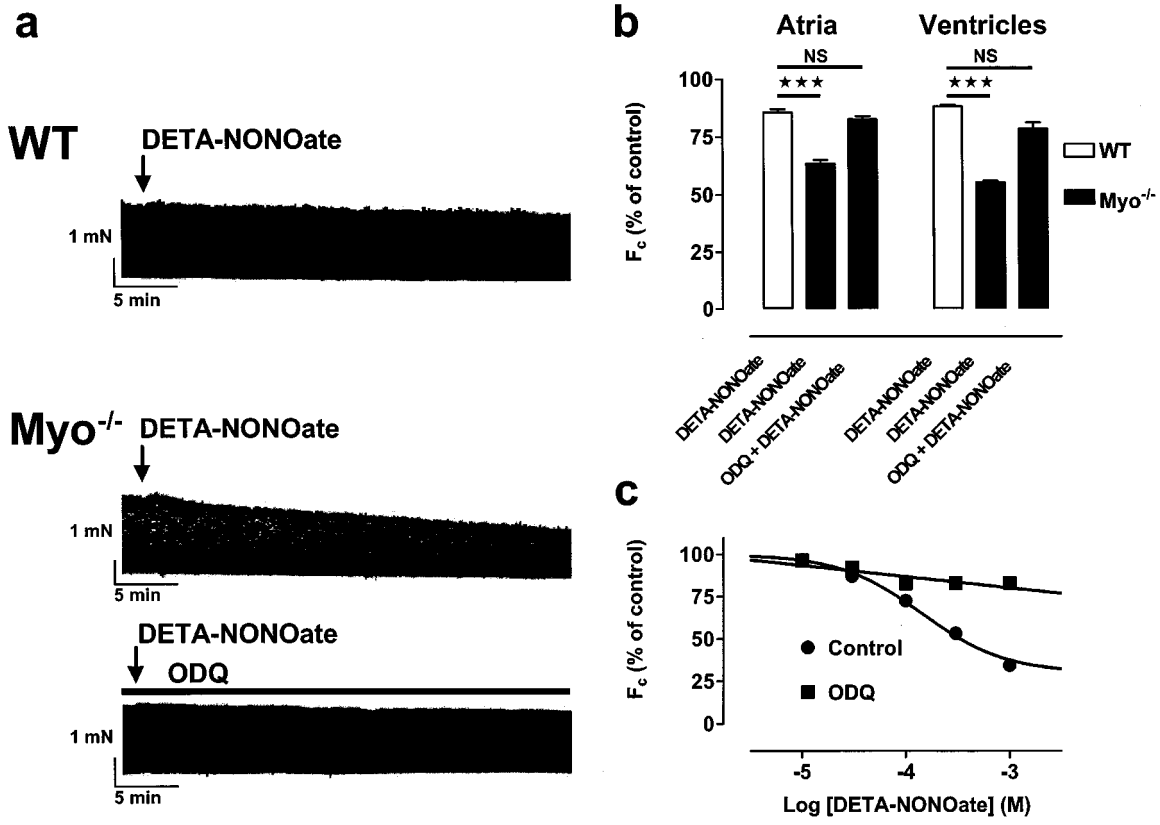


**Figure 3** Effects of DETA-NONOate on $F_C$ in cardiac muscle from WT and myo$^{-/-}$ mice. (a) Original recordings of $F_C$ from atrial preparations of WT (upper panel) and myo$^{-/-}$ mice (middle and lower panel). Arrows indicate the times at which DETA-NONOate (100 $\mu$M) was added. The bar indicates the presence of ODQ (100 $\mu$M). (b) Bars represent means$\pm$s.e.mean ($n=3-9$). Data were obtained 30 min after the addition of DETA-NONOate (100 $\mu$M). ODQ (100 $\mu$M) was applied 10 min before DETA-NONOate. NS and asterisks indicate the absence and presence of statistically significant differences, respectively. (c) Concentration-response curves of DETA-NONOate in atria from myo$^{-/-}$ mice in the absence and presence of ODQ (100 $\mu$M). Symbols represent means$\pm$s.e.mean ($n=3-5$).

open, whether these reactions have a bearing on the weak cardiac effects of SNAP.

The effects of DEA-NONOate and DETA-NONOate on $F_c$ had completely different time courses. The effect of DEA-NONOate reached a maximum within 2 min and returned to control values within 10 min. In contrast, the effect of DETA-NONOate reached its maximum within 30 min. An explanation for this difference may be the different time course of the NO release by each drug. The decomposition of DEA-NONOate and DETA-NONOate to release NO shows a half-life (at 37°C) of about 2 min and 20 h, respectively (Keefer *et al.*, 1996). Using these values, the concentration-time profile of the NO release from DEA-NONOate and from DETA-NONOate was calculated taking into account NO auto-oxidation as described (Schmidt *et al.*, 1997). According to this calculation, NO released by 100 $\mu$M DEA-NONOate produced a peak concentration of 10 $\mu$M within 80 s and returned to about 2 $\mu$M after 5 min. NO released by 100 $\mu$M DETA-NONOate produced a steady-state concentration of 0.6 $\mu$M within 1000 s during the calculated time interval of 40 min. These calculated profiles of [NO] fit well to and are, therefore, assumed to determine the time courses of the cardiac effects of DEA-NONOate and DETA-NONOate in myo$^{-/-}$ mice observed in the present study. Similarly, fast and slow time courses were also shown for the vasorelaxant effects of DEA-NONOate and SPER/NO ($t_{1/2}=39$ min) and related to the different time courses of NO release by each drug (Morley *et al.*, 1993).

Recently, myoglobin has been suggested to be involved in the inactivation of NO and to substantially determine the NO effects on coronary blood flow (Flögel *et al.*, 2001). In the latter study, NO decreased dp/dt in a Langendorff-type preparation, and this effect was slightly pronounced in hearts from myo$^{-/-}$ mice. The present study shows a more complete dissociation of the effects of NO donors in normal and myoglobin-deficient hearts. Most probably, the different techniques to assess contractility (whole heart vs isolated strips in isometric conditions) account for these differences. It seems likely, therefore, that the level of myoglobin (around 100–200 $\mu$M; (Wittenberg, 1970)) being present in the cardiomyocytes from WT mice acts as an intracellular scavenger for NO, as has been shown for extracellular haemoglobin (Martin *et al.*, 1985), reducing the effective NO concentration in the cardiomyocytes. As a consequence, only high levels of exogenously applied NO may reach intracellular target molecules to induce physiological effects, as seen for 100 $\mu$M DEA-NONOate (corresponding to about 10 $\mu$M NO) in the preparations from WT mice (Figure 2). On the

other hand, low levels of myoglobin may make it easier for exogenously derived NO to reach intracellular receptors in cardiomyocytes. Indeed, different levels of myoglobin were taken as evidence for the differences in NO donor-induced increases in cGMP levels in atrial and ventricular preparations from the rabbit (Ishibashi *et al*., 1993) and the differences of NO donor-dependent effects in cardiac muscle from neonatal and adult rats (Vulcu *et al*., 2000).

The effects of the NO donors DEA-NONOate and DETA-NONOate were absent in the presence of ODQ, an inhibitor of soluble guanylyl cyclase (sGC) (Garthwaite *et al*., 1995). These results suggest that the cGMP/sGC signalling pathway is involved in mediating the effects of NO in the murine myocardium. Intracellular application of cGMP has also been shown to induce similar effects on L-type $Ca^{2+}$ current ($I_{Ca}$) in rat cardiomyocytes (Vandecasteele *et al*., 2001) as did the NO donor SIN-1 (Méry *et al*., 1993). DEA-NONOate (100 $\mu$M) has been reported to reduce contractility in rat cardiomyocytes which was, however, not inhibited by 25 $\mu$M ODQ (Sandirasegarane & Diamond, 1999). Since ODQ is able to interact with other heme containing proteins besides sGC (Feelisch *et al*., 1999; Zhao *et al*., 2000), e.g. with intracellular myoglobin (100–200 $\mu$M) as suggested (Wegener *et al*., 1999), the ODQ concentration used in the former study may have been too low to result in a sufficient inhibition of sGC. However, concentrations of SNAP and DEA-NONOate above 100 $\mu$M reduced $F_c$ in preparations from myo$^{-/-}$ mice in the presence of 100 $\mu$M ODQ and even from WT mice. This finding indicates that, at these concentrations, effects besides activation of sGC are involved in the reduction of $F_c$, as for example inhibition of mitochondrial energy supply (Stumpe *et al*., 2001).

In summary, the present study has shown the absence of myocardial effects of exogenously applied NO, at least at concentrations below 10 $\mu$M. This has been explained by the presence of intracellular myoglobin acting as scavenger for NO. However, the *in vitro* findings do not unequivocally deny a physiological role for NO in the myocardium, if liberated from the cardiovascular endothelium or cardiomyocytes (Shah & Maccarthy, 2000). An intra-myocardial source for NO may be able to induce physiological functions in spite of the presence of myoglobin, if NO is generated in close vicinity to its target molecules. Indeed, an isoform of the NO synthase (endothelial NO synthase; eNOS) has been found closely associated to membrane caveolae in cardiomyocytes (Feron *et al*., 1999) and reported to mediate the stretch dependence of $Ca^{2+}$ release (Petroff *et al*., 2001). In addition, it has been shown that eNOS co-purifies with ryanodine receptor channel-containing sarcoplasmatic reticulum fractions from the myocardium (Zahradnikova *et al*., 1997). In cerebellar neurones, an NO synthase is co-localized with the NMDA receptor *via* an anchoring protein (Christopherson *et al*., 1999). A compartimentalization would escape the scavenge of NO by myoglobin in cardiomyocytes and may lead to local NO concentrations sufficient to exert effects on $\beta$-adrenergic signalling, $Ca^{2+}$ handling, or oxygen consumption.

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